Contribution to deciphering the complexity of mixed acidogenic fermentation of agroindustrial waste for the production of carboxylates

Doctoral Thesis

Anastasios Perimenis

Université catholique de Louvain, Earth & Life Institute,
Team Bioengineering

Supervisor

Prof. Patrick Gerin (Université catholique de Louvain, Belgium)

Accompanying committee

Prof. Spiros Agathos (Université catholique de Louvain, Belgium & Yachay Tech, Ecuador)

Prof. Jacques Mahillon (Université catholique de Louvain, Belgium)

Jury members

Prof. Claude Bragard (Université catholique de Louvain, Belgium)

Prof. Philippe Delfosse (Luxembourg Institute of Science and Technology, Luxembourg)

Prof. Frank Scholwin (University of Rostock and Institute of Biogas, Waste Management and Energy, Germany)

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Foreword

This thesis is situated within the context of sustainable exploitation of waste resources for the production of utilisable molecules for the benefit of the environment and the society.

The motivation for a PhD in the domain was a growing personal interest on better understanding the fundamentals of complex biological processes. This desire was fuelled by my scientific background in chemical and environmental engineering, my fascination of how processes at the micro-scale can have a tangible effect at the macro-scale and my personal conviction that organic waste is not at all waste; on the contrary it can be source of added value. I have decided to pursue this interest through a PhD in bioengineering in the Bioengineering Team (GEBI) of the Earth and Life Institute of UCL. I am grateful to have received funding by the Walloon Region, through two collaborative research projects:

- LIGNOFUEL (Convention Number: 716721). The purpose of the project was to develop integrated pathways (fermentation, catalysis, esterification) for the production of second generation biofuels from lignocellulosic residues.
- WAL-AID (Convention Number: 6089). The purpose of the project was to create a platform of expertise to support the sustainability and competitiveness of the agri-food sector in the Walloon Region through the development of innovative and sustainable process for the valorisation of co- and by-products of the sector.

As a consequence, certain scientific choices (e.g. types of waste tested, mechanical pre-treatment of waste) were partly imposed by the context of the projects and some analyses (e.g. waste composition) were conducted by the partners of the projects.

The work conducted within this thesis resulted in three publications in peer-reviewed scientific journals (i.e. *Waste Management*, *Biochemical Engineering Journal* and *Biomass Conversion and Biorefineries*), one manuscript submitted for publication (*Journal of the Institute of Brewing*), two manuscripts under preparation, two poster presentations (*National Symposium for Applied Biological Sciences 2013*, *9th International Conference on Renewable Resources and Biorefineries 2013*) and one oral presentation (*ENVITAM and GEPROC Graduate school PhD day 2012; best presentation award*).

This work also fed a number of project proposals for research funding, the most elaborate of which being a H2020 collaborative proposal aiming to demonstrate sustainable downstream processing of waste-derived VFA streams (Acronym *VOFAPURE*; Reference *SEP-210177779*; Topic *BIOTEC-*

4-2014). GEBI would focus on the control of the pilot scale acidogenic fermentation and also assumed the role of the coordinator of the proposal, bringing together a group of 14 academic and industrial partners. I was personally responsible for the development of the project idea, the assembly of the consortium and the editing of the proposal combining inputs from all partners. The proposal passed the first stage of evaluation but was not retained for funding at the second stage.

Abbreviations

COD: Chemical oxygen demand

CODs: soluble COD

CODt: total COD

DGGE: Denaturing gradient gel electrophoresis

DSP: Downstream processing

FM: Fresh matter

HRT: Hydraulic retention time

ML: Mixed liquor

OLR: Organic loading rate

OTU: Operational taxonomic unit

PCR: Polymerase chain reaction

TS: Total solids

VFA: Volatile fatty acids

tVFA: total VFA

VS: Volatile solids

Abstract

This thesis focused on the production of short chain carboxylates by mixed acidogenic fermentation, as a possible option for the sustainable valorisation of agroindustrial waste. Acidogenic fermentation is an intermediate step of standard anaerobic digestion, a process that sequentially converts organic matter mainly into methane. The product of acidogenic fermentation are short chain carboxylic acids, like acetic, propionic, butyric, caproic and lactic acids, with a variety of uses in green chemistry, renewable energy and other environmental applications. "Mixed fermentation" indicates the existence of natural consortia of microbial species treating composite substrates like lignocellulose, contrary to single, pure cultures treating simple substrates like glucose. The advantages of this approach include, among others, higher conversion capacities and increased flexibility in terms of substrate type, but the challenge of mastering the complexity of the fermentation system and the downstream processing of the products increases.

We examined a variety of agroindustrial waste, such as fruit pulps and brewery residues among others, to highlight the relevance of mixed acidogenic fermentation as a stand-alone bioconversion process. We employed a variety of techniques (including among others wet chemistry, chromatography, DNA extraction, amplification and sequencing) to analyse the metabolic and microbial profile of fermentations and decipher their complexity.

We were able to achieve carboxylate (mainly acetic, butyric and caproic acids) concentrations of approximately 41 $g_{COD}/kg_{mixed\_liquor}$ and substrate-to-carboxylate conversion yields of 0.64 $g_{COD\_carboxylates}/g_{COD\_substrate}$. We have shown that every substrate/inoculum combination leads to a unique and reproducible fermentation profile; a major factor for this uniqueness is the evolution and dynamics of the microbiome within each fermentation. We have highlighted, through the production of lactic acid from beer trub, the interest of mixed consortia not only as producers of target molecules but also as degraders of unwanted substances (inhibitory hop acids). We identified potential links between microbial species and metabolic products, but further research is needed to establish robust and direct correlations in a more systematic way. This would bring us a step closer to selective conversion of complex substrates into specific carboxylates and enable industrial use of mixed acidogenic fermentation.

Contents

1 Introduction

In this Chapter, the thesis is first briefly situated in the current Bioeconomy context. This is followed by the description of the theoretical background of mixed acidogenic fermentation and the scientific context within which this work has been conducted, i.e. the need to decipher the complexity of the fermentation system through a combination of metabolic and microbial analyses. Within this context, the specific objectives of the thesis are presented in the end of the introduction in the form of research questions.

1.1 Context and theoretical background

1.1.1 Bioeconomy & the biorefinery concept

With the adoption of the strategy "Innovation for Sustainable Growth: A Bioeconomy for Europe" in 2012[1], the European Commission has declared its commitment towards the development of a bio-based economy that would drive sustainable development and address five important societal challenges:

- Ensure food security for an ever-increasing population
- Manage natural resources efficiently and sustainably
- Reduce dependence on non-renewable resources
- Mitigate and adapting to climate change
- Create jobs and maintain European competitiveness

EU's commitment on bio-based development was obvious long before the adoption of this document, but we can consider this strategy as a milestone that officialises this commitment at the highest political level and consolidates a series of actions (i.e. "Bioeconomy Action Plan") to bring it forward. Some indicative initiatives for the promotion of the bio-based economy include:

- The Bio-based Industries Joint Undertaking in H2020 Framework Programme (https://www.bbi-europe.eu/)
- The Biotechnology Section of Leadership in Emerging and Industrial Technologies (LEIT) as well as the Societal Challenge "Food Security, Sustainable Agriculture and Forestry, Marine, Maritime and Inland Water Research and the Bioeconomy" in H2020 (https://ec.europa.eu/programmes/horizon2020/h2020-sections).

[1] http://ec.europa.eu/research/bioeconomy/pdf/bioeconomycommunicationstrategy_b5_brochure_web.pdf and http://ec.europa.eu/research/bioeconomy/pdf/review_of_2012_eu_bes.pdf#view=fit&pagemode=none

- The European Research Area Networks (ERA-NETs) under the Bioeconomy thematic (e.g. CoBioTech, https://www.cobiotech.eu/; BESTF3, http://eranetbestf.net/ and others)
- The Bioeconomy Knowledge Centre (https://biobs.jrc.ec.europa.eu/), a platform to support EU and national policy makers and stakeholders with science-based evidence, as well as the Bioeconomy Panel, a group of experts representing the views of different stakeholders in the field, aiming towards tangible policy actions.
- The Circular Economy Package[2] that englobes a series of legislative proposals on waste (e.g. landfills, packaging, electronic waste, end-of-life vehicles) and an Action Plan focusing on prevention, reduction, treatment, recycling & reuse of waste as well as protection of natural resources and sustainable use of primary resources.

The Bioeconomy sector is a complex and multidisciplinary sector spanning over a variety of industries (agriculture, biotechnology, forestry, fisheries, food and beverage manufacturing, chemical, etc.) and employing scientific knowledge from different disciplines (agronomy, biology & biotechnology, information & communication technologies, chemical engineering, social sciences, etc.). As such, it constitutes a vital part of Europe's economy. According to the latest Bio-Economy Report[3] of the Joint Research Centre (JRC), the EU Bioeconomy sector employed around 18.6 million people and generated approximately 2.2 trillion € in 2014.

Within this context and considering the variety of biomass sources and conversion processes, it has been clear that there can neither be only one technology that can treat all types of biomass streams nor only one biomass source that can cover the demand for bio-based products. Integrated concepts using different technologies to harness the potential from different parts of the same feedstock and/or from different feedstocks have been developed, under the general term "biorefinery" (Figure 1-1).

The parallelism with a classical refinery is helpful to understand the concept: biomass streams replace fossil resources as input, biomass conversion technologies replace standard refining technologies as processes and the generated biobased products replace fossil fuel products.

[2] http://ec.europa.eu/environment/circular-economy/
[3] https://biobs.jrc.ec.europa.eu/sites/default/files/files/JRC_Bioeconomy_Report2016.pdf

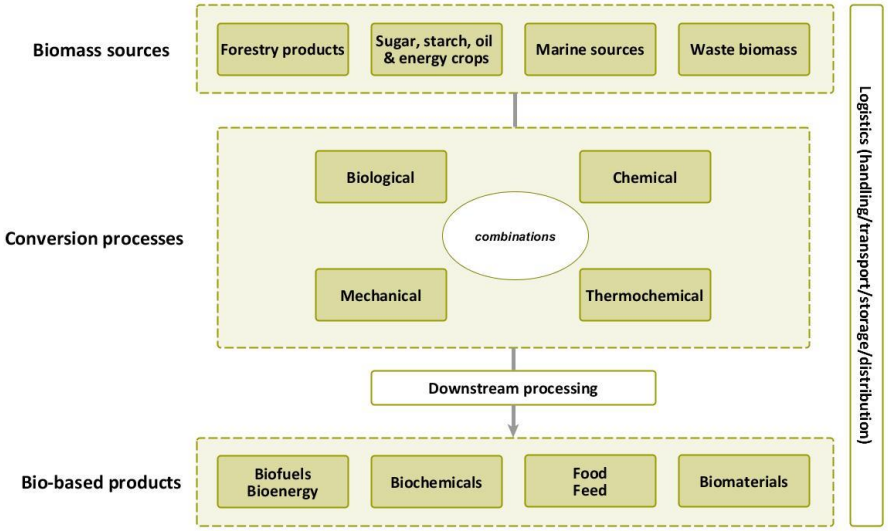

Figure 1-1: The biorefinery concept
Own scheme inspired by Cherubini et al. (2009), Demirbas (2010) and Mueller-Langer et al. (2018)

The emergence of the biorefinery concept has initiated numerous suggestions of possible sub-schemes, focusing on different parts of the process chain. Some researchers are referring to the type of feedstock, like waste biorefinery (Venkata Mohan *et al*., 2016), algae biorefinery (Yen *et al*., 2013), lignocellulose biorefinery (FitzPatrick *et al*., 2010) and food waste biorefinery (Dahiya *et al*., 2018); some are using the term "platform" to refer to the intermediate stage between feedstock and final product, like the sugar, syngas or carboxylate platforms (Agler *et al*., 2011; Arslan *et al*., 2016); some are referring to the processes involved, like the anaerobic biorefinery (Bastidas-Oyanedel *et al*., 2015; Sawatdeenarunat *et al*., 2016); and some are referring to the main final bioproduct, like the hydrogen biorefinery (Sarma *et al*., 2015). As a result of this diversity, a common classification of the biorefinery schemes is challenging (Cherubini *et al*., 2009). For the purposes of this thesis, it is not necessary to abide to a specific terminology, but the two types of schemes/platforms that are closer to the work performed are the ***biowaste biorefinery and the carboxylate platform***.

1.1.2 Waste biomass & agroindustrial waste

Biomass is a very heterogeneous and complex renewable material. As Fig.1 shows there are numerous biomass sources that can be converted to valuable biobased products, however focus is given nowadays to the sustainable management and utilisation of ***waste biomass sources***, for three basic reasons (Kaur *et al*., 2014):

- Avoiding competition with primary biomass resources used for food/feed purposes.
- Managing an ever-increasing volume of waste generated as a result of increasing population and improved living standards.
- Exploiting a hidden potential for material and energy recovery.

Similar to primary biomass resources, waste biomass resources also have a multitude of sources and can be classified into different categories (Table 1-1) (Kaur *et al*., 2014; Yang *et al*., 2015):

Table 1-1: Categories of waste biomass resources

Agricultural residues (e.g. straw, roots, shells, hulls, stalks)
Animal husbandry residues (e.g. manure)
Food and beverage manufacturing residues (e.g. fruit pulp, brewer's spent grain, whey wastewater)
Forestry residues (e.g. wood chips, sawdust)
Municipal waste (e.g. household & restaurant solid waste, sludges, wastewater)
Marine processing residues (e.g. fisheries' residues)
Industrial organic waste (e.g. glycerol, etc.)

Admittedly, the terminology used is not always consistent (e.g. waste biomass, bio-waste, biodegradable waste, organic waste, etc.). For example, the European Commission communication on future steps in bio-waste management in the European Union in 2010[4] refers to the definitions of the Waste Framework Directive, where bio-waste includes "*garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises as well as comparable waste from food processing plants. It does not cover forestry or agricultural residue and it should not be confused with the wider term "biodegradable waste" which includes also other biodegradable materials such as wood, paper, cardboard, sewage sludge.*". According to this Communication, about 118-138 million tonnes of bio-waste are produced every year with a projection for a 10% increase by 2020.

In the following chapters, we interchangeably use the terms "agroindustrial residues" and "agroindustrial waste" to refer to two types of waste, i.e. W091 (animal and mixed food waste) and W092 (vegetal waste) generated from two economic activities, as classified by NACE Rev.2, i.e. A (agriculture, forestry and fishing) and C10-C12 (manufacture of food products, beverages and tobacco products). According to EUROSTAT the corresponding amount of

[4] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52010DC0235&from=EN

waste in EU28 in 2014 was approximately 28.1 million tonnes.

Food processing waste for example, a substrate of focus for this thesis, is according to Ravindran and Jaiswal (2016) particularly interesting for bioproduct and bioenergy generation as it is often lignocellulosic in nature; various authors have reviewed its potential for value-added compounds (e.g. Galanakis, 2012; Mirabella *et al*., 2014; Van Dyk *et al*., 2013; Liguori *et al*., 2014). Indeed, the potential of exploitation of macromolecules like cellulose, hemicellulose and lignin -besides the easier digestible components like free saccharides and proteins- is high if the challenge of breaking-down the recalcitrant lignocellulosic structure is overcome (Kaparaju *et al*. 2009; Zhao *et al*., 2012b). To that end, numerous authors have researched different technologies for and/or the enhancement of the bioconvertibility of lignocellulosic residues and/or their fractionation into separate constituents (Hendriks and Zeeman, 2009; Nigam *et al*., 2009). According to Ravindran and Jaiswal (2016) the current logistic strategies in the food processing industry are not dealing efficiently enough with the problematic of waste generation (high accumulation rates, water content, disposal management, etc.). Integration of technologies to derive added-value products already at this stage is a promising solution, as waste composition is more consistent at this stage, compared to the final consumer stage where waste composition is more heterogeneous.

In general, researchers agree that biorefining of such types of biowaste represents an important opportunity to valorise residual organic streams into marketable biobased commodities. Major challenges for the up-scaling of such concepts are: (i) optimised collection, storage and stabilisation of waste in decentralised schemes to deal with waste variability and seasonal availability (Fava *et al*., 2015; Ravindran and Jaiswal, 2016) (ii) process intensification through optimisation of process inputs, better control of operational parameters, (bio)catalyst use and downstream processing to increase product yields and decrease operating costs (Yang *et al*., 2015) (iii) the integration of bioprocesses not only within the strict boundaries of a biorefinery but in the general (existing) design of waste management systems (Venkata Mohan *et al*., 2016) and (iv) market development through long-term demonstration activities for assessment of sustainability, sharing of infrastructure, improved of regulatory framework and co-operation of multiple actors along the value chain (Fava *et al*., 2015; Federici *et al*., 2009).

1.1.3 Anaerobic digestion

As Figure 1-1 shows, biological processes are among the possible processes that could be employed in biorefineries. One of the most common biological processes for the treatment of agroindustrial waste and an option that the EU

communication on bio-waste management brought forward is anaerobic digestion. Anaerobic digestion is a naturally occurring process (e.g. in landfills, rice fields, rumen), where organic material is decomposed through four sequential stages, in the absence of oxygen or other inorganic electron acceptors: hydrolysis, acidogenesis (or acidogenic fermentation or simply fermentation), acetogenesis, methanogenesis (Figure 1-2). Each stage is dependent on the execution of the previous stage. The products of each stage are consumed in the following stage to finally yield biogas, a substitute of natural gas containing primarily methane (CH_4) and carbon dioxide (CO_2). Different microbial communities are responsible for the different stages and they are either working in synergy or sometimes in competition for the same intermediate product. The four stages are briefly discussed below, while more focus is given on the acidogenic step in sections 1.1.4 - 1.1.7. (Angelidaki *et al*., 2011; Batstone and Jensen, 2011).

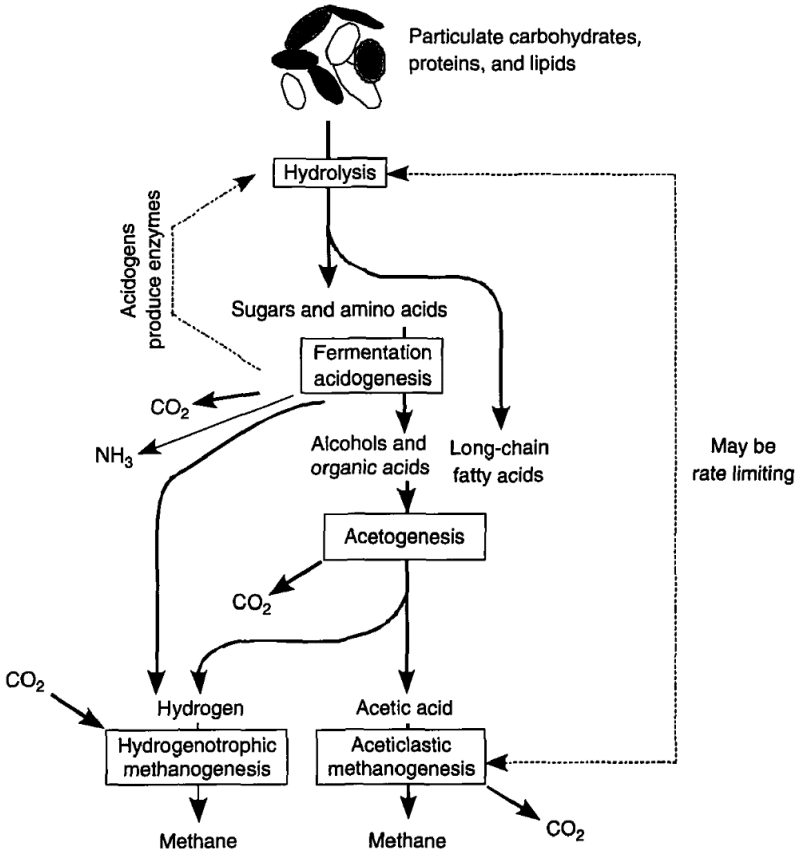

Figure 1-2: Anaerobic digestion
Adopted from Batstone and Jensen (2011); see also Annex 2 for metabolic pathways of metabolites of interest

- Hydrolysis is the stage where the soluble and insoluble macromolecules of the feedstock, primarily represented by proteins, carbohydrates and lipids, are broken down in their monomeric constituents, represented by amino acids, monosaccharides and long chain fatty acids, respectively. This break-down is achieved by means of enzymes produced from hydrolytic-acidogenic bacteria. Depending on the substrate and the reactor design, hydrolysis is often considered the rate-limiting step of anaerobic digestion and the stage that determines the retention time of the solid feedstock in the anaerobic digestion reactor.
- Acidogenesis is the stage where the soluble monomers released during hydrolysis are taken-up and metabolised by the same hydrolytic-acidogenic bacteria that were responsible for the hydrolysis stage. Main substrates here are monosaccharides and amino-acids. Long chain fatty acids degradation "by-passes" this stage by following the β-oxidation pathway directly to acetate. The pathways followed for the metabolism of the different monomers are quite different and complex. Monosaccharides ferment typically via the glycolytic cycle (Embden-Meyerhof-Parnas pathway) or alternative pathways (pentose-phosphate pathway, Entner-Doudoroff pathway) to pyruvate and then either directly to products like lactate, ethanol, CO_2, propionate or through acetyl-CoA to products like formate, acetate, butyrate, butanol, acetone, butanediol, hydrogen, etc. Amino acids ferment either through the Stickland reaction, a coupled oxidation/reaction process, where one amino acid is reduced and the other is oxidised or through an uncoupled oxidation. In general, this stage leads to short chain carboxylic acids, like acetic, propionic, butyric, valeric and caproic acids, also called volatile fatty acids (VFA); however, depending on the conditions it might also lead to the production of mixed alcohols and acetone (*solventogenic pathway*) or lactic acid (*lactic acid pathway*).
- In pure cultures using simple substrates, these pathways are well characterised and the product spectrum is well regulated and anticipated. ***However, when microorganisms co-exist in a natural environment and use complex substrates (i.e. mixed culture fermentation), the control of the product distribution is very challenging, despite the fact that the metabolic pathways are known*** (Angelidaki *et al*., 2011).
- Acetogenesis is the stage where products from the acidogenic stage are oxidised to acetate by hydrogen-producing acetogenic bacteria. Acetate can be also produced by homoacetogenic bacteria that reduce CO_2 to acetate with the use of H_2 (hydrogenotrophic pathway). Long chain fatty acids are sequentially converted directly into acetate through the b-oxidation pathway. Organic acid oxidation generally suffers from

unfavourable energetics, therefore the concentration of the product of the reaction must be kept rather low. Therefore, these reactions depend on hydrogen consuming reactions like the hydrogenotrophic pathway for acetate production mentioned before or more importantly the hydrogenotrophic methane formation explained below.

- Methanogenesis is the final product of anaerobic digestion leading to biogas, a mixture of CH_4 and CO_2. Methanogenic archaea are responsible for this step and they are producing methane by (i) cleaving acetate from the acetogenic stage before (aceticlastic methanogenesis), (ii) reducing CO_2 with hydrogen (hydrogenotrophic methanogenesis) and less often (iii) converting methylated C1 compounds (methylotrophic methanogenesis). Acetate can be either cleaved directly or used in a syntrophic way where it is oxidised by acetate oxidizers and the hydrogen produced is used by hydrogenotrophic archaea.

In anaerobic digestion, there are species that synergistically co-exist (e.g. the syntrophic methanogenesis pathway) but also microorganisms that compete for the same substrate (e.g. homoacetogenic bacteria and hydrogenotrophic archaea both use hydrogen; acetate oxidisers use the inverse pathway of homoacetogenic bacteria). Therefore, the control of anaerobic digestion is still challenging, even if the process is existing at commercial scale. Different technical configurations have been proposed and implemented across the years for biogas production (Bouallagui *et al*., 2004; Mata-Alvarez *et al*., 2000). Two-stage anaerobic systems, where hydrolysis and acidogenesis take place in the first reactor and acetogenesis and methanogenesis in the second reactor (as opposed to one-stage systems, where all processes happen in the same reactor) have been commonly used for a better control of the entire process. Anaerobic digestion is, nowadays, state-of-the-art for the treatment of a variety of agroindustrial waste (Nieto *et al*., 2012; Paritosh *et al*., 2017), nevertheless R&D needs are still existing, mainly in terms of process stabilisation (Xu *et al*., 2018).

Beside the general scheme of biogas production, different sub-schemes that build on anaerobic digestion but lead to other products are developed (Capson-Tojo *et al*., 2016). Apart from methane as the typical end-product, the anaerobic processes that were described above have a series of intermediate products that are of important industrial value (e.g. hydrogen, short chain organic acids, alcohols, ketones) and can be generated if we intervene in the process of anaerobic digestion in such a way that (i) those processes are favoured, i.e. the microorganisms that are responsible for them become dominant and follow the corresponding metabolic pathway, and (ii) undesired processes that would consume the target product (e.g. methanogenesis) are inhibited. ***Intervention in the fermentation system and monitoring of the process to ensure the generation of the target product is quite challenging.***

R&D needs are more marked for those processes in view of scale-up.

1.1.4 Mixed acidogenic fermentation and the carboxylate platform

Acidogenic fermentation is one of the processes within anaerobic digestion that could be considered as a stand-alone process for the generation of volatile fatty acids (Alkaya and Demirer, 2011), i.e. short chain carboxylic acids with a carbon number of C_2-C_6. It has been often studied in the past as a means to optimize biogas yields, but the concept of an independent process for the production of volatile fatty acids as group product or as monomers has gained increasing attention the last 15 years. Since the main product of acidogenesis is a mixture of carboxylic acids, it is often referred to as the "carboxylate platform" (Agler *et al*., 2011). Acidogenic fermentation is an electron neutral process since there is no external electron acceptor involved and the substrate is converted into both reduced and oxidised products. The products are volatile fatty acids like acetic, propionic, (iso-)butyric, (iso-)valeric and caproic acids, hydrogen and CO_2. Typical metabolic pathways for the production of VFA are shown in Annex 2, while typical reactions for their production are shown in Table 1-2.

Table 1-2: Carboxylic acids properties and stoichiometric reactions from glucose
ΔG°' values from Thauer et al. (1977) and, for caproic acid, from Spirito et al. (2014); COD equivalent represents the oxygen required from the stoichiometry of complete oxidation of 1 g carboxylic acid; MW: molecular weight. See also Annex 2.

Acetic acid Properties - MW: 60 Da, pKa: 4.79, COD: 1.067 g_{COD}/g_{VFA} $C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2$	$\Delta G°' = -206.3$ KJ
Lactic acid Properties - MW: 90 Da, pKa: 3.86, COD: 1.067 g_{COD}/g_{VFA} $C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOO^- + 2H^+$	$\Delta G°' = -198.3$ KJ
Propionic acid Properties - MW: 74 Da, pKa: 4.87, COD: 1.512 g_{COD}/g_{VFA} $C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COO^- + 2H^+ + 2H_2O$	$\Delta G°' = -358.1$ KJ
Butyric acid Properties - MW: 88 Da, pKa: 4.82, COD: 1.816 g_{COD}/g_{VFA} $C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2$	$\Delta G°' = -254.8$ KJ
Valeric acid Properties - MW: 102 Da, pKa: 4.87, COD: 2.036 g_{COD}/g_{VFA} $C_6H_{12}O_6 + H_2 \rightarrow CH_3CH_2CH_2CH_2COO^- + H_2O + HCO_3^- + 2H^+$	$\Delta G°' = -330.5$ KJ
Caproic acid Properties - MW: 116 Da, pKa: 4.88, COD: 2.204 g_{COD}/g_{VFA} $6CH_3CH_2OH + 5CH_3CH_2CH_2COO^- \rightarrow 5CH_3CH_2CH_2CH_2CH_2COO^- + CH_3COO^- + 2H_2 + H^+ + 4H_2O$	$\Delta G°' = -590.2$ KJ

As mentioned in chapter 1.1.3, the complexity of acidogenic fermentation is increased when the microbial communities responsible for it originate from natural environments and are not pure cultures. The advantages of using mixed microbial consortia are important (Kleerebezem and van Loosdrecht, 2007; Temudo *et al*., 2008):

- Reinforcing the microbial diversity and hence the metabolic capacity of the inherent microbial communities of the substrate.
- Benefiting from a diverse microbial activity that can treat a variety of different substrates or a larger part of the same feedstock.
- Removing the necessity of working under sterile conditions and the associated costs
- Being able to work under continuous conditions without considering the seasonal availability of feedstock sources.

On the downside, this lack of specificity between microbial culture and substrate leads to decreased product yields while the general complexity of the system is not yet mastered well enough to be able to work at industrial scale. ***Currently, research in acidogenic fermentation is mainly focused on two basic areas***:

1. Deciphering the complexity of the fermentation system through the investigation of the wealth of the microbiota, their function and the effect of experimental parameters on them and hence on the acidogenic performance (see Table 1-3 for a list of studies with a microbial analysis component).
2. Developing innovative and robust downstream processes to recover, concentrate, separate and purify VFA from the fermentation broth (indicatively Andersen *et al*., 2014; Arslan *et al*., 2017; Kucek *et al*., 2016; López-Garzón and Straathof, 2014; Zacharof and Lovitt, 2014).

1.1.5 Applications of volatile fatty acids

The properties of volatile fatty acids are presented in Table 1-2. These carboxylic acids have a multitude of potential uses for energy and material purposes and this versatility makes them an attractive bio-based product for different applications:

- Production of hydrocarbon fuels and industrial chemicals via VFA esterification and/or catalytic hydrogenation to mixed alcohols (Chang *et al*., 2010; Holtzapple *et al*., 2015)
- Lipid synthesis through assimilation by oleaginous yeast cultures and subsequent biodiesel production. Indeed, oleaginous yeast (e.g. of the genus *Cryptococcus, Candida, Yarrowia*), are microorganisms with the

capacity to accumulate lipids up to 72% of their biomass. Waste-derived VFA act as substrate for lipid synthesis by replacing more costly sugar monomers. Lipids can then be extracted and used for biodiesel synthesis (Johnravindar *et al*., 2017; Liu *et al*., 2016; Papanikolaou and Aggelis, 2001)

- Production of esters for use in the green chemistry or as biofuels either through chemical or biological esterification (Layton and Trinh, 2016; Cabrera-Rodríguez *et al.*, 2015; Kandylis *et al.*, 2016; Lange *et al*., 2010)
- Photofermentative hydrogen production by phototrophic (purple non-sulfur) bacteria. Indeed, VFA can serve as carbon source for phototrophic bacteria (e.g. of the genus *Rhodobacter*, *Rhodopseudomonas*) that can produce hydrogen under light source. This is a potential option to increase hydrogen yield by combining dark fermentation leading to hydrogen and VFA followed by photofermentation that further converts VFA into hydrogen. (Lazaro *et al*., 2012; Sakurai *et al*., 2013; Tekucheva and Tsygankov, 2012)
- Production of biodegradable bioplastics called polyhydroxyalkanoates (PHA). Under excess of carbon source (e.g. waste-derived VFA), certain bacteria (e.g. phosphorus accumulating organisms, PAO) can accumulate VFA and polymerise them intracellularly as reserve carbon molecules. PHA in mixed cultures can represent up to 60% of the biomass dry weight (up to 80% of the cell dry weight in case of pure cultures). (Albuquerque *et al*., 2010; Reis *et al*., 2003; Shen *et al*., 2014)
- Enhanced biological nutrient (N, P) removal in wastewaters. Indeed, denitrification and phosphorus removal in wastewater is dependent on a sufficient but not excessive availability of COD that can be readily assimilated (like VFA) by denitrifying and phosphorus removal bacteria. (Lim *et al*., 2000)
- Production of electricity through microbial fuel cells (MFC) or hydrogen through microbial electrolysis cell (MEC). Both are bioelectrochemical systems comprised of an anode, a cathode and a proton exchange membrane between them. The VFA stream is oxidized by dedicated bacteria (e.g. of the genus *Geobacter, Shewanella*) in the anode creating an electron and proton flow. Electrons flowing towards the cathode generate electricity (MFC) and end their way into the cathode where they can reduce the protons that passed through the membrane to yield hydrogen (MEC). (Freguia *et al*., 2010; Zhao *et al*., 2012a)

For further discussion on the variety of applications of volatile fatty acids, the reader is referred to the very good reviews of Bastidas-Oyanedel *et al*. (2015),

Jang *et al*. (2012), Lee *et al*. (2014a) and Singhania *et al*. (2013). Of note, the importance of VFA is not only limited to environmental and biobased applications: recent studies have highlighted the key role of VFA in health-related issues (Puddu *et al*., 2014; Vonk and Reckman, 2017).

1.1.6 Factors influencing acidogenic fermentation

A series of factors are influencing the performance of mixed acidogenic fermentation of organic waste fractions. Among them:

The substrate: Acidogenic fermentation has been studied for a variety of organic residue of low or high solids content (Rajagopal and Béline, 2011; Silva *et al*., 2013). Some authors have also tried to correlate the composition of the substrate with the product distribution. Indicatively, Chen *et al*. (2007), Ma *et al*. (2017) and Parawira *et al*. (2004) suggested that protein-rich substrates tend to favour odd carbon number VFA (propionate, valerate), while Parawira *et al*. (2004) suggested that carbohydrate-rich substrates tend to favour VFA with even carbon number (acetate, butyrate). Garcia-Aguirre *et al*. (2017) also noted that fermentation of proteinaceous substrates is more resilient to changes in operational parameters (pH, temperature) than that of carbohydrate-rich substrates. Inversely, Jankowska *et al*. (2017) claimed that variations in the composition of the substrate have only minor effect on mixed acidogenesis, when all other process parameters remain constant.

Substrate pre-treatment weakens the structure of recalcitrant substrates, like lignocellulosic materials, so that hydrolytic and acidogenic bacteria can access the digestible fractions more easily (Hendriks and Zeeman, 2009; Kandylis *et al*., 2016; Modenbach and Nokes, 2012). Different pre-treatment strategies have been tested to enhance acidogenic conversion, namely acid treatment, alkaline treatment, enzymatic treatment, sonication, hydrothermal treatment, steam explosion and combinations thereof (Espinoza-Escalante *et al*., 2008; Guo *et al*., 2011; Kim *et al*., 2005). While the majority of them leads indeed to an improvement of the acidogenic performance (i.e. product concentration and substrate conversion yield) at laboratory scale, it is important to consider the cost/benefit ratio of such a process for integrated biorefineries of larger scale.

The external inoculum: The addition of an external inoculum aims at increasing the hydrolytic and metabolic capacity of the inherent flora existing in the waste, thus improving its degradability (Forrest *et al*., 2012). Most commonly used external inocula are anaerobic sludge from wastewater anaerobic reactors, waste activated sludge and less often rumen fluid (Infantes *et al*., 2012; Jiang *et al*., 2013; Lim *et al*., 2000; Wang *et al*., 2014; Yin *et al*., 2016; Zhao *et al*., 2016). Often, the inoculum itself receives a pre-treatment that permits the selection of acidogenic species (Akutsu *et al*., 2009; Liu *et*

al., 2012). The feed-to-microorganism ratio (F/M) has been investigated by some authors and is a compromise between the need for sufficient digestible material that will be rapidly converted to VFA and sufficient hydrolytic/acidogenic microbial capacity to treat the substrate (Wang *et al*., 2012; Xu *et al*., 2012).

The pH: Due to its direct effect on microorganism specific growth rate, pH is one of the most influential parameters in acidogenic fermentation (Cheng *et al*., 2014; Horiuchi *et al*., 2002). The range of pH values examined in literature spans from 3 to 12. Very acidic pH is often reported in fermentations leading to lactic acid accumulation (Itoh *et al*., 2012), as this is the preferred pathway for easily digestible substrates because it allows rapid disposal of reducing equivalents (Agler *et al*., 2011). Most acidogenic studies are focusing on pH values from 5 to 8, which according to Batstone and Jensen (2011) is an optimal range for growth of acidogenic microorganisms. Nevertheless, several researchers have reported better acidogenic performance at more alkaline conditions (Chen *et al*., 2007; Lin *et al*., 2013). A way to inhibit methanogenesis is to avoid pH values of 7-8, which are considered optimal for methanogenic archaea (Batstone and Jensen, 2011).

The temperature: While the majority of acidogenic studies of organic waste have been conducted in the mesophilic range (20-45 °C), several authors have investigated the effect of different temperatures in the acidogenic conversion of organic waste. Indicatively, Jiang *et al*. (2013), Lee *et al*. (2014b) and Luo *et al*. (2014) reported higher VFA concentration at mesophilic (35-45 °C) compared to thermophilic (55 °C) or even psychrophilic (10 °C) conditions, irrespectively of the substrate's nature (palm oil mill effluent, food waste and waste activated sludge with rice, respectively). Nevertheless, other researchers pointed out a comparative advantage of thermophilic temperatures in acidogenic fermentation of organic residues, often wastewaters or slurries (Garcia-Aguirre *et al*., 2017; Kim *et al*., 2010; Yu and Fang, 2003).

Further influencing factors are the gas pressure and composition in the reactor's headspace (Arslan *et al*., 2012; de Kok *et al*., 2013), the hydraulic retention time (HRT) and organic loading rate (OLR) for (semi-)continuous systems (Argelier *et al*., 1998; Doğan and Demirer, 2009; Jiang *et al*., 2013; Rincón *et al*., 2008), the product concentration (Infantes *et al*., 2012; Xiao *et al*., 2016), the oxidation/reduction potential (ORP) of the fermentation broth (Yin *et al*., 2016) and the carbon-nitrogen (C/N) ratio (Smith and Holtzapple, 2011).

For a more detailed discussion on the effect of operating parameters, the reader is referred to the very good reviews of Arslan *et al*. (2016), Lee *et al*. (2014) and Zhou *et al*. (2018).

It is interesting to note that the large number of parameters influencing mixed fermentations and the sometimes divergent results presented by different authors render confident correlations between operational parameters and fermentation products a very challenging task. The reason of this challenge is the common denominator that is ultimately affected by all the aforementioned factors and is, in turn, determining the performance of acidogenic fermentation: the microbiota of the fermentation system. ***Deciphering how the diverse microbiota evolve during mixed acidogenic fermentation is a major milestone in mastering the process towards a target product***.

1.1.7 Microbial diversity

By definition, mixed fermentations are fermentations where the microbial communities responsible for the metabolic activity are very diverse, adding to the difficulty of controlling the fermentation output. All factors mentioned in chapter 1.1.5 affect, in the first line, the dynamics and evolution of the microbial consortia, which in turn have an effect on the product concentration and distribution (Figure 1-3): a change in an operational parameter may (i) induce an adaptive change in the metabolism of the same microorganism (e.g. acidogenic, solventogenic) leading to a different metabolic product, (ii) favour one group of microorganisms over others leading to the dominance of a different metabolic product, (iii) have a negligible effect on the microbial structure and dynamics (i.e. resistance), (iv) lead to a reversible change in the microbial structure (i.e. resilience) (iv) have a considerable effect on the microbial structure but no effect on the performance stability (i.e. functional redundancy) (Carballa *et al.*, 2015; Ye *et al*., 2007).

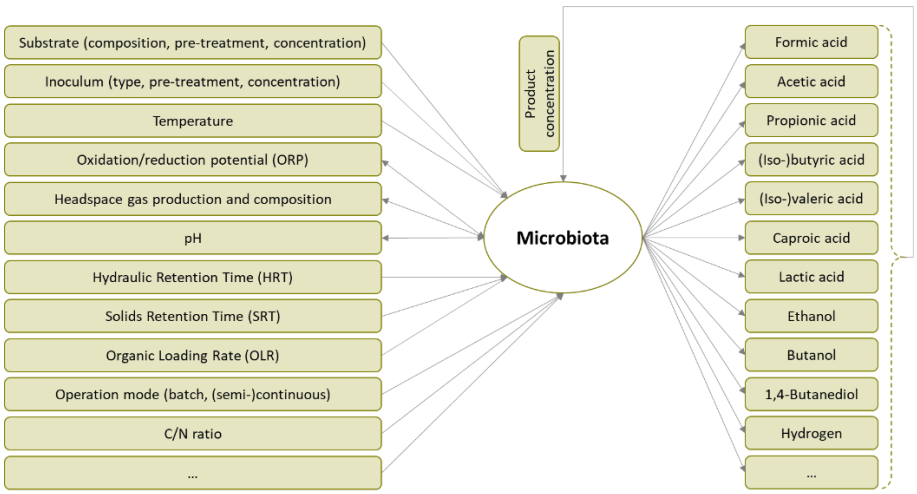

Figure 1-3: Relationship between operational parameters, microbiota and metabolic products

Several authors have tried to elucidate the wealth of the microbiota of acidogenic fermentation by employing standard and modern molecular biology techniques (Table 1-3). The number of microbial species involved can be overwhelming (see Table A.1-1 in Annex 1, corresponding to the references of Table 1-3). Of note, Table 1-3 and Table A.1-1 include only studies where VFA were explicitly mentioned as desired product. Other references of general appearance of acidogenic species (i.e. processes such as biohydrogen production by dark fermentation, or one-/two-stage anaerobic digesters) are, in principle, not included, with limited exceptions (e.g. Kim *et al*., 2011).

Table 1-3: Mixed acidogenic fermentation studies with a microbial analysis component

For a list of species reported in the studies, see Table A.1-1 in Annex 1; AGS: Anaerobic granular sludge; AS; Anaerobic sludge; C: Concentration; DGGE: Denaturing Gradient Gel Electrophoresis; ES: Excess sludge; FISH: Fluorescence In Situ Hybridization; FW: Food waste; HRT; Hydraulic Retention Time; MFC: Microbial fuel cells; OLR: Organic Loading Rate; PS: Pyrosequencing; SRT: Solids Retention Time; T: Temperature; TGGE: Temperature Gradient Gel Electrophoresis; TRFLP: Terminal Restriction Fragment Length Polymorphism; WAS: Waste activated sludge; WW: Wastewater

Source	Substrate	Inoculum	Technique	Focus
1	FW and ES	WAS	Illumina	pH
2	WAS	-	Illumina	pH
3	Different WW	AS	DGGE	Start-up
4	Rice straw	AS	PS	T, pH, C
5	FW	-	DGGE	pH
6	FW-recycling WW	Anaerobic sludge	DGGE	HRT, pH, T
7	Alginate	AS	DGGE	pH, C
8	Swine WW	AS	DGGE	T, HRT
9	Cheese whey WW	Rumen	FISH, TGGE, Illumina, Cloning	Microbiome
10	Microalgae	AS	DGGE	T
11	WAS	-	FISH	Substrate
12	Wheat straw	Rumen	Illumina	Enzymatic activity
13	Molasses WW	AS	Ion Torrent	OLR
14	Recycled office paper	Soil samples & pure culture	PS	Inoculum
15	Sorghum	Marine sediment	PS	T
16	Sorghum	Marine	PS	Scale-up

		sediment		
17	S. Japonica	AS	PS	T, methane inhibition
18	WAS	-	FISH, PS	pH
19	WAS and corn stover	-	FISH, PS	Substrate
20	WAS	-	Illumina	pH
21	WAS, potato peel waste, FW	WAS	Illumina	Substrate
22	Tofu & egg white (commercial)	AGS	Illumina	Substrate
23	FW and ES	-	PS	T, substrate, SRT, OLR
24	Thin stillage	-	Illumina	Product recovery
25	Excess sludge	-	DGGE	pH
26	WAS	-	Illumina	T
27	Different WW glycerol, ES	Anaerobic sludge	PS	Substrate
28	Swine manure	-	DGGE	Initial pH
29	Proteinaceous sewage sludge	Sewage sludge	TRFLP	pH
30	FW	WAS, AGS	Illumina	ORP, inoculum
31	WAS	-	Cloning	pH, kinetics
32	Sewage sludge	AS	PS	pH
33	Cattle dung	AS	DGGE	MFC
34	Maize silage	Anaerobic percolation liquid	TRFLP	Microbial dynamics

1: Cheng et al. (2014); 2: Yuan et al. (2015); 3: Kim et al. (2011); 4: Park et al. (2015); 5: Ye et al. (2007); 6: Han et al. (2016); 7: Seon et al. (2014); 8: Kim et al. (2013); 9: Ntougias et al. (2015); 10: Cho et al. (2015); 11: Zhang et al. (2016); 12: Lazuka et al. (2015); 13: Yun and Cho (2016); 14: Forrest et al. (2012); 15: Hollister et al. (2010); 16: Hollister et al. (2011); 17: Jung et al. (2015); 18: Zheng et al. (2013); 19: Zhou et al. (2016); 20: Chen et al. (2017); 21: Ma et al. (2017); 22: Shen et al. (2017); 23: Wu et al. (2016); 24: Andersen et al. (2015); 25: Jie et al. (2014); 26: Yuan et al. (2016); 27: Shen et al. (2014); 28: Lin et al. (2013); 29: Liu et al. (2012); 30: Yin et al. (2016); 31: Zhang et al. (2010); 32: Maspolim et al. (2015); 33: Zhao et al. (2012); 34: Sträuber et al. (2012)

Generally, the authors of the abovementioned studies converge to the conclusion that microbial analysis is essential to dissect the complexity of the fermentation system. Nevertheless, the microbial diversity is so high that this

task is very challenging. The challenge is all the more increasing since we only have knowledge of a small fraction of the existing bacterial diversity in engineered environments like anaerobic reactors (Briones and Raskin, 2003; Nelson *et al*., 2011). With the development of culture-independent techniques, it has become clear that the role of unclassified, uncultured or unknown bacterial species is very important. Indeed, in the abovementioned studies, an important share (often more than 50%) of entries reported belonged to unclassified, uncultured or unknown bacteria. Furthermore, only few of the studies mentioned in Table 1-3 have tried to correlate the microbial species identified with the metabolic product observed. ***The lack of direct & robust correlations between microbial species and metabolic products in mixed systems remains a scientific challenge***. Given the variety and complexity the fermentation system and the high bacterial diversity, every scientific contribution in building a systematic overview of species-product linkages is essential.

1.2 Objectives and research questions

This thesis is situated within the following research frame: on the one hand, the need for sustainable exploitation of agroindustrial residues, resources with significant potential due to their organic content, their large production volumes and their low cost; on the other hand, the possibility to achieve this through mixed acidogenic fermentation, a process with significant potential of producing a multitude of marketable products, but also with open research challenges before scale-up.

This thesis, therefore, aims at contributing to the knowledge on mixed acidogenic fermentation of complex substrates in order to improve its control towards selective carboxylate production. It does so by providing input to answer the following research questions:

<u>Appropriateness of mixed acidogenic fermentation in biorefining concepts</u>

1. Can mixed acidogenic fermentation efficiently treat complex agroindustrial waste?
    a. What level of **product concentrations and substrate conversion yields** can be achieved and under what conditions?
    b. What are the **predominant products** and how **reproducible** is the process?
    c. Are residues with a **high lignocellulosic content** an appropriate substrate for this process?
2. Is the typically used biomethane potential of agroindustrial waste a representative assessment of their **acidogenic** potential? Why could there be a difference?

3. Is there a **residue that is particularly promising** for mixed fermentation as a result of its properties and its fermentation profile?

System complexity and microbial diversity

4. Is the addition of a **mixed external inoculum** useful to improve the fermentation performance?
5. How **unique** is in terms of metabolic performance a given combination between substrate-inoculum-experimental conditions? What could this uniqueness be attributed to?
6. Can a robust and direct **link** between the metabolic products and microbial species be established?

1.3 Methodology outline

To answer to the research questions, this thesis combined batch fermentations of various agroindustrial residues, with or without the addition of external inocula, complemented by metabolic and microbial analysis of the fermentation system. Some limited resources have also been dedicated to semi-continuous operation of the most promising substrate suggested from batch operation.

Analysis of gaseous and soluble fermentation metabolites like VFA, lactic acid, ethanol, hydrogen, carbon dioxide was conducted by Gas Chromatography (GC) and High-Performance Liquid Chromatography (HPLC).

Analysis of the microbial communities was conducted by DNA extraction, DNA amplification through the Polymerase Chain Reaction (PCR), culture independent techniques like Denaturing Gradient Gel Electrophoresis (DGGE) or next generation sequencing (Illumina) as well as standard bacterial cultures.

1.4 Structure

This thesis follows a funnel structure from broader to more specific aspects of mixed acidogenic fermentation. Besides the introduction, each chapter of the thesis together with the annexes provide elements to answer the research questions set in section 1.2.

Chapter 1 briefly introduced the current European Bioeconomy context, in which this thesis is situated. The description of the theoretical background of mixed acidogenic fermentation was followed by the description of the current scientific state-of the-art and the challenges therein, i.e. the need to decipher the complexity of the fermentation system. This description set the frame for the specific objectives of this thesis that were presented in the form of research

questions.

Chapter 2 deals with the overall pertinence of acidogenic fermentation as a stand-alone process compared to standard anaerobic digestion. It does so through a systematic comparison of the acidogenic and methanogenic potential (defined as the yield of conversion of the substrate to the respective product, i.e. VFA or methane) of different agroindustrial residues. *(contribution to research questions 1, 2, 3, 4)*

Chapter 3 deals with a more specific biorefinery scenario, where acidogenic fermentation is employed as part of an integrated scheme of substrate fractionation and conversion that aims to valorise the largest possible fraction of lignocellulosic substrates. *(contribution to research questions 1, 4)*

Chapter 4 goes further into the detailed analysis of the influence of two basic parameters of acidogenic fermentation: the inoculum origin and the substrate type. It determines how unique, from a statistical point of view, nine substrate/inoculum combinations are in terms of acidogenic performance. *(contribution to research questions 1, 5)*

Chapter 5 attributes this uniqueness to the microbiota in the fermentation system and uses a combined approach of microbial and metabolic analysis to investigate the complexity of mixed fermentation of apple pulp. *(contribution to research questions 4, 5, 6)*

Chapter 6 investigates the capacity of mixed fermentations to yield lactic acid from the, otherwise inhibiting for lactic acid bacteria, beer hot trub. It employs the same combined metabolic and microbial approach as chapter 5. *(contribution to research questions 1, 3, 4, 6)*

Chapter 7 presents a synthetic discussion of the results and perspectives for further work on the basis of the research questions set.

Annex 1 - “Acidogenic bacteria reported in mixed acidogenic fermentation studies” and Annex 2 - “Metabolic pathways of short chain carboxylic acids” complement the fundamental information that is provided in Chapter 1.

Annex 3 - “Preliminary results of metagenomic approach” presents work in progress on the metabolic and microbial analysis of acidogenic fermentation of chicory roots and the effect of pH and initial COD concentration on the microbial evolution and acidogenic performance. (*contribution to research questions 1, 6).*

Annex 4 - “Preliminary results of semi-continuous experiments” presents work in progress on semi-continuous operation of acidogenic fermentation of hot trub. *(contribution to research questions 1, 6).*

Annex 3 and Annex 4 both contribute to the synthetic discussion of Chapter

7, but are placed in the annex because the degree of maturity of data analysis and interpretation as well as manuscript preparation is lower compared to Chapters 2-6.

1.5 References

Agler, M.T., Wrenn, B.A., Zinder, S.H., Angenent, L.T., 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends Biotechnol. 29, 70–78. https://doi.org/10.1016/j.tibtech.2010.11.006

Akutsu, Y., Lee, D.-Y., Li, Y.-Y., Noike, T., 2009. Hydrogen production potentials and fermentative characteristics of various substrates with different heat-pretreated natural microflora. International Journal of Hydrogen Energy 34, 5365–5372. https://doi.org/10.1016/j.ijhydene.2009.04.052

Albuquerque, M.G.E., Concas, S., Bengtsson, S., Reis, M.A.M., 2010. Mixed culture polyhydroxyalkanoates production from sugar molasses: The use of a 2-stage CSTR system for culture selection. Bioresource Technology 101, 7112–7122. https://doi.org/10.1016/j.biortech.2010.04.019

Alkaya, E., Demirer, G.N., 2011. Anaerobic acidification of sugar-beet processing wastes: Effect of operational parameters. Biomass and Bioenergy 35, 32–39. https://doi.org/10.1016/j.biombioe.2010.08.002

Andersen, S.J., Hennebel, T., Gildemyn, S., Coma, M., Desloover, J., Berton, J., Tsukamoto, J., Stevens, C., Rabaey, K., 2014. Electrolytic Membrane Extraction Enables Production of Fine Chemicals from Biorefinery Sidestreams. Environ. Sci. Technol. 48, 7135–7142. https://doi.org/10.1021/es500483w

Andersen, S.J., Candry, P., Basadre, T., Khor, W.C., Roume, H., Hernandez-Sanabria, E., Coma, M., Rabaey, K., 2015. Electrolytic extraction drives volatile fatty acid chain elongation through lactic acid and replaces chemical pH control in thin stillage fermentation. Biotechnology for Biofuels 8. https://doi.org/10.1186/s13068-015-0396-7

Angelidaki, I., Karakashev, D., Batstone, D.J., Plugge, C.M., Stams, A.J.M., 2011. Biomethanation and its potential. Meth. Enzymol. 494, 327–351. https://doi.org/10.1016/B978-0-12-385112-3.00016-0

Argelier, S., Delgenes, J.-P., Moletta, R., 1998. Design of acidogenic reactors for the anaerobic treatment of the organic fraction of solid food waste. Bioprocess Engineering 18, 309–315. https://doi.org/10.1007/s004490050448

Arslan, D., Steinbusch, K.J.J., Diels, L., De Wever, H., Buisman, C.J.N., Hamelers, H.V.M., 2012. Effect of hydrogen and carbon dioxide on carboxylic acids patterns in mixed culture fermentation. Bioresource Technology 118, 227–234. https://doi.org/10.1016/j.biortech.2012.05.003

Arslan, D., Steinbusch, K.J.J., Diels, L., Hamelers, H.V.M., Strik, D.P.B.T.B., Buisman, C.J.N., De Wever, H., 2016. Selective short-chain carboxylates production: A review of control mechanisms to direct mixed culture fermentations. Critical Reviews in Environmental Science and Technology 46, 592–634. https://doi.org/10.1080/10643389.2016.1145959

Arslan, D., Zhang, Y., Steinbusch, K.J.J., Diels, L., Hamelers, H.V.M., Buisman, C.J.N., De Wever, H., 2017. In-situ carboxylate recovery and simultaneous pH control with tailor-configured bipolar membrane electrodialysis during continuous mixed culture fermentation. Separation and Purification Technology 175, 27–35. https://doi.org/10.1016/j.seppur.2016.11.032

Bastidas-Oyanedel, J.-R., Bonk, F., Thomsen, M.H., Schmidt, J.E., 2015. Dark fermentation biorefinery in the present and future (bio)chemical industry. Reviews in Environmental Science and Bio/Technology 14, 473–498. https://doi.org/10.1007/s11157-015-9369-3

Batstone, D.J., Jensen, P.D., 2011. Anaerobic Processes, in: Wilderer, P. (Ed.), Treatise on Water Science. Elsevier, Oxford, pp. 615–639. https://doi.org/10.1016/B978-0-444-53199-5.00097-X

Bouallagui, H., Torrijos, M., Godon, J.J., Moletta, R., Ben Cheikh, R., Touhami, Y., Delgenes, J.P., Hamdi, M., 2004. Two-phases anaerobic digestion of fruit and vegetable wastes: bioreactors performance. Biochemical Engineering Journal 21, 193–197. https://doi.org/10.1016/j.bej.2004.05.001

Briones, A., Raskin, L., 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Current Opinion in Biotechnology 14, 270–276. https://doi.org/10.1016/S0958-1669(03)00065-X

Cabrera-Rodríguez, C.I., van der Wielen, L.A.M., Straathof, A.J.J., 2015. Separation and Catalysis of Carboxylates: Byproduct Reduction during the Alkylation with Dimethyl Carbonate. Industrial & Engineering Chemistry Research 54, 10964-10973. https://doi.org/10.1021/acs.iecr.5b02911

Capson-Tojo, G., Rouez, M., Crest, M., Steyer, J.-P., Delgenès, J.-P., Escudié, R., 2016. Food waste valorization via anaerobic processes: a review. Reviews in Environmental Science and Bio/Technology 15, 499–547. https://doi.org/10.1007/s11157-016-9405-y

Carballa, M., Regueiro, L., Lema, J.M., 2015. Microbial management of anaerobic digestion: exploiting the microbiome-functionality nexus. Current Opinion in Biotechnology 33, 103-111. http://dx.doi.org/10.1016/j.copbio.2015.01.008

Chang, H.N., Kim, N.-J., Kang, J., Jeong, C.M., 2010. Biomass-derived volatile fatty acid platform for fuels and chemicals. Biotechnol. Bioproc. Eng. 15, 1–10. https://doi.org/10.1007/s12257-009-3070-8

Chen, Y., Jiang, S., Yuan, H., Zhou, Q., Gu, G., 2007. Hydrolysis and acidification of waste activated sludge at different pHs. Water Research 41, 683–689. https://doi.org/10.1016/j.watres.2006.07.030

Chen, Y., Jiang, X., Xiao, K., Shen, N., Zeng, R.J., Zhou, Y., 2017. Enhanced volatile fatty acids (VFAs) production in a thermophilic fermenter with stepwise pH increase – Investigation on dissolved organic matter transformation and microbial community shift. Water Research 112, 261–268. https://doi.org/10.1016/j.watres.2017.01.067

Cheng, W., Chen, H., Yan, S., Su, J., 2014. Illumina sequencing-based analyses of bacterial communities during short-chain fatty-acid production from food waste

and sewage sludge fermentation at different pH values. World Journal of Microbiology and Biotechnology 30, 2387–2395. https://doi.org/10.1007/s11274-014-1664-6

Cherubini, F., Jungmeier, G., Wellisch, M., Willke, T., Skiadas, I., Van Ree, R., de Jong, E., 2009. Toward a common classification approach for biorefinery systems. Biofuels, Bioproducts and Biorefining 3, 534–546. https://doi.org/10.1002/bbb.172

Cho, H.U., Kim, Y.M., Choi, Y.-N., Kim, H.G., Park, J.M., 2015. Influence of temperature on volatile fatty acid production and microbial community structure during anaerobic fermentation of microalgae. Bioresource Technology 191, 475–480. https://doi.org/10.1016/j.biortech.2015.03.009

Dahiya, S., Kumar, A.N., Shanthi Sravan, J., Chatterjee, S., Sarkar, O., Mohan, S.V., 2018. Food waste biorefinery: Sustainable strategy for circular bioeconomy. Bioresource Technology 248, 2–12. https://doi.org/10.1016/j.biortech.2017.07.176

de Kok, S., Meijer, J., van Loosdrecht, M.C.M., Kleerebezem, R., 2013. Impact of dissolved hydrogen partial pressure on mixed culture fermentations. Appl. Microbiol. Biotechnol. 97, 2617–2625. https://doi.org/10.1007/s00253-012-4400-x

Demirbas, A., 2010. Fuels from Biomass. In: Biorefineries. Springer London, London, pp. 33–73. https://doi.org/10.1007/978-1-84882-721-9_2

Doğan, E., Demirer, G.N., 2009. Volatile Fatty Acid Production from Organic Fraction of Municipal Solid Waste Through Anaerobic Acidogenic Digestion. Environmental Engineering Science 26, 1443–1450. https://doi.org/10.1089/ees.2009.0062

Espinoza-Escalante, F.M., Pelayo-Ortiz, C., Gutiérrez-Pulido, H., González-Alvarez, V., Alcaraz-González, V., Bories, A., 2008. Multiple response optimization analysis for pretreatments of Tequila's stillages for VFAs and hydrogen production. Bioresour. Technol. 99, 5822–5829. https://doi.org/10.1016/j.biortech.2007.10.008

Fava, F., Totaro, G., Diels, L., Reis, M., Duarte, J., Carioca, O.B., Poggi-Varaldo, H.M., Ferreira, B.S., 2015. Biowaste biorefinery in Europe: opportunities and research & development needs. N Biotechnol 32, 100–108. https://doi.org/10.1016/j.nbt.2013.11.003

Federici, F., Fava, F., Kalogerakis, N., Mantzavinos, D., 2009. Valorisation of agro-industrial by-products, effluents and waste: concept, opportunities and the case of olive mill wastewaters. J. Chem. Technol. Biotechnol. 84, 895–900. https://doi.org/10.1002/jctb.2165

FitzPatrick, M., Champagne, P., Cunningham, M.F., Whitney, R.A., 2010. A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products. Bioresource Technology 101, 8915–8922. https://doi.org/10.1016/j.biortech.2010.06.125

Forrest, A.K., Hollister, E.B., Gentry, T.J., Wilkinson, H.H., Holtzapple, M.T., 2012. Comparison of mixed-acid fermentations inoculated with six different mixed

cultures. Bioresource Technology 118, 343–349. https://doi.org/10.1016/j.biortech.2012.05.043

Freguia, S., Teh, E.H., Boon, N., Leung, K.M., Keller, J., Rabaey, K., 2010. Microbial fuel cells operating on mixed fatty acids. Bioresource Technology 101, 1233–1238. https://doi.org/10.1016/j.biortech.2009.09.054

Galanakis, C.M., 2012. Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. Trends in Food Science & Technology 26, 68–87. https://doi.org/10.1016/j.tifs.2012.03.003

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

Guo, P., Mochidzuki, K., Cheng, W., Zhou, M., Gao, H., Zheng, D., Wang, X., Cui, Z., 2011. Effects of different pretreatment strategies on corn stalk acidogenic fermentation using a microbial consortium. Bioresour. Technol. 102, 7526–7531. https://doi.org/10.1016/j.biortech.2011.04.083

Han, G., Shin, S.G., Lee, J., Lee, C., Jo, M., Hwang, S., 2016. Mesophilic Acidogenesis of Food Waste-Recycling Wastewater: Effects of Hydraulic Retention Time, pH, and Temperature. Applied Biochemistry and Biotechnology 180, 980–999. https://doi.org/10.1007/s12010-016-2147-z

Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology 100, 10–18. https://doi.org/10.1016/j.biortech.2008.05.027

Hollister, E.B., Forrest, A.K., Wilkinson, H.H., Ebbole, D.J., Malfatti, S.A., Tringe, S.G., Holtzapple, M.T., Gentry, T.J., 2010. Structure and dynamics of the microbial communities underlying the carboxylate platform for biofuel production. Applied Microbiology and Biotechnology 88, 389–399. https://doi.org/10.1007/s00253-010-2789-7

Hollister, E.B., Hammett, A.M., Holtzapple, M.T., Gentry, T.J., Wilkinson, H.H., 2011. Microbial community composition and dynamics in a semi-industrial-scale facility operating under the MixAlco™ bioconversion platform. Journal of Applied Microbiology 110, 587–596. https://doi.org/10.1111/j.1365-2672.2010.04919.x

Holtzapple, M., Lonkar, S., Granda, C., 2015. Producing Biofuels via the Carboxylate Platform. Chemical Engineering Progress 111, 3, 52-57.

Horiuchi, J.-I., Shimizu, T., Tada, K., Kanno, T., Kobayashi, M., 2002. Selective production of organic acids in anaerobic acid reactor by pH control. Bioresource Technology 82, 209–213. https://doi.org/10.1016/S0960-8524(01)00195-X

Infantes, D., González del Campo, A., Villaseñor, J., Fernández, F.J., 2012. Kinetic model and study of the influence of pH, temperature and undissociated acids on acidogenic fermentation. Biochemical Engineering Journal 66, 66–72. https://doi.org/10.1016/j.bej.2012.04.017

Itoh, Y., Tada, K., Kanno, T., Horiuchi, J.-I., 2012. Selective production of lactic acid in continuous anaerobic acidogenesis by extremely low pH operation. J. Biosci.

Bioeng. 114, 537–539. https://doi.org/10.1016/j.jbiosc.2012.05.020

Jang, Y.-S., Kim, B., Shin, J.H., Choi, Y.J., Choi, S., Song, C.W., Lee, J., Park, H.G., Lee, S.Y., 2012. Bio-based production of C2-C6 platform chemicals. Biotechnol. Bioeng. 109, 2437–2459. https://doi.org/10.1002/bit.24599

Jankowska, E., Chwialkowska, J., Stodolny, M., Oleskowicz-Popiel, P., 2017. Volatile fatty acids production during mixed culture fermentation – The impact of substrate complexity and pH. Chemical Engineering Journal 326, 901–910. https://doi.org/10.1016/j.cej.2017.06.021

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

Jie, W., Peng, Y., Ren, N., Li, B., 2014. Volatile fatty acids (VFAs) accumulation and microbial community structure of excess sludge (ES) at different pHs. Bioresource Technology 152, 124–129. https://doi.org/10.1016/j.biortech.2013.11.011

Johnravindar, D., Karthikeyan, O.P., Selvam, A., Murugesan, K., Wong, J.W.C., 2017. Lipid accumulation potential of oleaginous yeasts: A comparative evaluation using food waste leachate as a substrate. Bioresource Technology. https://doi.org/10.1016/j.biortech.2017.06.151

Jung, K., Kim, W., Park, G.W., Seo, C., Chang, H.N., Kim, Y.-C., 2015. Optimization of volatile fatty acids and hydrogen production from Saccharina japonica: acidogenesis and molecular analysis of the resulting microbial communities. Applied Microbiology and Biotechnology 99, 3327–3337. https://doi.org/10.1007/s00253-015-6419-2

Kandylis, P., Bekatorou, A., Pissaridi, K., Lappa, K., Dima, A., Kanellaki, M., Koutinas, A.A., 2016. Acidogenesis of cellulosic hydrolysates for new generation biofuels. Biomass and Bioenergy 91, 210–216. https://doi.org/10.1016/j.biombioe.2016.05.006

Kaparaju, P., Serrano, M., Thomsen, A.B., Kongjan, P., Angelidaki, I., 2009. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresour. Technol. 100, 2562–2568. https://doi.org/10.1016/j.biortech.2008.11.011

Kaur, S., Dhillon, G.S., Sarma, S.J., Brar, S.K., Misra, K., Oberoi, H.S., 2014. Waste Biomass: A Prospective Renewable Resource for Development of Bio-Based Economy/Processes, in: Brar, S.K., Dhillon, G.S., Soccol, C.R. (Eds.), Biotransformation of Waste Biomass into High Value Biochemicals. Springer New York, New York, NY, pp. 3–28. https://doi.org/10.1007/978-1-4614-8005-1_1

Kim, W., Yahaya, M. s., Goto, M., 2005. Effects of steam explosion on the chemical composition and rumen degradability of rice (Oryza sativa L.) straw. Grassland Science 51, 139–144. https://doi.org/10.1111/j.1744-697X.2005.00019.x

Kim, W., Hwang, K., Shin, S.G., Lee, S., Hwang, S., 2010. Effect of high temperature on bacterial community dynamics in anaerobic acidogenesis using mesophilic sludge inoculum. Bioresour. Technol. 101 Suppl 1, S17-22.

https://doi.org/10.1016/j.biortech.2009.03.029

Kim, J., Shin, S.G., Han, G., O'Flaherty, V., Lee, C., Hwang, S., 2011. Common key acidogen populations in anaerobic reactors treating different wastewaters: Molecular identification and quantitative monitoring. Water Research 45, 2539–2549. https://doi.org/10.1016/j.watres.2011.02.004

Kim, W., Shin, S.G., Lim, J., Hwang, S., 2013. Effect of temperature and hydraulic retention time on volatile fatty acid production based on bacterial community structure in anaerobic acidogenesis using swine wastewater. Bioprocess and Biosystems Engineering 36, 791–798. https://doi.org/10.1007/s00449-013-0905-7

Kleerebezem, R., van Loosdrecht, M.C., 2007. Mixed culture biotechnology for bioenergy production. Current Opinion in Biotechnology 18, 207–212. https://doi.org/10.1016/j.copbio.2007.05.001

Kucek, L.A., Xu, J., Nguyen, M., Angenent, L.T., 2016. Waste Conversion into n-Caprylate and n-Caproate: Resource Recovery from Wine Lees Using Anaerobic Reactor Microbiomes and In-line Extraction. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01892

Lange, J.-P., Price, R., Ayoub, P.M., Louis, J., Petrus, L., Clarke, L., Gosselink, H., 2010. Valeric Biofuels: A Platform of Cellulosic Transportation Fuels. Angewandte Chemie International Edition 49, 4479–4483. https://doi.org/10.1002/anie.201000655

Layton, D.S., Trinh, C.T., 2016. Expanding the modular ester fermentative pathways for combinatorial biosynthesis of esters from volatile organic acids. Biotechnol. Bioeng. 113, 1764–1776. https://doi.org/10.1002/bit.25947

Lazaro, C.Z., Vich, D.V., Hirasawa, J.S., Varesche, M.B.A., 2012. Hydrogen production and consumption of organic acids by a phototrophic microbial consortium. International Journal of Hydrogen Energy 37, 11691–11700. https://doi.org/10.1016/j.ijhydene.2012.05.088

Lazuka, A., Auer, L., Bozonnet, S., Morgavi, D.P., O'Donohue, M., Hernandez-Raquet, G., 2015. Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium. Bioresource Technology 196, 241–249. https://doi.org/10.1016/j.biortech.2015.07.084

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

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014b. Influence of temperature on the bioconversion of palm oil mill effluent into volatile fatty acids as precursor to the production of polyhydroxyalkanoates. J. Chem. Technol. Biotechnol. 89, 1038–1043. https://doi.org/10.1002/jctb.4197

Liguori, R., Amore, A., Faraco, V., 2013. Waste valorization by biotechnological conversion into added value products. Appl. Microbiol. Biotechnol. 97, 6129–6147. https://doi.org/10.1007/s00253-013-5014-7

Lim, S.-J., Choi, D.W., Lee, W.G., Kwon, S., Chang, H.N., 2000. Volatile fatty acids production from food wastes and its application to biological nutrient removal.

Bioprocess Engineering 22, 543–545. https://doi.org/10.1007/s004499900109

Lin, L., Wan, C., Liu, X., Lee, D.-J., Lei, Z., Zhang, Y., Tay, J.H., 2013. Effect of initial pH on mesophilic hydrolysis and acidification of swine manure. Bioresource Technology 136, 302–308. https://doi.org/10.1016/j.biortech.2013.02.106

Liu, H., Wang, J., Liu, X., Fu, B., Chen, J., Yu, H.-Q., 2012. Acidogenic fermentation of proteinaceous sewage sludge: Effect of pH. Water Research 46, 799–807. https://doi.org/10.1016/j.watres.2011.11.047

Liu, J., Yuan, M., Liu, J.-N., Lu, L.-J., Peng, K.-M., Huang, X.-F., 2016. Microbial conversion of mixed volatile fatty acids into microbial lipids by sequencing batch culture strategy. Bioresource Technology 222, 75–81. https://doi.org/10.1016/j.biortech.2016.09.100

López-Garzón, C.S., Straathof, A.J.J., 2014. Recovery of carboxylic acids produced by fermentation. Biotechnology Advances 32, 873–904. https://doi.org/10.1016/j.biotechadv.2014.04.002

Luo, J., Feng, L., Zhang, W., Li, X., Chen, H., Wang, D., Chen, Y., 2014. Improved production of short-chain fatty acids from waste activated sludge driven by carbohydrate addition in continuous-flow reactors: Influence of SRT and temperature. Applied Energy 113, 51–58. https://doi.org/10.1016/j.apenergy.2013.07.006

Ma, H., Liu, He, Zhang, L., Yang, M., Fu, B., Liu, Hongbo, 2017. Novel insight into the relationship between organic substrate composition and volatile fatty acids distribution in acidogenic co-fermentation. Biotechnology for Biofuels 10, 137. https://doi.org/10.1186/s13068-017-0821-1

Maspolim, Y., Zhou, Y., Guo, C., Xiao, K., Ng, W.J., 2015. The effect of pH on solubilization of organic matter and microbial community structures in sludge fermentation. Bioresource Technology 190, 289–298. https://doi.org/10.1016/j.biortech.2015.04.087

Mata-Alvarez, J., Macé, S., Llabrés, P., 2000. Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. Bioresource Technology 74, 3–16. https://doi.org/10.1016/S0960-8524(00)00023-7

Mirabella, N., Castellani, V., Sala, S., 2014. Current options for the valorization of food manufacturing waste: a review. Journal of Cleaner Production 65, 28–41. https://doi.org/10.1016/j.jclepro.2013.10.051

Modenbach, A.A., Nokes, S.E., 2012. The use of high-solids loadings in biomass pretreatment—a review. Biotechnol. Bioeng. 109, 1430–1442. https://doi.org/10.1002/bit.24464

Mueller-Langer, F., Majer, S., Perimenis, A., 2018. Biofuels: A Technical, Economic, and Environmental Comparison, in: Meyers, D.R.A. (Ed.), Encyclopedia of Sustainability Science and Technology. Springer New York, pp. 1–30. https://doi.org/10.1007/978-1-4939-2493-6_257-3

Nelson, M.C., Morrison, M., Yu, Z., 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. 102, 3730–3739. https://doi.org/10.1016/j.biortech.2010.11.119

Nieto, P.P., Hidalgo, D., Irusta, R., Kraut, D., 2012. Biochemical methane potential (BMP) of agro-food wastes from the Cider Region (Spain). Water Sci. Technol. 66, 1842–1848. https://doi.org/10.2166/wst.2012.372

Nigam, P.S. nee', Gupta, N., Anthwal, A., 2009. Pre-treatment of Agro-Industrial Residues, in: Nigam, P.S. nee', Pandey, A., (Eds), Biotechnology for Agro-Industrial Residues Utilisation. Springer, Dordrecht, pp. 13–33. https://doi.org/10.1007/978-1-4020-9942-7_2

Ntougias, S., Tsiamis, G., Soultani, D., Melidis, P., 2015. Dominance of rumen microorganisms during cheese whey acidification: acidogenesis can be governed by a rare Selenomonas lacticifex-type fermentation. Applied Microbiology and Biotechnology 99, 9309–9318. https://doi.org/10.1007/s00253-015-6827-3

Papanikolaou, S., Aggelis, G., 2011. Lipids of oleaginous yeasts. Part II: Technology and potential applications. European Jounral of Lipid Science Technology 113, 8, 1052-1073, https://doi.org/ 10.1002/ejlt.201100015

Parawira, W., Murto, M., Read, J.S., Mattiasson, B., 2004. Volatile fatty acid production during anaerobic mesophilic digestion of solid potato waste. J. Chem. Technol. Biotechnol. 79, 673–677. https://doi.org/10.1002/jctb.1012

Paritosh, K., Kushwaha, S.K., Yadav, M., Pareek, N., Chawade, A., Vivekanand, V., 2017. Food Waste to Energy: An Overview of Sustainable Approaches for Food Waste Management and Nutrient Recycling. BioMed Research International 2017, 1–19. https://doi.org/10.1155/2017/2370927

Park, G.W., Seo, C., Jung, K., Chang, H.N., Kim, W., Kim, Y.-C., 2015. A comprehensive study on volatile fatty acids production from rice straw coupled with microbial community analysis. Bioprocess and Biosystems Engineering 38, 1157–1166. https://doi.org/10.1007/s00449-015-1357-z

Puddu, A., Sanguineti, R., Montecucco, F., Viviani, G.L., 2014. Evidence for the gut microbiota short-chain fatty acids as key pathophysiological molecules improving diabetes. Mediators Inflamm. 2014, 162021. https://doi.org/10.1155/2014/162021

Rajagopal, R., Béline, F., 2011. Anaerobic hydrolysis and acidification of organic substrates: determination of anaerobic hydrolytic potential. Bioresour. Technol. 102, 5653–5658. https://doi.org/10.1016/j.biortech.2011.02.068

Ravindran, R., Jaiswal, A.K., 2016. Exploitation of Food Industry Waste for High-Value Products. Trends in Biotechnology 34, 58–69. https://doi.org/10.1016/j.tibtech.2015.10.008

Reis, M. a. M., Serafim, L.S., Lemos, P.C., Ramos, A.M., Aguiar, F.R., Loosdrecht, M.C.M.V., 2003. Production of polyhydroxyalkanoates by mixed microbial cultures. Bioprocess Biosyst Eng 25, 377–385. https://doi.org/10.1007/s00449-003-0322-4

Rincón, B., Sánchez, E., Raposo, F., Borja, R., Travieso, L., Martín, M.A., Martín, A., 2008. Effect of the organic loading rate on the performance of anaerobic acidogenic fermentation of two-phase olive mill solid residue. Waste Management 28, 870–877. https://doi.org/10.1016/j.wasman.2007.02.030

Sakurai, H., Masukawa, H., Kitashima, M., Inoue, K., 2013. Photobiological hydrogen production: Bioenergetics and challenges for its practical application.

Journal of Photochemistry and Photobiology C: Photochemistry Reviews 17, 1–25. https://doi.org/10.1016/j.jphotochemrev.2013.05.001

Sarma, S.J., Pachapur, V., Brar, S.K., Le Bihan, Y., Buelna, G., 2015. Hydrogen biorefinery: Potential utilization of the liquid waste from fermentative hydrogen production. Renewable and Sustainable Energy Reviews 50, 942–951. https://doi.org/10.1016/j.rser.2015.04.191

Sawatdeenarunat, C., Nguyen, D., Surendra, K.C., Shrestha, S., Rajendran, K., Oechsner, H., Xie, L., Khanal, S.K., 2016. Anaerobic biorefinery: Current status, challenges, and opportunities. Bioresource Technology 215, 304–313. https://doi.org/10.1016/j.biortech.2016.03.074

Seon, J., Lee, T., Lee, S.C., Pham, H.D., Woo, H.C., Song, M., 2014. Bacterial community structure in maximum volatile fatty acids production from alginate in acidogenesis. Bioresource Technology 157, 22–27. https://doi.org/10.1016/j.biortech.2014.01.072

Shen, L., Hu, H., Ji, H., Cai, J., He, N., Li, Q., Wang, Y., 2014. Production of poly(hydroxybutyrate–hydroxyvalerate) from waste organics by the two-stage process: Focus on the intermediate volatile fatty acids. Bioresource Technology 166, 194–200. https://doi.org/10.1016/j.biortech.2014.05.038

Shen, D., Yin, J., Yu, X., Wang, M., Long, Y., Shentu, J., Chen, T., 2017. Acidogenic fermentation characteristics of different types of protein-rich substrates in food waste to produce volatile fatty acids. Bioresource Technology 227, 125–132. https://doi.org/10.1016/j.biortech.2016.12.048

Silva, F.C., Serafim, L.S., Nadais, H., Arroja, L., Capela, I., 2013. Acidogenic Fermentation Towards Valorisation of Organic Waste Streams into Volatile Fatty Acids. Chem. Biochem. Eng. Q. 27, 467–476.

Singhania, R.R., Patel, A.K., Christophe, G., Fontanille, P., Larroche, C., 2013. Biological upgrading of volatile fatty acids, key intermediates for the valorization of biowaste through dark anaerobic fermentation. Bioresour. Technol. 145, 166–174. https://doi.org/10.1016/j.biortech.2012.12.137

Smith, A.D., Holtzapple, M.T., 2011. Investigation of the optimal carbon–nitrogen ratio and carbohydrate–nutrient blend for mixed-acid batch fermentations. Bioresource Technology 102, 5976–5987. https://doi.org/10.1016/j.biortech.2011.02.024

Spirito, C.M., Richter, H., Rabaey, K., Stams, A.J., Angenent, L.T., 2014. Chain elongation in anaerobic reactor microbiomes to recover resources from waste. Current Opinion in Biotechnology 27, 115–122. https://doi.org/10.1016/j.copbio.2014.01.003

Sträuber, H., Schröder, M., Kleinsteuber, S., 2012. Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a leach-bed process. Energ Sustain Soc 2, 13. https://doi.org/10.1186/2192-0567-2-13

Tekucheva, D., N., Tsygankov, A., A., 2012. Combined biological hydrogen-producing systems: A review. Applied Biochemistry and Microbiology 48, 4, 319-337

Temudo, M.F., Muyzer, G., Kleerebezem, R., van Loosdrecht, M.C.M., 2008. Diversity of microbial communities in open mixed culture fermentations: impact of the pH and carbon source. Appl. Microbiol. Biotechnol. 80, 1121–1130. https://doi.org/10.1007/s00253-008-1669-x

Thauer, R.K., Jungermann, K., Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41, 100–180.

Van Dyk, J.S., Gama, R., Morrison, D., Swart, S., Pletschke, B.I., 2013. Food processing waste: Problems, current management and prospects for utilisation of the lignocellulose component through enzyme synergistic degradation. Renewable and Sustainable Energy Reviews 26, 521–531. https://doi.org/10.1016/j.rser.2013.06.016

Venkata Mohan, S., Nikhil, G.N., Chiranjeevi, P., Nagendranatha Reddy, C., Rohit, M.V., Kumar, A.N., Sarkar, O., 2016. Waste biorefinery models towards sustainable circular bioeconomy: Critical review and future perspectives. Bioresource Technology 215, 2–12. https://doi.org/10.1016/j.biortech.2016.03.130

Vonk, R.J., Reckman, G., 2017. Progress in the biology and analysis of short chain fatty acids. J Physiol 595, 419–420. https://doi.org/10.1113/JP273260

Wang, X., Zhang, S., Wang, J., Yu, X., Lu, X., 2012. Exploring optimal feed to microbes ratio for anaerobic acidogenic femrentation of cassava residue from brewery. BioResources 7, 1111–1122. https://doi.org/10.15376/biores.7.1.1111-1122

Wang, K., Yin, J., Shen, D., Li, N., 2014. Anaerobic digestion of food waste for volatile fatty acids (VFAs) production with different types of inoculum: Effect of pH. Bioresource Technology 161, 395–401. https://doi.org/10.1016/j.biortech.2014.03.088

Wu, Q.-L., Guo, W.-Q., Zheng, H.-S., Luo, H.-C., Feng, X.-C., Yin, R.-L., Ren, N.-Q., 2016. Enhancement of volatile fatty acid production by co-fermentation of food waste and excess sludge without pH control: The mechanism and microbial community analyses. Bioresource Technology 216, 653–660. https://doi.org/10.1016/j.biortech.2016.06.006

Xiao, K., Zhou, Y., Guo, C., Maspolim, Y., Ng, W.J., 2016. Impact of undissociated volatile fatty acids on acidogenesis in a two-phase anaerobic system. Journal of Environmental Sciences 42, 196–201. https://doi.org/10.1016/j.jes.2015.06.015

Xu, S.Y., Karthikeyan, O.P., Selvam, A., Wong, J.W.C., 2012. Effect of inoculum to substrate ratio on the hydrolysis and acidification of food waste in leach bed reactor. Bioresource Technology 126, 425–430. https://doi.org/10.1016/j.biortech.2011.12.059

Xu, F., Li, Y., Ge, X., Yang, L., Li, Y., 2018. Anaerobic digestion of food waste – Challenges and opportunities. Bioresource Technology 247, 1047–1058. https://doi.org/10.1016/j.biortech.2017.09.020

Yang, X., Choi, H.S., Park, C., Kim, S.W., 2015. Current states and prospects of organic waste utilization for biorefineries. Renewable and Sustainable Energy Reviews 49, 335–349. https://doi.org/10.1016/j.rser.2015.04.114

Ye, N.-F., Lü, F., Shao, L.-M., Godon, J.-J., He, P.-J., 2007. Bacterial community dynamics and product distribution during pH-adjusted fermentation of vegetable wastes. J. Appl. Microbiol. 103, 1055–1065. https://doi.org/10.1111/j.1365-2672.2007.03321.x

Yen, H.-W., Hu, I.-C., Chen, C.-Y., Ho, S.-H., Lee, D.-J., Chang, J.-S., 2013. Microalgae-based biorefinery – From biofuels to natural products. Bioresource Technology 135, 166–174. https://doi.org/10.1016/j.biortech.2012.10.099

Yin, J., Yu, X., Zhang, Y., Shen, D., Wang, M., Long, Y., Chen, T., 2016. Enhancement of acidogenic fermentation for volatile fatty acid production from food waste: Effect of redox potential and inoculum. Bioresource Technology 216, 996–1003. https://doi.org/10.1016/j.biortech.2016.06.053

Yu, H.Q., Fang, H.H.P., 2003. Acidogenesis of gelatin-rich wastewater in an upflow anaerobic reactor: influence of pH and temperature. Water Res. 37, 55–66.

Yuan, Y., Wang, S., Liu, Y., Li, B., Wang, B., Peng, Y., 2015. Long-term effect of pH on short-chain fatty acids accumulation and microbial community in sludge fermentation systems. Bioresource Technology 197, 56–63. https://doi.org/10.1016/j.biortech.2015.08.025

Yuan, Y., Liu, Y., Li, B., Wang, B., Wang, S., Peng, Y., 2016. Short-chain fatty acids production and microbial community in sludge alkaline fermentation: Long-term effect of temperature. Bioresource Technology 211, 685–690. https://doi.org/10.1016/j.biortech.2016.03.138

Yun, J., Cho, K.-S., 2016. Effects of organic loading rate on hydrogen and volatile fatty acid production and microbial community during acidogenic hydrogenesis in a continuous stirred tank reactor using molasses wastewater. Journal of Applied Microbiology 121, 1627–1636. https://doi.org/10.1111/jam.13316

Zacharof, M.-P., Lovitt, R.W., 2014. Recovery of volatile fatty acids (VFA) from complex waste effluents using membranes. Water Sci. Technol. 69, 495–503. https://doi.org/10.2166/wst.2013.717

Zhang, P., Chen, Y., Zhou, Q., Zheng, X., Zhu, X., Zhao, Y., 2010. Understanding short-chain fatty acids accumulation enhanced in waste activated sludge alkaline fermentation: kinetics and microbiology. Environ. Sci. Technol. 44, 9343–9348. https://doi.org/10.1021/es102878m

Zhang, D., Fu, X., Dai, X., Chen, Y., Dai, L., 2016. A new biological process for short-chain fatty acid generation from waste activated sludge improved by Clostridiales enhancement. Environ Sci Pollut Res 23, 23972–23982. https://doi.org/10.1007/s11356-016-7579-z

Zhao, G., Ma, F., Wei, L., Chua, H., Chang, C.-C., Zhang, X.-J., 2012a. Electricity generation from cattle dung using microbial fuel cell technology during anaerobic acidogenesis and the development of microbial populations. Waste Management 32, 1651–1658. https://doi.org/10.1016/j.wasman.2012.04.013

Zhao, X., Zhang, L., Liu, D., 2012b. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. Biofuels, Bioprod. Bioref. 6, 465–482. https://doi.org/10.1002/bbb.1331

Zhao, B., Liu, J., Frear, C., Holtzapple, M., Chen, S., 2016. Consolidated bioprocessing of microalgal biomass to carboxylates by a mixed culture of cow rumen bacteria using anaerobic sequencing batch reactor (ASBR). Bioresource Technology 222, 517–522. https://doi.org/10.1016/j.biortech.2016.09.120

Zheng, X., Su, Y., Li, X., Xiao, N., Wang, D., Chen, Y., 2013. Pyrosequencing Reveals the Key Microorganisms Involved in Sludge Alkaline Fermentation for Efficient Short-Chain Fatty Acids Production. Environmental Science & Technology 47, 4262–4268. https://doi.org/10.1021/es400210v

Zhou, A., Zhang, J., Wen, K., Liu, Z., Wang, G., Liu, W., Wang, A., Yue, X., 2016. What could the entire cornstover contribute to the enhancement of waste activated sludge acidification? Performance assessment and microbial community analysis. Biotechnology for Biofuels 9. https://doi.org/10.1186/s13068-016-0659-y

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

2 Comparison of acidogenic and methanogenic potential of agroindustrial residues

Comment

Chronologically the third study of this thesis, this work spanned a long period due to the seasonal availability of the substrates tested and practical reasons of managing a large number of experiments. Having in mind that, with no external intervention, methanogenesis is the natural continuation of acidogenesis, the motivation behind this study was the question of whether the biomethane potential of a substrate, a parameter that is very commonly used, is an accurate representation also of the acidogenic potential of the same substrate, a parameter that is less commonly used. Following the experience of Chapter 4 (pH adjusted to 6.5 leading to VFA loss) and Chapter 3 (no pH adjustment leading to strong reactor acidification), the pH selected for the acidogenic experiments was kept in the area of 5. The metabolic analysis and comparison of the two potentials were followed by a simplified economic analysis, which only focused on product quantities and typical prices, without considering total production costs, downstream processing costs or any kind of premiums. Although acknowledging that this is a major simplification, the purpose was only to provide a rough estimation of the economic potential of VFA as marketable products, compared to methane, a natural gas substitute.

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*Corresponding author

Specific AP contribution: experimental work, data analysis, data interpretation, article compilation

Abstract

The methanogenic and acidogenic potentials of six different agroindustrial residues, i.e. of fruit pulps and brewery residues, were determined. For all substrates, the methanogenic conversion yield was systematically higher than the acidogenic one in Chemical Oxygen Demand (COD) terms, ranging from 0.46 to 0.87 $g_{COD\_CH4}/g_{COD\_substrate\_fed}$ and from 0.24 to 0.56 $g_{COD\_tVFA}/g_{COD\_substrate\_fed}$, respectively. During methanogenic conversion, brewery trub exhibited the highest methane potential (304 $ml_{CH4}/g_{COD\_substrate}$). Trub also exhibited the highest total volatile fatty acids (tVFA) concentrations in the mixed liquor (ML) during acidogenic conversion (29.7 g_{COD_tVFA}/kg_{ML}). Acetic, butyric and caproic acids were the main carboxylates produced by the different substrates. Despite the lower conversion yields, the economic value of the acidogenic product (carboxylate streams) is higher than that of methanogenic conversion (methane) due to the higher value of carboxylates and their potential use in finer applications (e.g. bio-based products) compared to energy production form methane.

Keywords

Agroindustrial residues, acidogenic fermentation, mixed culture, anaerobic digestion, volatile fatty acids, carboxylates

2.1 Introduction

Agroindustrial residues like fruit, vegetable or brewery waste have attracted increasing attention during the last decade owing to the potential of valorising their organic fraction for various energetic or material applications, thus bringing added value to the entire process from which they are generated (Oreopoulou and Tzia, 2007). According to Eurostat, the amount of the waste categories (i) animal & mixed food waste and (ii) vegetal waste (classification W091 and W092, respectively) generated by the economic activity "Manufacture of food products, beverages and tobacco products (activity C10-C12 according to NACE-Rev2 classification) amounted to 22.1 Mt in 2014 in the EU-28 (EUROSTAT, 2017). The valorisation potential of these residues is, therefore, attractive as suggested by several authors. Indicatively, for the type of waste that are of interest in this study, Ravindran and Jaiswal (2016) and Federici *et al*. (2009) both highlighted the potential of food processing waste and commented on the technological challenges that need to be overcome to increase product yield and decrease operating costs for their conversion. Mussatto (2009) and Mathias *et al*. (2014) also reviewed the biotechnological potential of the brewing industry by-products and their possible applications.

Among other options for the treatment of agroindustrial waste, anaerobic digestion has been widely implemented at industrial scale, typically, for the production of methane (biogas), a substitute of natural gas (Angelidaki *et al*., 2011). Anaerobic digestion is a sequence of four naturally occurring and interdependent processes i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis. Methane has been the main focus in literature, as the final product of anaerobic digestion. Nevertheless, short chain carboxylic acids deriving from the intermediate acidogenic phase like acetic, propionic, butyric valeric and caproic acids, otherwise called volatile fatty acids (VFA) or carboxylates, are also of high industrial interest. Acidogenesis (or acidogenic fermentation) has been mostly studied in view of increasing biogas (e.g. two-stage anaerobic digestion systems) or biohydrogen (i.e. dark fermentation) yields, but it can be envisaged as a stand-alone process with a product of higher added value (Singhania *et al*., 2013; Alkaya and Demirer, 2011; Zacharof and Lovitt, 2013). VFA have a variety of potential end-uses, for example as monomers for polyhydroxyalkanoates (PHA) production, for bio-based solvent production, for biological nutrient removal, etc. Interest in VFA has increased during the last years in the context of the carboxylate platform, a term used in the biorefinery context highlighting the multitude of production processes and potential applications of VFA (Jang *et al*., 2012; Agler *et al*., 2011).

Each phase in the sequence of anaerobic digestion is realised by a different

group of microorganisms (i.e. acidogenic bacteria, acetogenic bacteria, methanogenic archaea) working in synergy but often also competition (Angelidaki *et al*., 2011). While methane production occurs naturally under anaerobic conditions, separating acidogenesis requires a more careful control of the process conditions. Acetogenesis and methanogenesis must be avoided to increase the net conversion yield of the substrate into VFA. This control is especially challenging for mixed culture fermentations, i.e. fermentation by natural microbial consortia, not pure cultures (Arslan *et al*., 2016; Rodríguez *et al*., 2006). Some advantages of using mixed cultures are: the ability to treat various types of complex substrates owing to the microbial diversity, no need for sterile conditions, lower costs of production and potential for a continuous process (Kleerebezem and van Loosdrecht, 2007). On the other hand, the lack of product specificity and the complexity of the medium are still a bottleneck in terms of product yields and recovery and prevent mixed culture fermentation from wide implementation at industrial scale.

While methane production is already established as an industrial process, acidogenic fermentation is still under research. Research is focusing mainly on (i) the influence of process parameters of batch or continuous operation (pH, substrate concentration, HRT, OLR) on the concentration and composition of VFA (Arslan *et al*., 2016; Lee *et al*., 2014; Gameiro *et al*., 2016; Jiang *et al*., 2013) and (ii) downstream processing for the concentration, recovery, separation and purification of VFA streams (Zacharof and Lovitt, 2014; López-Garzón and Straathof, 2014; Arslan *et al*., 2017).

Keeping in mind the difference in maturity between the two processes, the purpose of this study is to highlight the interest of mixed acidogenic fermentation as a possible option for organic solid waste treatment. For that we determined and compared both the acidogenic and methanogenic potential of a number of organic residues of agroindustrial interest. The present paper presents the results and discusses the observed differences. The decision over the one or the other process will ultimately depend on the product yields, the economic potential deriving thereof and the investment required to harness it. To the best of our knowledge, this is the first study where these two processes are systematically examined as two separate processes for the same waste fraction.

2.2 Materials and Methods

2.2.1 Substrates

Two types of agroindustrial residues were investigated: (i) fruit pulps after juice extraction and water washing, more specifically date, pear and apple pulp from a syrup producing industry (Aubel, Walloon Region, Belgium), (ii)

brewery residues, more specifically hot trub, spent yeast, and spent grain from a small-scale brewery (Ath, Walloon Region, Belgium). The properties of the substrates are resumed in Table 2-1.

Table 2-1 : Composition of the residues from brewery and fruit syrup activities, used as substrates
FM: Fresh Matter; TS: Total Solids; VS: Volatile Solids; COD: Chemical Oxygen Demand;

Parameter	Unit	Trub	Spent yeast	Spent grain	Pear pulp	Date pulp	Apple pulp
Total solids	%FM, w/w	23.8	13.8	25.0	32.4	24.5	22.2
Volatile solids	%TS, w/w	98.1	93.2	96.5	99.2	98.1	99.1
COD	g_{COD}/g_{FM}	0.294	0.289	0.316	0.427	0.317	0.360
COD	g_{COD}/g_{VS}	1.26	2.24	1.31	1.33	1.32	1.64
Cellulose	% TS, w/w	8.2	1.0	19.4	32.9	22.6	38.9
Hemicellulose	% TS, w/w	5.6	14.7	28.8	22.1	11.5	13.5
Lignin	% TS, w/w	8.0	0.3	4.2	17.0	32.5	12.2
Pectin	% TS, w/w	-	-	-	2.0	2.9	2.4
Sugar	% TS, w/w	57.4	38.6	12.4	7.6	12.3	10.2
Proteins	% TS, w/w	15.0	35.9	18.0	5.9	11.0	7.1
Lipid	% TS, w/w	3.0	3.9	10.0	1.8	0.7	2.9
Ash	% TS, w/w	2.5	5.6	5.0	1.0	1.9	1.2

TS, VS, COD values were determined by the authors for all substrates. For the other parameters i.e. cellulose, hemicellulose, lignin, sugar, lipid, protein, pectin and ash content we refer the reader to Aguedo et al. (2012) in the case of fruit pulps, since they used the same substrates as this study. For the respective parameters of brewery residues, the results have been delivered by Celabor, Belgium whom we gratefully acknowledge.

2.2.2 Concentrated activated sludge

Activated sludge from the municipal wastewater treatment plant in Mont-St-Guibert, Belgium was used as primary inoculum for both methanogenic and acidogenic conversions. The sludge was left to settle in the dark at 4°C for two days and the supernatant was removed. Average properties of the concentrated sludge were: Total Solids (TS) in the fresh matter (FM) at 0.024 ± 0.005 g_{TS}/g_{FM} (8 samples from 4 experiments), Volatile Solids (VS) at 0.40 ± 0.08 g_{VS}/g_{TS} (10 samples from 4 experiments) and COD at 20.7 ± 1.2 g_{COD}/kg_{FM} (13 samples from 4 experiments. The treatment of the inoculum prior to each conversion is presented in sections 2.2.3.1 and 2.2.4.1, respectively

2.2.3 Methanogenic conversion

2.2.3.1 Inoculum preparation

For the preparation of the methanogenic inoculum, the primary inoculum was maintained at 35°C under anaerobic conditions for at least four weeks and fed

every week with fresh activated sludge, concentrated as described in section 2.2.2 at a ratio of 0.1 $g_{COD\_concentrated\_sludge}/g_{COD\_primary\_inoculum}$. The container of the primary inoculum was closed with a rubber cap. One extremity of a PVC tube was inserted through the rubber cap into the headspace of the container, while the other extremity was immersed into Erlenmeyer flask filled with water. This allowed the release of biogas while preventing air contact with the container (Angelidaki *et al*., 2011). Ten days before the start of the experiment, the inoculum received a last feed at a ratio of 0.3 $g_{COD\_concentrated\_sludge}/g_{COD\_primary\_inoculum}$ (Donoso-Bravo *et al*., 2015).

2.2.3.2 Bioreactor preparation

The anaerobic digestion took place in 1 l Schott Duran GL45 bottles with a lateral glass tube of 4 cm long placed at an angle of 45° upwards. A 2-way Luer polycarbonate valve (Fisher Scientific) was connected via a short PVC tube to the extremity of the glass tube. Gas sampling and pressure measurements was achieved through this valve. The bioreactor-bottle was closed with a PBT screw-cap, containing a PTFE coated silicone seal.

At the start of the experiment, the methanogenic inoculum was introduced in the reactors and the substrate was subsequently added, for a mixed liquor (ML) volume of approximately 570 ml. Experiments were conducted in triplicates. The quantities of each substrate added in the reactors are shown in Table 2-2. Three reactors contained the inoculum and water instead of substrate and served as a control. After adding the inoculum and the substrate, the headspace of the reactor was flushed with N_2 for one minute before closing the reactor, in order to remove oxygen and establish anaerobic conditions. The volume of each reactors' headspace was determined by comparing the weight of each reactor (i) empty, (ii) full of water, (iii) filled with the final volume of mixed liquor.

Table 2-2: Initial COD concentrations in anaerobic digestion experiments
Mean values and standard deviation of three reactors. ML: mixed liquor; COD; Chemical Oxygen Demand

Substrate	Substrate (g_{COD})	Inoculum (g_{COD})	Reactor concentration (g_{COD}/kg_{ML})
Trub[1]	0.75 ± 0.00	5.00 ± 0.01	15.50 ± 0.02
Spent yeast[2]	1.00 ± 0.00	5.32 ± 0.03	16.17 ± 0.52
Spent grain[2]	1.00 ± 0.00	5.20 ± 0.08	16.15 ± 0.27
Pear pulp[3]	1.20 ± 0.00	7.50 ± 0.00	15.31 ± 0.00
Date pulp[1]	0.75 ± 0.00	5.00 ± 0.01	15.48 ± 0.02
Apple pulp[3]	1.50 ± 0.00	7.50 ± 0.00	15.81 ± 0.00

[1, 2, 3] *Experiments took place at different times, due to seasonal substrate availability. Substrates*

sharing the same superscript were tested in the same experiment.

2.2.3.3 Monitoring of methanogenic conversion

Depending on the substrate, the anaerobic digestion lasted between 20 and 50 days. At regular intervals, the pressure of the reactor's headspace was measured with a manometer (UNIK 5000 PTX5072-TA-A3-CA-H0-PA, GE Measurement & Control Solutions) in order to determine the quantity of biogas produced, through the ideal gas law. The manometer was connected to the reactor with a 3-way valve, permitting the connection of a 50 ml polypropylene syringe to sample the gas phase. After sampling, the reactor was depressurized to atmospheric pressure that was also recorded. The gas phase sample was immediately analysed by GC-TCD (see 2.2.5) to determine its composition in CH_4, CO_2, H_2, O_2 and N_2.

The total volume of biogas produced and its composition permitted to calculate the amount of CH_4 produced. The amount of methane produced in the control reactors was subtracted from the amount of methane produced in the reactors with the substrate, in order to provide the net amount of methane produced by the substrate. The conversion yield of the substrate to methane (methanogenic potential) is determined by Eq. 1.

$$R_{CH4} = \frac{V_{CH4} * F}{Q_{substrate}} \qquad \text{Eq. 1,} \quad \text{where}$$

R_{CH4}: yield of substrate conversion to methane ($g_{COD\_CH4}/g_{COD\_substrate}$)
V_{CH4}: cumulated methane production at normal conditions (l_{CH4})
F: stoichiometric COD factor of methane (2.86 g_{COD_CH4}/l_{CH4})
$Q_{substrate}$: initial COD quantity of the substrate fed to the reactor ($g_{COD\_substrate}$)

2.2.4 Acidogenic conversion

2.2.4.1 Inoculum preparation

The concentrated sludge (see section 2.2.2) was incubated under anaerobic conditions at 35°C for five days. The pH of the concentrated sludge was then adjusted to 5.5 with HCl and the sludge was maintained for 1 hour at 70°C in a shaking water bath, to select spore-forming acidogenic species (Arslan *et al*., 2016). It was left to cool down to room temperature and the pH was re-adjusted to 5.5 with HCl just before use as acidogenic inoculum.

2.2.4.2 Bioreactor preparation

Acidogenic fermentation was performed in the same type of reactors as for methanogenic conversion (Scott Duran GL 45 bottles), though smaller in size (250ml). The substrate and the inoculum were introduced in the reactors (triplicate for spent grain, apple pulp and date pulp; duplicate for spent yeast

and trub) at a COD ratio of 4 $g_{COD\_substrate}/g_{COD\_inoculum}$ (Perimenis *et al*., 2016) and water was added to reach a mixed liquor volume of 100ml (Table 2-3). The substrate to inoculum ratio (S/I) was higher than in the methanogenic conversion, to ensure the presence of sufficient digestible fraction allowing acidogenic bacteria to rapidly produce VFA and inhibit development of methanogens (van Aarle *et al*., 2015). Control reactors, where either the inoculum or the substrate was replaced by deionised water, were also prepared. Before hermetic closure, the bioreactor headspace was flushed for 1 minute with N_2.

Table 2-3: Initial COD concentration in acidogenic fermentation experiments
Mean values and standard deviation or deviation from the mean of three or two reactors, respectively; ML: mixed liquor; COD; Chemical Oxygen Demand

Substrate	Substrate (g_{COD})	Inoculum (g_{COD})	Reactor concentration (g_{COD}/kg_{ML})
Trub	4.81 ± 0.00	1.16 ± 0.01	60.72 ± 0.16
Spent yeast [1]	4.80 ± 0.00	1.22 ± 0.00	60.41 ± 0.02
Spent grain [2]	8.00 ± 0.02	2.00 ± 0.00	80.97 ± 0.27
Pear pulp [1]	4.80 ± 0.00	1.20 ± 0.00	59.64 ± 0.18
Date pulp [2]	8.01 ± 0.02	2.00 ± 0.00	80.57 ± 0.19
Apple pulp [2]	8.01 ± 0.01	2.00 ± 0.00	82.84 ± 0.48

[1, 2] Experiments took place at different times, due to seasonal substrate availability. Substrates sharing the same superscript were tested in the same experiment.

2.2.4.3 Monitoring of acidogenic conversion

The batch incubation was conducted at 35°C in the dark. When necessary, the pH of the mixed liquor was adjusted to the desired value of 4.5-5.5 with the addition of 1M HCl or 1M NaOH solution. Depending on the substrate, fermentation lasted between 22 and 66 days. Samples of the gas phase and the mixed liquor were taken at regular intervals for analysis. Flushing with nitrogen took place each time the reactors were opened. The parameters monitored were: pH, soluble COD (CODs), biogas production and composition and the concentration of metabolites (e.g. VFA, ethanol).

Monitoring of the gas production and composition were performed in the same way as described for the methanogenic conversion. The samples of the mixed liquor were firstly centrifuged at 15,300 x g for 10 min. The supernatant was separated and stored at -20 °C for the metabolic analysis. The solid remainder was also stored at -20 °C.

It was assumed that the inoculum has the same contribution in the inoculated reactors as in the control reactors. The contribution of the inoculum to the final tVFA concentration was then subtracted from the results of the inoculated reactors. The acidogenic potential was then determined by Eq. 2:

$$R_{tVFA\_substrate} = \frac{C_{tVFA\_inoculated} - C_{tVFA\_inoculum}}{C_{substrate}}$$ Eq. 2, where

R_{tVFA}: conversion yield of substrate fed to tVFA ($g_{COD\_tVFA}/g_{COD\_substrate}$)
$C_{tVFA\_inoculated}$: tVFA concentration in inoculated substrate series (g_{COD_tVFA}/kg_{ML})
$C_{tVFA\_inoculum}$: tVFA concentration in inoculum control series (g_{COD_tVFA}/kg_{ML})
$C_{substrate}$: Initial COD concentration of the substrate fed in the reactor ($g_{COD\_substrate}/kg_{ML}$)

2.2.5 Analytical methods

Total solids (TS), volatile solids (VS) and total COD (CODt) were measured according to Standard Methods (APHA, 1998). Soluble COD (CODs) was measured with the COD Cell Test method (Spectroquant® kits 1.14541.0001 and 1.14555.0001, Spectroquant® ThermoReactor 620, Photometer SQ200, Merck Germany) according to the provider's instructions.

Analysis of brewery residues was based on the hypothesis that the sample was composed of fibres (cellulose, hemicellulose and lignin), proteins, fat, ash and sugars. Fibre content was determined according to [AOAC]. Proteins were determined according to [ISO 1871:2009], lipid content according to [ISO 1443:1973] and ash content according to [ISO 936]. Sugar content was calculated by difference.

The composition of the biogas was analysed using a gas chromatograph (compact GC, Interscience) with a thermal conductivity detector (GC-TCD). The chromatograph combined two channels. The front channel was equipped with a Rt-QBond column (10 m x 0.32 mm), which separates CO_2 from the other gases. The back channel includes two columns in series: one similar to the front channel (Rt-QBond, 2 m x 0.32 mm) which retains CO_2, followed by a Molsieve 5A column (7 m x 0.32 mm) that separates the other gases. A valve between the two columns, allows to back-flush CO_2 to the injection port so that it doesn't enter in the Molsieve column. The elution was performed under isotherm conditions at 60 °C with helium as carrier gas at 20 ml/min and a pressure of 80 kPa for the front channel and at 70 °C with argon as carrier gas at 10ml/min and a pressure of 70 kPa for the back channel. The detector was heated at 90 °C and the filament at 170 °C.

VFA and ethanol were analysed using a gas chromatograph (Thermo Finnigan, Trace) with a flame ionisation detector (GC-FID). The column was packed with Carbowax (2m x 1/8', 4% Carbowax 20M on Carbograph 1-DA, 80/120 mesh, Alltech). The temperature of the injector and the detector was 250 °C. The sequence for the temperature of the oven started at 140 °C at injection, rising to 180 °C at 4 °C/min and maintained for 10 min, rising to 190 °C at 10 °C/min and maintained for 3 min and decreasing to 140 °C at 10 °C/min and maintained for 1 min. The carrier gas was helium at 1.5 ml/min.

The stoichiometric COD factors for soluble metabolites are 2.087 $g_{COD}/g_{ethanol}$, 1.067g_{COD}/g_{acetic_acid}, 1.512 $g_{COD}/g_{propionic\_acid}$, 1.813 $g_{COD}/g_{butyric\_acid}$, 1.813 $g_{COD}/g_{iso\text{-}butyric\_acid}$, 2.036 $g_{COD}/g_{valeric\_acid}$, 2.036 $gCOD/g_{iso\text{-}valeric\_acid}$ and 2.207 $g_{COD}/g_{caproic\_acid}$.

2.3 Results & Discussion

2.3.1 Methanogenic conversion

Figure 2-1 presents the production of methane. Fruit residues produce less methane than brewery residues most likely due to their higher content in less easily digestible fibres (i.e. lignin and cellulose combined content of 50, 50 and 55 %TS for apple, pear and date pulp, respectively). The highest production is observed for trub (304 $ml_{CH4}/g_{COD\_substrate}$), most likely due to its high sugar content (59% of TS). Indeed, this trub (hot trub) is formed through the precipitation of insoluble substances (e.g. polyphenols and proteins) during wort boiling and thus contains a substantial amount of free sugars. Large scale breweries have systems in place to recover wort from the trub and further ferment it to increase beer yield, but these techniques are not economically viable for micro-breweries.

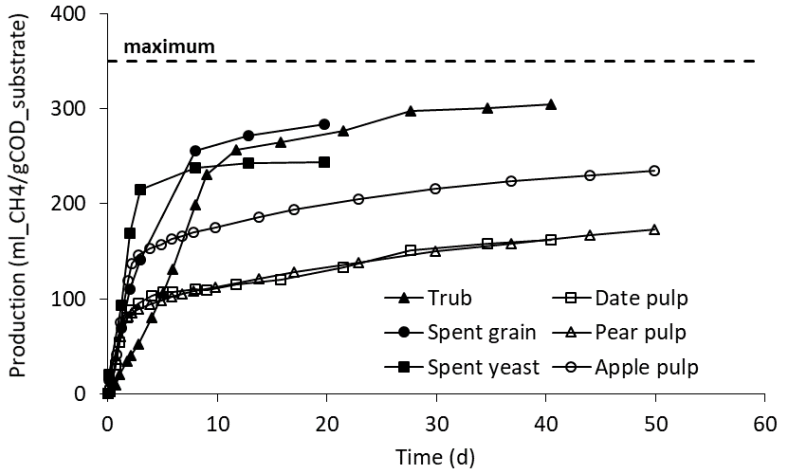

Figure 2-1 : Methane production
The dashed line indicates the stoichiometric methane production from 1 g_{COD} (theoretical maximum). Coefficients of variation between triplicates at the end of experiment: 9.3% for date pulp; 1.6% for pear pulp; 3.3% for apple pulp; 1.0% for trub; 6.2% for spent yeast; 2.0% for spent grain.

As indicated by the steep curves of Figure 2-1, the kinetics of methanogenic conversion are fast as there is sufficient readily digestible substances to be converted. A small delay was observed in the case of trub that can be attributed

to the high sugar content that was readily digested in the acidogenic phase, leading to acidification of the broth, which in turn prevented methanogens from developing as fast as in the case of the other substrates (Labatut *et al.*, 2011). In all cases, more than 75% of the final methane production has been reached within the first 17 days of the experiment; in the case of brewery residues this percentage increased to 85% in the first 10 days.

2.3.2 Acidogenic conversion

Figure 2-2 shows the evolution of the concentration of tVFA for the substrates tested.

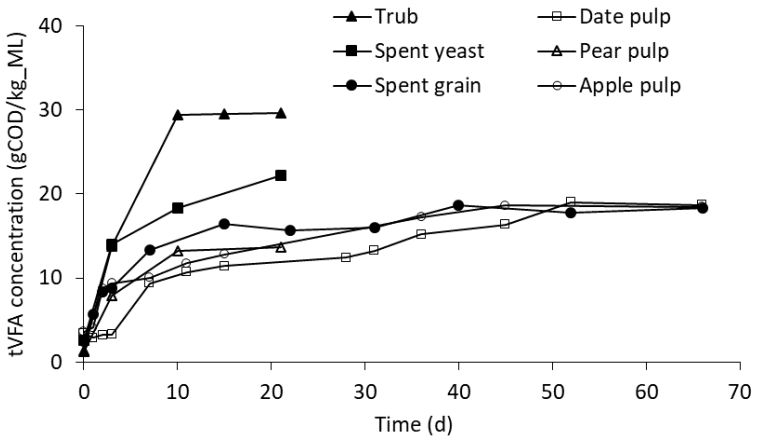

Figure 2-2: Total VFA concentrations
Coefficients of variation between reactors at the day of the highest concentration: 8.8% for date pulp at day 52; 4.0% for pear pulp at day 21; 18.4% for apple pulp at day 45; 6.1% for trub at day 21; 3.0% for spent yeast at day 21; 3.9% for spent grain at day 40.

The first experiments (date, spent grain and apple pulp) showed that about 60% to 90% of the highest concentration is obtained within 15 to 35 days of fermentation, therefore the subsequent experiments had a shorter duration. Similar to the methanogenic conversion, trub exhibits the highest tVFA concentration of approximately 30 g_{COD_tVFA}/kg_{ML}, followed by spent yeast at approximately 22 g_{COD_tVFA}/kg_{ML}. Fruit pulps resulted in lower tVFA concentration values than brewery residues. The highest tVFA concentrations in the control series are presented in Table 2-4.

Table 2-4 : tVFA and ethanol concentration of control series in acidogenic fermentation experiments
tVFA: total volatile fatty acids; COD: chemical oxygen demand; ML: mixed liquor

Series	tVFA Concentration (g_{COD_tVFA}/kg_{ML})	Ethanol concentration (g_{COD}/kg_{ML})
Inoculum control A	3.56 ± 0.95	

Inoculum control B	2.25 ± 0.03	
Inoculum control C	2.04 ± 0.09	
Apple pulp control	8.15 ± 0.67	
Date pulp control	4.36 ± 0.12	
Spent grain control	12.35 ± 0.48	
Trub control	0.69 ± 0.15	28.48 ± 2.26
Spent yeast control	5.59 ± 0.27	20.85 ± 1.80

A: for substrates apple pulp, date pulp, spent grain; B: for substrate trub; C: for substrates spent yeast, pear pulp

In the inoculum controls, tVFA concentrations ranged between 2 and 3.6 g_{COD_tVFA}/kg_{ML}, which corresponds at maximum to 19% of the tVFA concentration of the inoculated series (for apple pulp). In the substrate controls, tVFA concentrations ranged between 2% (for trub) and 66% (for spent grain) of the tVFA concentration in the inoculated series. It can be concluded that the presence and conversion of the substrate contributes the most to the final tVFA concentration of the inoculated series; addition of the inoculum effectively enhances acidogenic fermentation performance.

Butyric and acetic acid were the two dominant products in almost all cases (Figure 2-3), a result that is in accordance with many acidogenic studies using similar type of substrates (Cysneiros *et al*., 2012; Han and Shin, 2002; Parawira *et al*., 2004; Rincón *et al*., 2008). Butyric acid ranged from 5.5 to 13.2 g_{COD}/kg_{ML} for date pulp and trub, respectively, while acetic acid ranges from 2.9 to 9.8 g_{COD}/kg_{ML} for trub and spent yeast, respectively. Interestingly, trub shows a considerable production of caproic acid (up to 13 g_{COD}/kg_{ML}), a result which coincides with the recent interest in production of caproic acid from biomass waste. Caproic acid has a higher carbon number and is easier to separate from water than acetic and butyric acid (Agler *et al*., 2014; Grootscholten *et al*., 2013; Kucek *et al*., 2016; Spirito *et al*., 2014; Vasudevan *et al*., 2014). Caproic acid is produced through the chain elongation process, a process where short-chain carboxylic acids like acetic and butyric are elongated with two carbons from a reduced molecule like ethanol (Angenent *et al*., 2016). In our experiments, ethanol was the main product of the non-inoculated trub series (up to 28.5 g_{COD}/kg_{ML}, see Table 2-4), which is an indication that production of ethanol could also have taken place in the inoculated reactors and permitted the natural chain elongation process without external ethanol addition. COD conversion to hydrogen was limited (maximum 0.07 g_{COD_H2}/g_{CODt} for trub and less than 0.03 g_{COD_H2}/g_{CODt} for the other substrates)

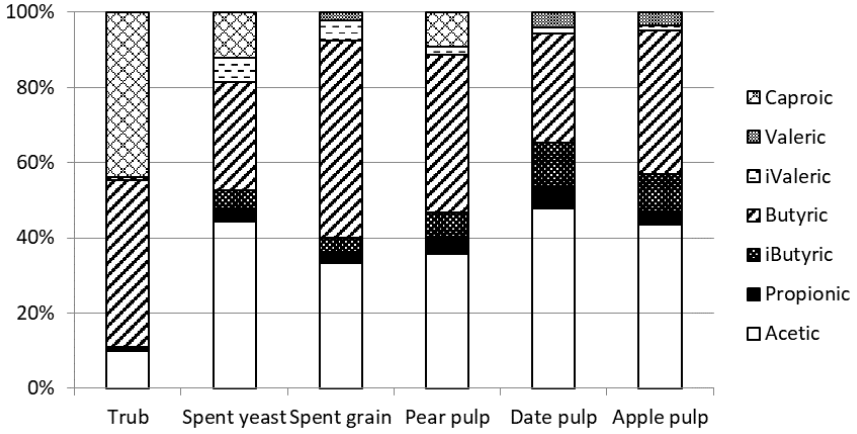

Figure 2-3: Volatile fatty acid profile of acidogenic fermentation experiments

2.3.3 Substrate conversion yield

The conversion yields of the substrates into product (methane or tVFA), calculated following Equations 1 and 2, are presented in Figure 2-4 in terms of COD.

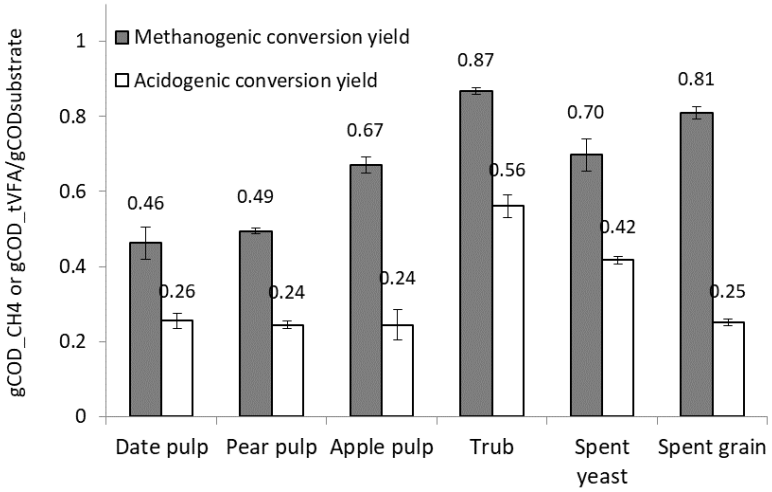

Figure 2-4 : Acidogenic and methanogenic conversion yield

Irrespectively of the type of anaerobic conversion, substrates with a higher lignocellulosic content (like fruit pulps) are more recalcitrant to conversion. This is reflected in the product/substrate COD ratio (conversion yield), which is systematically lower than the one of the other residues. The methanogenic conversion yield is significantly higher than the acidogenic potential, for all

the substrates tested. Since (i) acidogenic fermentation is an intermediate stage of anaerobic digestion, i.e. methane production goes through VFA production and (ii) the processes do not involve an external electron acceptor, i.e. the COD value of methane as a final product is equal to the COD value of its precursors, the difference in conversion yield is striking. It appears that when the natural process of anaerobic digestion is stopped at the stage of acidogenesis, i.e. inhibition of methanogenesis through the combination of inoculum pre-treatment, pH adjustment and high initial substrate concentration, no further conversion of the substrate to VFA takes place after a certain point, probably due to inhibition effects. On the opposite, when the anaerobic digestion process is naturally left to continue, the produced VFA are not accumulating but are further consumed until the majority of the digestible fraction of the substrate is converted into methane (Agler *et al*., 2014).

Arslan *et al*. (2016) suggest overloading (substrate concentration) and product inhibition as the major causes of limitations in acidogenic conversion. The mechanism of product inhibition is summarised in Batstone and Jensen (2011): in the bulk solution, acids are mainly in their undissociated form since their pKa (4.8) is similar to the acidic pH; in this form, they can pass through the cell membrane and dissociate in the cell where the pH is neutral. Cells are then forced to divert energy from growth and metabolism into maintaining homeostasis by exporting protons formed by acid dissociation. Maximum concentrations reported in the comprehensive reviews of Lee *et al*. (2014) and Arslan *et al*. (2016) for batch acidogenic reactors are in line with the ones reported in this study. Indicatively, approximately 30 g_{COD_tVFA}/l for food waste (Kim *et al*., 2011a) and 19 g_{COD_tVFA}/l for rice waste (Dong *et al*., 2009). Furthermore, Veeken *et al*. (2000) report that high VFA concentration in the area of 40-50 g_{COD}/l lead to severe inhibition of hydrolysis, which is performed by the same acidogenic microorganisms. Findings of Jiang *et al*. (2013) confirm this threshold, as VFA production in their experiments on food waste reached a plateau at around 40 g/l. On the substrate concentration, Arslan *et al*. (2016) suggest that at a level of 40 g_{COD}/l, no further increase of carboxylate concentration is observed. In our study, initial substrate concentration was higher than this threshold and comparable to the ones of Kim *et al*. (2011b) (approximately 56 g_{COD}/l of a mixture of food waste and sewage sludge leading to approximately 22 g_{COD}/l tVFA concentration) and Komemoto *et al*. (2009) (approximately 93 g_{COD}/l of food waste leading to 11 g_{COD}/l tVFA concentration). Overall, the range of tVFA concentrations in this study is of the highest reported in literature for stand-alone acidogenic batch reactors and it is plausible to assume that the lower conversion yield (compared to methanogenic conversion) can, indeed, be attributed to overloading and product inhibition.

Acidogenic conversion yields are comparable to the ones in the literature for similar type of substrates. Traverso *et al*. (2000) reported a COD conversion yield of total COD into tVFA of approximately 24% for mixed vegetable waste, while Espinoza-Escalante *et al*. (2008) reported a value of 16% for tequila stillage. Higher values of approximately 55%, comparable to the ones of trub in this study, are reported from Shen *et al*. (2014) for waste molasses. Given that acetic and butyric acid are in all cases the dominant VFA products, there is no specific correlation between the distribution of VFA and the substrate composition.

Concerning the methanogenic conversion, a 24% substrate-to-methane COD conversion yield for spent yeast (corresponding to 74.2 $ml_{CH4}/g_{COD\_substrate}$) was reported by Sosa-Hernández *et al*. (2016), which is considerably lower that the approximately 70% yield reported in this study (see Fig. 4). A possible explanation is the high substrate-to-inoculum (S/I) ratio that they used (1.2 compared to 0.19 g_{COD}/g_{COD} in the present study) that could have led to a strong initial acidification of the mixed liquor that prevented methanogenic microorganisms from developing properly and producing methane; a delayed methane production of approximately 10 days, as reported by the authors, supports this interpretation

Table 2-5 summarises the methane potential determined for the substrates in this study and compares it to other studies in literature, despite the differences in starting conditions, for example in terms of substrate-to-inoculum (S/I) ratio.

Table 2-5: Biomethane potential in $ml_{CH4}/g_{VS\_substrate}$ and corresponding S/I on VS basis (values in parenthesis) of residues tested

Trub	Spent yeast	Spent grain	Pear pulp	Date pulp	Apple pulp	Source[#]
382 (0.21)	548 (0.15)	370 (0.26)	230 (0.20)	214 (0.20)	385 (0.22)	This study
			426 (0.5)			1
					297 (0.21-1.50)	2
		429 (0.03-0.08)				3
	313 (0.53)*					*4*

[#]1: Ariunbaatar et al. (2014); 2: Nieto et al. (2012); 3: Colussi et al. (2016); 4: Sosa-Hernández et al. (2016)

**Own calculation based on information available in the study*

2.3.4 Economic potential

Table 2-6 presents the annual economic value of the residues, indicatively calculated for the case of brewery residues for both types of anaerobic conversion. For the acidogenic conversion the following were considered:

- the annual tVFA production which is calculated by multiplying the amount of residues from an artisanal brewery of approximately 6200 hl/a beer production (Table 2-6) with the COD content of the residue (Table 2-1) and the respective acidogenic conversion yield (Figure 2-4).
- the annual economic value of the VFA streams based on the tVFA production previously calculated, the VFA distribution from Figure 2-3 (we only consider acetic, butyric and caproic acid with COD values of 1.067, 1.816 and 2.204 g_{COD}/g_{VFA}, respectively) and commercial VFA prices from Zacharof and Lovitt (2013):

For the methanogenic conversion the following were considered:

- the annual methane production which is calculated by multiplying the amount of residues (Table 2-6) with the COD content of the residue (Table 2-1) and the respective methanogenic conversion yield (Figure 2-4).
- The annual energetic and economic value of methane based on the methane production previously calculated as well as the energy content (LHV: 9.97 kWh/m^3) and the residential D3 price (approximately 32€/MWh; only considering energy price without transmission, distribution and taxes; D3 corresponds to consumption of 23.3 MWh/a for heating) of natural gas in the Walloon Region, respectively (CWAPE, 2016).

Table 2-6: Economic value of products from brewery residues on an annual basis

Residue	**Production** ($t_{residue}$/a)	**Acidogenic conversion**		**Methanogenic conversion**		
		tVFA production (kg_{COD_tVFA}/a)	**Value** (€/a)	**CH_4 production** (m^3_{CH4}/a)	**Energetic output** (MWh/a)	**Value** (€/a)
Trub	80	13,190	13,709	7,141	71	2,294
Spent grain	200	15,847	13,315	17,870	178	5,742
Spent yeast	24	2,900	2,228	1,694	17	544
Total	304	31,930	29,252	26,705	266	8,580

a: annual

Table 2-6 shows that the potential value of the residues of this small artisanal brewery amounts to around 8,600 €/a if they are valorised for the production of methane and approximately 29,250 €/a if they are valorised for the production of VFA. VFA are indeed a higher added value product (in this case

more than three times higher) due to their potential use in finer applications (e.g. green chemistry) compared to energy production from methane.

This constitutes an oversimplified calculation that only considers the value of the final product. The fermentation and downstream processing costs that are required to harness it in its usable form are not considered. Indicatively, downstream costs for pure fermentations are estimated to be approximately 30-40% of the total production costs (Straathof, 2011); this value is expected to increase for mixed fermentations, due to the complexity of the fermentation broth. On the other hand, potential price premiums or other type of state support for bio-based products is also not included. In a biorefinery context, the residues' added value could increase even more if the non-converted COD of the substrate after acidogenic conversion is further digested for methane production.

In the entire Walloon Region, Belgium, artisanal breweries like the one in this study have an annual production capacity of approximately 778,200 hl[5]. Assuming, for simplification, a similar ratio of residue production, the value of residues could amount to up to 3.7 M€/a for the entire region in the case of VFA and 1.1 M€ in the case of methane. The respective values for the entire Belgium territory are approximately 2,488,600 hl production capacity, 11.7 M€/a VFA potential and 3.4 M€/a methane potential.

As a benchmarking value we refer to the PRODCOM annual data 2015 of Eurostat for two product categories that are the closest to the ones of this study, but still not fully representative: (i) product code 20143220: mono-, di- or tri-chloroacetic acids; *propionic, butanoic and pentanoic acids*; their salts and esters; and (ii) product code 20143271: *acetic acid*). The value of sold production for those product codes in Belgium amounts to 36.5 M€ (EUROSTAT, 2016).

2.4 Conclusions

This study highlighted the relevance of both conversion processes for organic waste valorisation of substrates with high solids content. For all substrates, acidogenic conversion is systematically lower that methanogenic conversion (from 35 to 65%), indicating an inhibition of the process of acidogenesis, probably as a result of substrate overloading and product concentration. Nevertheless, the product of acidogenic conversion (VFA streams) has a higher economic value, but also a costlier and technically more demanding

[5]Dr. Gifty Agboton, Université de Namur – Personnal Communication

downstream processing requirement.

The tVFA concentrations and substrate-to-tVFA conversion yields presented here are among the highest reported in literature for batch experiments, with trub performing better than any other substrate. Particularly interesting in trub is the production of caproic acid, a VFA with a high economic value due to its potential applications. Therefore, trub is a substrate that merits further research and together with the other brewery residues, they exhibit a considerable valorisation potential, basing on product value.

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2.6 References

Agler, M.T., Wrenn, B.A., Zinder, S.H., Angenent, L.T., 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends Biotechnol. 29, 70–78. https://doi.org/10.1016/j.tibtech.2010.11.006

Agler, M.T., Spirito, C.M., Usack, J.G., Werner, J.J., Angenent, L.T., 2014. Development of a highly specific and productive process for n-caproic acid production: applying lessons from methanogenic microbiomes. Water Sci. Technol. 69, 62–68. https://doi.org/10.2166/wst.2013.549

Aguedo, M., Kohnen, S., Rabetafika, N., Vanden Bossche, S., Sterckx, J., Blecker, C., Beauve, C., Paquot, M., 2012. Composition of by-products from cooked fruit processing and potential use in food products. Journal of Food Composition and Analysis 27, 61–69. https://doi.org/10.1016/j.jfca.2012.04.005

Alkaya, E., Demirer, G.N., 2011. Anaerobic acidification of sugar-beet processing wastes: Effect of operational parameters. Biomass and Bioenergy 35, 32–39. https://doi.org/10.1016/j.biombioe.2010.08.002

Angelidaki, I., Karakashev, D., Batstone, D.J., Plugge, C.M., Stams, A.J.M., 2011. Biomethanation and its potential. Meth. Enzymol. 494, 327–351. https://doi.org/10.1016/B978-0-12-385112-3.00016-0

Angenent, L.T., Richter, H., Buckel, W., Spirito, C.M., Steinbusch, K.J.J., Plugge, C.M., Strik, D.P.B.T.B., Grootscholten, T.I.M., Buisman, C.J.N., Hamelers, H.V.M., 2016. Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology to Produce Biochemicals. Environ. Sci. Technol. 50, 2796–2810. https://doi.org/10.1021/acs.est.5b04847

Ariunbaatar, J., Panico, A., Frunzo, L., Esposito, G., Lens, P.N.L., Pirozzi, F., 2014. Enhanced anaerobic digestion of food waste by thermal and ozonation

pretreatment methods. Journal of Environmental Management 146, 142–149. https://doi.org/10.1016/j.jenvman.2014.07.042

Arslan, D., Steinbusch, K.J.J., Diels, L., Hamelers, H.V.M., Strik, D.P.B.T.B., Buisman, C.J.N., De Wever, H., 2016. Selective short-chain carboxylates production: A review of control mechanisms to direct mixed culture fermentations. Critical Reviews in Environmental Science and Technology 46, 592–634. https://doi.org/10.1080/10643389.2016.1145959

Arslan, D., Zhang, Y., Steinbusch, K.J.J., Diels, L., Hamelers, H.V.M., Buisman, C.J.N., De Wever, H., 2017. In-situ carboxylate recovery and simultaneous pH control with tailor-configured bipolar membrane electrodialysis during continuous mixed culture fermentation. Separation and Purification Technology 175, 27–35. https://doi.org/10.1016/j.seppur.2016.11.032

Batstone, D.J., Jensen, P.D., 2011. Anaerobic Processes, in: Wilderer, P. (Ed.), Treatise on Water Science. Elsevier, Oxford, pp. 615–639. https://doi.org/10.1016/B978-0-444-53199-5.00097-X

Colussi, I., Cortesi, A., Gallo, V., Vitanza, R., 2016. Biomethanization of Brewer's spent grain evaluated by application of the Anaerobic Digestion Model No.1. Environ. Prog. Sustainable Energy 35, 1055–1060. https://doi.org/10.1002/ep.12326

CWAPE (Commission Wallone pour l'Energie), 2016. Price Observatory, http://www.compacwape.be/proc/simulation;jsessionid=CC97C882B88AB3640A64216C70B0F576?execution=e1s2 (accessed 29/04/2017).

Cysneiros, D., Banks, C.J., Heaven, S., Karatzas, K.-A.G., 2012. The effect of pH control and "hydraulic flush" on hydrolysis and Volatile Fatty Acids (VFA) production and profile in anaerobic leach bed reactors digesting a high solids content substrate. Bioresource Technology 123, 263–271. https://doi.org/10.1016/j.biortech.2012.06.060

Dong, L., Zhenhong, Y., Yongming, S., Xiaoying, K., Yu, Z., 2009. Hydrogen production characteristics of the organic fraction of municipal solid wastes by anaerobic mixed culture fermentation. International Journal of Hydrogen Energy 34, 812–820. https://doi.org/10.1016/j.ijhydene.2008.11.031

Donoso-Bravo, A., Bindels, F., Gerin, P.A., Vande Wouwer, A., 2015. Anaerobic biodegradability of fish remains: experimental investigation and parameter estimation. Water Science and Technology 71, 922–928. https://doi.org/10.2166/wst.2015.047

Espinoza-Escalante, F.M., Pelayo-Ortiz, C., Gutiérrez-Pulido, H., González-Alvarez, V., Alcaraz-González, V., Bories, A., 2008. Multiple response optimization analysis for pretreatments of Tequila's stillages for VFAs and hydrogen production. Bioresour. Technol. 99, 5822–5829. https://doi.org/10.1016/j.biortech.2007.10.008

EUROSTAT, PRODCOM - Annual Data 2015, 2016. http://ec.europa.eu/eurostat/web/prodcom/data/excel-files-nace-rev.2, accessed 29/04/2017.

EUROSTAT, Waste Statistics, 2017.

http://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do, accessed 26/08/2017.

Federici, F., Fava, F., Kalogerakis, N., Mantzavinos, D., 2009. Valorisation of agro-industrial by-products, effluents and waste: concept, opportunities and the case of olive mill wastewaters. J. Chem. Technol. Biotechnol. 84, 895–900. https://doi.org/10.1002/jctb.2165

Gameiro, T., Lopes, M., Marinho, R., Vergine, P., Nadais, H., Capela, I., 2016. Hydrolytic-Acidogenic Fermentation of Organic Solid Waste for Volatile Fatty Acids Production at Different Solids Concentrations and Alkalinity Addition. Water Air Soil Pollut 227, 391. https://doi.org/10.1007/s11270-016-3086-6

Grootscholten, T.I.M., Kinsky dal Borgo, F., Hamelers, H.V.M., Buisman, C.J.N., 2013. Promoting chain elongation in mixed culture acidification reactors by addition of ethanol. Biomass and Bioenergy 48, 10–16. https://doi.org/10.1016/j.biombioe.2012.11.019

Han, S.-K., Shin, H.-S., 2002. Enhanced acidogenic fermentation of food waste in a continuous-flow reactor. Waste Manag Res 20, 110–118. https://doi.org/10.1177/0734242X0202000202

Jang, Y.-S., Kim, B., Shin, J.H., Choi, Y.J., Choi, S., Song, C.W., Lee, J., Park, H.G., Lee, S.Y., 2012. Bio-based production of C2-C6 platform chemicals. Biotechnol. Bioeng. 109, 2437–2459. https://doi.org/10.1002/bit.24599

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

Kim, D.-H., Kim, S.-H., Jung, K.-W., Kim, M.-S., Shin, H.-S., 2011a. Effect of initial pH independent of operational pH on hydrogen fermentation of food waste. Bioresource Technology 102, 8646–8652. https://doi.org/10.1016/j.biortech.2011.03.030

Kim, D.-H., Kim, S.-H., Kim, H.-W., Kim, M.-S., Shin, H.-S., 2011b. Sewage sludge addition to food waste synergistically enhances hydrogen fermentation performance. Bioresource Technology 102, 8501–8506. https://doi.org/10.1016/j.biortech.2011.04.089

Kleerebezem, R., van Loosdrecht, M.C., 2007. Mixed culture biotechnology for bioenergy production. Current Opinion in Biotechnology 18, 207–212. https://doi.org/10.1016/j.copbio.2007.05.001

Komemoto, K., Lim, Y.G., Nagao, N., Onoue, Y., Niwa, C., Toda, T., 2009. Effect of temperature on VFA's and biogas production in anaerobic solubilization of food waste. Waste Management 29, 2950–2955. https://doi.org/10.1016/j.wasman.2009.07.011

Kucek, L.A., Xu, J., Nguyen, M., Angenent, L.T., 2016. Waste Conversion into n-Caprylate and n-Caproate: Resource Recovery from Wine Lees Using Anaerobic Reactor Microbiomes and In-line Extraction. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01892

Labatut, R.A., Angenent, L.T., Scott, N.R., 2011. Biochemical methane potential and

biodegradability of complex organic substrates. Bioresource Technology 102, 2255–2264. https://doi.org/10.1016/j.biortech.2010.10.035

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

López-Garzón, C.S., Straathof, A.J.J., 2014. Recovery of carboxylic acids produced by fermentation. Biotechnology Advances 32, 873–904. https://doi.org/10.1016/j.biotechadv.2014.04.002

Mathias, R. dos S., Pedro, P.M. de M., Eliana, F.C.S., 2014. Solid wastes in brewing process: A review. Journal of Brewing and Distilling 5, 1–9. https://doi.org/10.5897/JBD2014.0043

Mussatto, S.I., 2009. Biotechnological Potential of Brewing Industry By-Products, in: Singh nee' Nigam, P., Pandey, A. (Eds.), Biotechnology for Agro-Industrial Residues Utilisation. Springer Netherlands, Dordrecht, pp. 313–326. https://doi.org/10.1007/978-1-4020-9942-7_16

Nieto, P.P., Hidalgo, D., Irusta, R., Kraut, D., 2012. Biochemical methane potential (BMP) of agro-food wastes from the Cider Region (Spain). Water Sci. Technol. 66, 1842–1848. https://doi.org/10.2166/wst.2012.372

Oreopoulou, V., Tzia, C., 2007. Utilization of Plant By-products for the recovery of Proteins, Dietary Fibers, Antioxidants and Colorants, in: Oreopoulou, V., Russ, W., (Eds), Utilization of By-Products and Treatment of Waste in the Food Industry. Springer, New York, pp. 209-232.

Parawira, W., Murto, M., Read, J.S., Mattiasson, B., 2004. Volatile fatty acid production during anaerobic mesophilic digestion of solid potato waste. J. Chem. Technol. Biotechnol. 79, 673–677. https://doi.org/10.1002/jctb.1012

Perimenis, A., van Aarle, I.M., Nicolay, T., Jacquet, N., Meyer, L., Richel, A., Gerin, P.A., 2016. Metabolic profile of mixed culture acidogenic fermentation of lignocellulosic residues and the effect of upstream substrate fractionation by steam explosion. Biomass Conversion and Biorefinery 6, 25–37. https://doi.org/10.1007/s13399-015-0164-8

Ravindran, R., Jaiswal, A.K., 2016. Exploitation of Food Industry Waste for High-Value Products. Trends in Biotechnology 34, 58–69. https://doi.org/10.1016/j.tibtech.2015.10.008

Rincón, B., Sánchez, E., Raposo, F., Borja, R., Travieso, L., Martín, M.A., Martín, A., 2008. Effect of the organic loading rate on the performance of anaerobic acidogenic fermentation of two-phase olive mill solid residue. Waste Management 28, 870–877. https://doi.org/10.1016/j.wasman.2007.02.030

Rodríguez, J., Kleerebezem, R., Lema, J.M., van Loosdrecht, M.C.M., 2006. Modeling product formation in anaerobic mixed culture fermentations. Biotechnol. Bioeng. 93, 592–606. https://doi.org/10.1002/bit.20765

Shen, L., Hu, H., Ji, H., Cai, J., He, N., Li, Q., Wang, Y., 2014. Production of poly(hydroxybutyrate–hydroxyvalerate) from waste organics by the two-stage process: Focus on the intermediate volatile fatty acids. Bioresource Technology 166, 194–200. https://doi.org/10.1016/j.biortech.2014.05.038

Singhania, R.R., Patel, A.K., Christophe, G., Fontanille, P., Larroche, C., 2013. Biological upgrading of volatile fatty acids, key intermediates for the valorization of biowaste through dark anaerobic fermentation. Bioresour. Technol. 145, 166–174. https://doi.org/10.1016/j.biortech.2012.12.137

Sosa-Hernández, O., Parameswaran, P., Alemán-Nava, G.S., Torres, C.I., Parra-Saldívar, R., 2016. Evaluating biochemical methane production from brewer's spent yeast. J Ind Microbiol Biotechnol 43, 1195–1204. https://doi.org/10.1007/s10295-016-1792-0

Spirito, C.M., Richter, H., Rabaey, K., Stams, A.J., Angenent, L.T., 2014. Chain elongation in anaerobic reactor microbiomes to recover resources from waste. Current Opinion in Biotechnology 27, 115–122. https://doi.org/10.1016/j.copbio.2014.01.003

Straathof AJJ, 2011. The proportion of downstream costs in fermentative production processes. In: Moo-Young M, editor. Comprehensive biotechnology. 2nd ed. Elsevier, p. 811–4.

Traverso, P., Pavan, P., Bolzonella, D., Innocenti, L., Cecchi, F., Mata-Alvarez, J., 2000. Acidogenic fermentation of source separated mixtures of vegetables and fruits wasted from supermarkets. Biodegradation 11, 407–414.

van Aarle, I.M., Perimenis, A., Lima-Ramos, J., de Hults, E., George, I.F., Gerin, P.A., 2015. Mixed inoculum origin and lignocellulosic substrate type both influence the production of volatile fatty acids during acidogenic fermentation. Biochemical Engineering Journal 103, 242–249. https://doi.org/10.1016/j.bej.2015.07.016

Vasudevan, D., Richter, H., Angenent, L.T., 2014. Upgrading dilute ethanol from syngas fermentation to n-caproate with reactor microbiomes. Bioresour. Technol. 151, 378–382. https://doi.org/10.1016/j.biortech.2013.09.105

Veeken, A., Kalyuzhnyi, S., Scharff, H., Hamelers, B., 2000. Effect of pH and VFA on Hydrolysis of Organic Solid Waste. Journal of Environmental Engineering 126, 1076–1081. https://doi.org/10.1061/(ASCE)0733-9372(2000)126:12(1076)

Zacharof, M.-P., Lovitt, R.W., 2013. Complex Effluent Streams as a Potential Source of Volatile Fatty Acids. Waste Biomass Valor 4, 557–581. https://doi.org/10.1007/s12649-013-9202-6

Zacharof, M.-P., Lovitt, R.W., 2014. Recovery of volatile fatty acids (VFA) from complex waste effluents using membranes. Water Sci. Technol. 69, 495–503. https://doi.org/10.2166/wst.2013.717

3 Metabolic profile of mixed culture acidogenic fermentation of lignocellulosic residues and the effect of upstream substrate fractionation by steam explosion

Comment

This study was part of a biocascading scheme to exploit the largest possible fraction of a given feedstock. Steam explosion was used upstream (and independently of this study) to fractionate the lignocellulosic material and recover the hemicellulose for further processing into food applications. The solid remainder after fractionation was the material used for acidogenic fermentation. The motivation was to examine whether acidogenic fermentation is suitable as complementary process in biorefining concepts where upstream treatment has decreased the available digestible fraction of the substrate. Chronologically the second study in this thesis, the pH in this study was not adjusted, following the experience of Chapter 4 (first study chronologically), where adjustment to 6.5 led to VFA loss. The basic mechanical treatment for size reduction before steam explosion was hammer milling and similar to Chapter 4, substrates were tested in the form in which they were delivered.

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*Corresponding author

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Abstract

Lignocellulosic biomass residues have attracted attention for the sustainable production of molecules for material and energetic use through biochemical conversion. Their recalcitrant structure prevents a broader use and asks for the development of sustainable techniques that can efficiently separate, recover and valorize the constituting components. In a cascading concept, residual streams of such processes can be further exploited in an attempt to valorise the largest possible fraction of the initial material. Three lignocellulosic substrates, namely dried sugar beet pulp, wheat bran and miscanthus straw were upstream fractionated by steam explosion to extract the hemicellulose fraction. This study evaluated the valorisation of the residual solid fraction through mixed acidogenic fermentation for the production of volatile fatty acids (VFA) as platform chemicals. Batch experiments have been conducted for the reference material (non-treated) and the solid fraction remaining after steam explosion, with and without the addition of an external mixed inoculum. Steam explosion residues contained less hemicellulose than the initial materials. The difference in the fermentation profile between steam explosion residues and non-treated substrates is dependent on the substrate. Maximum total VFA (tVFA) concentration was 18.8 $g_{COD}/kg_{mixed\_liquor}$ and maximum yield of chemical oxygen demand (COD) conversion into tVFA was 33% for the case of non-treated inoculated beet pulp.

Keywords

Steam explosion, mixed culture fermentation, acidogenesis, lignocellulose, volatile fatty acids, cascade biorefining

Abbreviations

W: wheat bran; B: dried sugar beet pulp; M: miscanthus straw; VFA: volatile fatty acids; tVFA: total VFA; CODs: soluble chemical oxygen demand; CODt: total chemical oxygen demand; ML: mixed liquor; TS: total solids; VS: volatile Solids

3.1 Introduction

During the last 15 years, the concept of biorefining has received increasing attention as an opportunity to produce an array of marketable products from different types of biomass by integrated processes (Kaparaju *et al*., 2009). Special attention has been given to the efficient utilisation of lignocellulosic feedstock, including agroindustrial residues, in light of general concerns on biomass availability, competition with food and sustainability of production. Treatment of biomass residual fractions for the production of "green" products increases their value and reduces the amount of waste (Federici *et al*., 2009).

The challenge for a broad use of lignocellulosic residues as a source of bio-based products lies in the inherent complexity and recalcitrance of the lignocellulosic structure (Zhao *et al*., 2012). In order to deal with this challenge, a series of techniques have been developed that will either break-down the entire structure or selectively extract some of its components and thus permit further valorisation (Hendriks and Zeeman, 2009; Mosier *et al*., 2005; Taherzadeh and Karimi, 2008). Steam explosion can be applied in order to fractionate lignocellulosic materials, recover the soluble fraction for further exploitation and/or improve the digestibility of the materials. It involves the heating of the material at elevated steam temperatures (in the area of 160-240°C) and limited residence times (up to 10 min) followed by sudden pressure release that causes the "explosion" of the material by internal steam expansion and the disruption of its structure (Guo *et al*., 2011; Han *et al*., 2010; Ramos, 2003; Viola *et al*., 2008; Wang *et al*., 2010). Steam explosion has been reported as an efficient method for the selective recovery of hemicelluloses (Sabiha-Hanim *et al*., 2015), a biopolymer with diverse applications (Rabetafika *et al*., 2014). Within the cascading principle, the remainder solid material after steam explosion, a cellulose-lignin-rich fraction, can be further valorised through biochemical processes for the production of bio-based products.

Anaerobic digestion has been widely used for the treatment of organic residual fractions mainly for the production of methane (Angelidaki *et al*., 2011). It is a complex process involving a large diversity of microorganisms in different stages. Acidogenic fermentation (acidogenesis) is the second intermediate stage of anaerobic digestion, where soluble substances are converted to molecules like volatile fatty acids (VFA), ethanol, lactic acid, hydrogen and carbon dioxide. Acidogenic fermentation has been primarily studied as part of the anaerobic fermentation (mostly of wastewaters) in order to enhance the production of methane (De la Rubia *et al*., 2009; Doğan and Demirer, 2009; Kim *et al*., 2010). It has been less often investigated as a stand-alone process, though it could be seen individually as part of a biorefining concept for the production of chemical building blocks (Alkaya and Demirer, 2011). VFA

could be used in a variety of applications (e.g. platform chemicals for the "green" chemistry, production of bioplastics such as polyhydroxyalkanoates, biological nutrient removal in wastewaters, monomers for biofuel production) (Lee *et al*., 2014; Singhania *et al*., 2013; Zacharof and Lovitt, 2013).

Acidogenic fermentation is a natural process that involves a wide range of microorganisms that, under natural conditions in the environment, grow as mixed consortia and not as pure cultures. Using mixed culture fermentations provides a high microbial diversity, which allows treating different types and mixtures of substrates under non-sterile conditions for a variety of possible products. On the other side, the synergistic and/or competitive conditions between microorganisms in such systems and the variety of metabolic pathways render the control of the profile of mixed culture fermentations very challenging. (Angelidaki *et al*., 2011; Schmidt *et al*., 2011; Temudo *et al*., 2008)

The present study focuses on the valorisation of the solid residual fraction after steam explosion of three lignocellulosic substrates. Steam explosion was implemented upstream, in the context of a separate investigation that focused on the recovery of the hemicellulose fraction from the substrates. It was not specifically used in order to enhance the digestibility of the material. The steam explosion residues were submitted to acidogenic conversion by mixed microbial cultures. The main goal was to determine the effect of the upstream steam explosion on the digestibility of the residual fractions, on the possibility to achieve an acidogenic conversion of those residues and on their fermentation profiles, i.e., the evolution of pH, VFA concentration, biogas production and composition). As a reference, the fermentation profile of the starting raw materials, i.e. before steam explosion, was also investigated.

3.2 Materials and Methods

3.2.1 Substrate

Three typical agroindustrial lignocellulosic substrates of importance in the Belgian context and with distinctive properties with respect to their polysaccharide and lignin content were examined (Kracher *et al*., 2014; Mayer *et al*., 2014; Reisinger *et al*., 2013): (i) wheat bran substrate, rich in hemicelluloses and residual starch (code: *W*; produced by "les Moulins de Statte", Huy, Belgium), (ii) dried sugar beet pulp substrate mechanically pressed and air-dried in a rotating drum drier at the sugar factory, rich in pectins (code: *B*; produced by "Raffinerie Tirlemontoise", Tienen, Belgium), (iii) miscanthus x giganteus straw substrate, rich in lignocellulose, stripped of leaves, air dried and ensiled, (code: *M*; winter harvest in Tournai, Belgium).

The substrates were first hammer-milled with a 1mm mesh. The milled

substrates will be further referred to as non-treated (code: *nt*). The steam-explosion treatment consisted of heating the milled substrates with steam under a pressure of 18 bar and a temperature of 207 °C for 2 minutes (after reaching the desired pressure and temperature conditions). These conditions are within the range suggested in literature for the recovery of the hemicellulose fraction (Vignon *et al*., 1995; Wu *et al*., 1999) and correspond to a severity factor of approximately 3.6 (Viola *et al*., 2008). Approximately 5.5 kg of steam for 1kg of fresh substrate were used during the process. A sudden release of the pressure at atmospheric levels caused the water solubilised within the particles to vaporize and expand (substrate "explosion"). The solid fraction of the steam exploded substrate (code: *se*) was separated from the liquid through a 25µm nylon filter and dried at 50°C.

3.2.2 Inoculum

An acidogenic mixed culture inoculum impoverished in methanogens was developed from aerobic activated sludge collected at the Chastre municipal wastewater treatment plant (Mont-Saint-Guibert, Belgium). Upon arrival in the laboratory, the sludge was left to settle in the dark at 4°C for two days. The clear supernatant was removed once a day. The concentrated sludge was transferred to a smaller container and kept under anaerobic conditions at 35°C for five days. The pH of the concentrated sludge was then adjusted at 5.5 with 1M HCl. The sludge was then transferred into Scott Duran GL 45 bottles, tightly sealed with a PBT screw-cap containing a PFTE coated silicone seal, and incubated for 1 hour at 70°C in a shaking water bath. It was then left to cool down to room temperature under anaerobic conditions and regular agitation. The pH was re-adjusted at 5.5 just before use as acidogenic inoculum. This inoculum pre-treatment (i.e. acidification, heating) served to favour sporulated acidogenic populations (De la Rubia *et al*., 2009; Liu *et al*., 2012). The three substrates were not tested at the same time, so three different batches of inoculum (from the same source and receiving the same pre-treatment) were used.

3.2.3 Equipment

Bioreactors consisted of 250 mL Schott Duran GL 45 bottle with a lateral glass tube at the level of filling for 250 mL. A 2-way Luer polycarbonate valve (Fisher Scientific) was connected at the extremity of the glass tube. The bioreactor-bottle was closed with a PBT screw-cap, containing a PTFE coated silicone seal. Each bioreactor was checked to be airtight and resistant to internal pressure before each experimental use.

3.2.4 Experimental procedure

Three identical experiments were performed, one for each substrate. Each

experiment included five series of triplicate reactors (15 reactors for each experiment). The five series represent different combinations of substrate and inoculum, namely: (i) mix of steam-exploded substrate and inoculum (code: *Sse_X*), (ii) mix of non-treated substrate and inoculum, as a reference (code: *Snt_X*), (iii) control of inoculum (code: X_S), (iv) control of steam-exploded substrate (code: *Sse*), (v) control of non-treated substrate (code: *Snt*). *X* represents the inoculum and *S* the substrate, i.e., W, B and M for wheat bran, sugar beet pulp and miscanthus, respectively.

Each bioreactor was filled with substrate and/or inoculum and completed with distilled H_2O to 100 ml, in order to reach 48 g_{CODt}/kg_{ML} of substrate and 12 g_{CODt}/kg_{ML} of inoculum in the respective bioreactors. The reactors with the mix contained, therefore, 60 g_{CODt}/kg_{ML} with a COD ratio of 4 $g_{CODt\_substrate}/g_{CODt\_inoculum}$. Before hermetic closure, the bioreactor headspace was flushed for 1 minute with a constant flow of nitrogen gas in order to ensure the absence of oxygen in the bioreactors.

The bioreactors were incubated at 35°C in the dark under batch conditions. The fermentation was monitored for 22 days and samples for analysis were taken at days 0, 1, 2, 3, 7, 10, 15 and 22. Flushing with nitrogen took place each time the reactors were opened (e.g. for sampling). The parameters monitored in order to establish the fermentation profile were: pH, CODs, quantity and composition of biogas (i.e., H_2, CO_2 and CH_4), concentration of metabolites (e.g., ethanol, lactic acid, VFA) and yield of substrate conversion (i.e., g_{COD} of gaseous or soluble metabolite per g_{CODt} of initial substrate).

The samples collected were frozen at -20°C overnight, then thawed and centrifuged (Beckmann Coulter TJ-25 centrifuge) at 15,300 x g for 15 min at 4°C. The supernatant was collected and re-centrifuged under the same conditions and used to measure the CODs and VFA. At each sampling day, the gas produced in each bioreactor was collected in a 50 mL syringe (Terumo Europe N.V., Leuven, Belgium) equipped with a polycarbonate 2-way Luer valve (Fischer Scientific) and analysed within one hour. The biogas production was determined by the volume accumulated in the syringe until the piston reached an equilibrium position against the atmospheric pressure. For certain reactors, more than one syringe were extracted from the bioreactor.

3.2.5 Analytical methods

Total solids (TS), volatile solids (VS) and total COD (CODt) were measured according to Standard Methods (Clescerl *et al.*, 1999). Soluble COD (CODs) was measured with the COD Cell Test method (Spectroquant® kits 1.14541.0001 and 1.14555.0001, Spectroquant® ThermoReactor 620, Photometer SQ200, Merck Germany) according to the provider's instructions.

The methods used to analyse cellulose, hemicelluloses and lignin contents are

extensively described in Escarnot *et al.* (2010). Cellulose and lignin content were measured by removal through two non-enzymatic gravimetric methods: Acid Detergent Fibre (ADF; includes cellulose and lignin) and Acid Detergent Lignin (ADL). Then, the Neutral Detergent Fibre (NDF) method provided data on lignin, hemicellulose and cellulose content by subtraction with ADF and ADL. Protein content was determined by the Kjeldahl procedure using a conversion factor of 6.25. For extractives determination, samples were successively extracted with hot water (100 ◦C) and hot ethanol in a Soxhlet extractor (Vanderghem *et al*., 2012). The percentage of ashes was determined after combustion of the samples at 525°C for 4 h (Vanderghem *et al*., 2012).

Extraction and pectin characterization is described in (Combo *et al.*, 2013). Galacturonic acid content was determined by a high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The chromatographic system was a Dionex ICS-3000 model (Sunnyvale, CA, USA) equipped with an ED-3000 electrochemical detector, and a SP gradient pump. The column was a Dionex CarboPac PA-100 (250 mm×4 mm) coupled to a CarboPac guard column (40 mm × 4 mm). The mobile phase consisted of 100 mM sodium hydroxide (eluent A), 600 mM sodium acetate in 100 mM sodium hydroxide (eluent B) and 500 mM sodium hydroxide (eluent C). The flow-rate was 1 ml/min and the injection volume was 25 µl.

Free sugars were determined as alditol acetates by GC. Reduction of monosaccharides and acetylation was performed according to the procedure described in (Blakeney *et al.*, 1983). Analyses were carried out with a Hewlett-Packard (HP 6890) gas chromatograph equipped with a flame ionization detector (GC-FID). The components were separated using a high performance capillary column, HP1-methylsiloxane (30 cm×320 µm, 0.25 µm, Scientific Glass Engineering, S.G.E. Pty. Ltd., Melbourne, Australia). Data were analyzed using ChemHP software. 2-Deoxyglucose (internal standard), glucose, xylose, arabinose, mannose and galactose were obtained from Sigma–Aldrich (St. Louis, USA). Solutions of known concentrations were used as standards (Vanderghem *et al*., 2012).

Composition of the produced biogas was analysed by gas chromatography (Trace GC, ThermoFinnigan, Interscience, Belgium) with a thermal conductivity detector (GC-TCD). The chromatograph combined two columns: the first column (Porapak Q; 3m x 1/8'; 50/80 mesh; Chrompack NR-62337-9) permitted to separate CO_2 from the other gases and was followed by a second column (MolecularSieve 5A; 2m x 2m; Restek 80429-800) which separated and analysed H_2, O_2, N_2 and CH_4. The elution was performed under isotherm conditions at 70 °C with argon as carrier gas at 30 ml/min. The detector was heated at 220 °C and the filament at 350 °C.

VFA were analysed by gas chromatography (Trace GC, ThermoFinnigan, Interscience, Belgium) with a flame ionisation detector (GC-FID). The column was packed with Carbowax (2m x 1/8', 4% Carbowax 20M on Carbograph 1-DA, 80/120 mesh, Alltech). The temperature of the injector and the detector was 250 °C. The sequence for the oven temperature started with an initial temperature of 140 °C at injection, then rising up to 180 °C at a rate 4 °C/min and maintained for 10 min, then rising up to 190 °C at a rate of 10 °C/min and maintained for 3 min and decreasing back to 140 °C at 10 °C/min and maintained for 1 min. The carrier gas was helium at 1.5 ml/min.

For the statistical treatment of the results, the mean value and the standard deviation were used when three values were available and the mean value and the deviation from the mean when two values were available.

3.3 Results

3.3.1 Substrate characterisation

The composition and basic properties of the substrates are summarised in Table 3-1.

Table 3-1: Characterisation of the dry substrates

W: wheat bran; B: sugar beet pulp; M: miscanthus; se: steam-exploded; nt: non-treated; TS: Total Solids; FM: Fresh Matter; VS: Volatile solids; CODt: Total chemical oxygen demand; Average values and standard deviations of 3 determinations

Parameter	W_{nt}	W_{se}	B_{nt}	B_{se}	M_{nt}	M_{se}
TS ($\%_{FM}$)	89.4 ± 0.5	96.1 ± 0.5	90.4 ± 0.6	95.3 ± 0.5	91.1 ± 0.2	94.7 ± 0.2
VS ($\%_{TS}$)	93.7 ± 0.1	94.5 ± 0.0	94.0 ± 0.1	92.4 ± 0.0	97.1 ± 0.0	97.4 ± 0.1
CODt (g_{COD}/g_{VS})	1.33 ± 0.01	1.38 ± 0.01	1.19 ± 0.01	1.32 ± 0.02	1.27 ± 0.01	1.31 ± 0.01
Cellulose ($\%_{TS}$)	11.0 ± 0.8	13.3 ± 0.4	20.6 ± 0.2	31.4 ± 0.2	52.2 ± 1.2	59.4 ± 14.3
Hemicellulose ($\%_{TS}$)	44.1 ± 1.4	25.3 ± 0.1	32.1 ± 0.9	7.3 ± 0.3	29.4 ± 0.5	13.6 ± 2.7
Lignin ($\%_{TS}$)	7.8 ± 0.1	10.0 ± 0.7	2.7 ± 0.3	8.9 ± 0.2	14.4 ± 0.7	15.4 ± 2.5
Proteins ($\%_{TS}$)	17.8 ± 0.1	19.4 ± 0.1	9.6 ± 0.1	12.6 ± 0.1	1.3 ± 0.0	1.5 ± 0.0
Ash ($\%_{TS}$)	5.6 ± 0.1	5.2 ± 0.1	5.1 ± 0.1	7.2 ± 0.1	2.5 ± 0.1	2.4 ± 0.1
Starch ($\%_{TS}$)	15.4 ± 0.7	0.3 ± 0.1	-	-	-	-
Free sugars ($\%_{TS}$)	0.9 ± 0.1	1.1 ± 0.1	0.1 ± 0.1	3.4 ± 0.1	0.3 ± 0.1	0.9 ± 0.1
Pectin ($\%_{TS}$)	-	-	11.2 ± 0.1	3.5 ± 0.1	-	-

3.3.2 pH

Figure 3-1 presents the evolution of pH during the fermentation under the different conditions tested. All substrates subjected to steam explosion were more acid at the beginning of the fermentation, as compared to their non-treated forms. All substrates tended to acidify and remain acid (pH 3.5-4.5)

with fermentation. The pH of the inoculated substrate was generally slightly less acidic that the corresponding non-inoculated substrate; the pH of non-treated miscanthus even increased after 10 days. The pH of the inocula alone increased immediately after the start of the fermentation and showed a similar evolution for all three inocula.

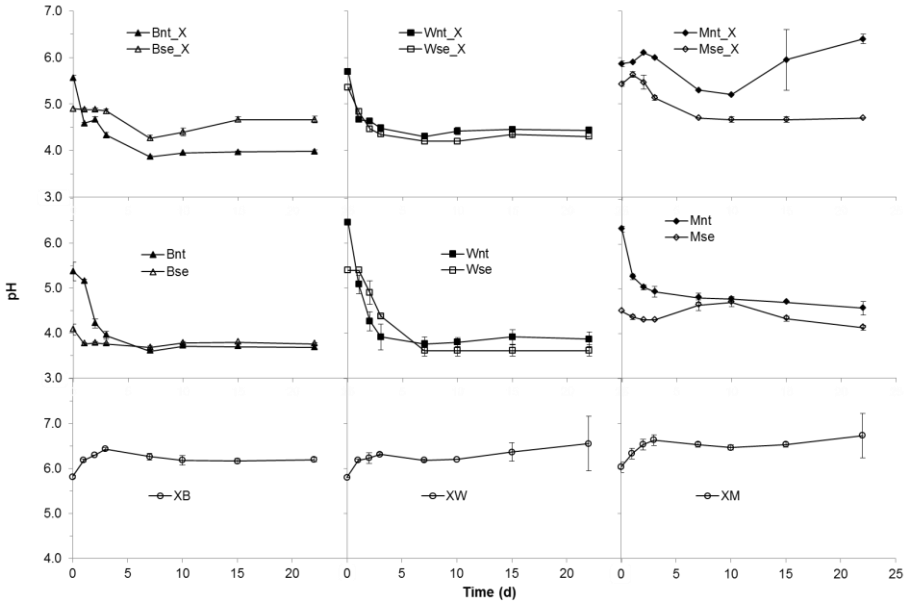

Figure 3-1: Evolution of pH during fermentation of investigated substrates

Left column: Beet pulp // Middle column: Wheat Bran // Right column: Miscanthus; Top row: inoculated substrates // middle row: non-inoculated substrates // bottom row: inoculum control; W: wheat bran; B: sugar beet pulp; M: miscanthus; X: inoculum; se: steam-exploded; nt: non-treated

3.3.3 Gas production

The cumulated biogas production is presented in Figure 3-2 as a function of fermentation time. Carbon dioxide and hydrogen are the main gaseous products for beet pulp and wheat bran. Methane is produced only in the inoculum controls and with miscanthus as substrate. Substrate COD conversion to hydrogen was limited to less than 4 % of the initial CODt (dashed lines referring to the right axis in Figure 3-2). When methane is produced (inocula and miscanthus), higher values are found but remain between 3.5 and 7%. Compared to the other substrates, miscanthus produces smaller amounts of biogas. In most cases, the steam exploded substrate produced less gas than the corresponding non-treated substrate. In most cases also, the addition of the inoculum affected the gas production rate and composition.

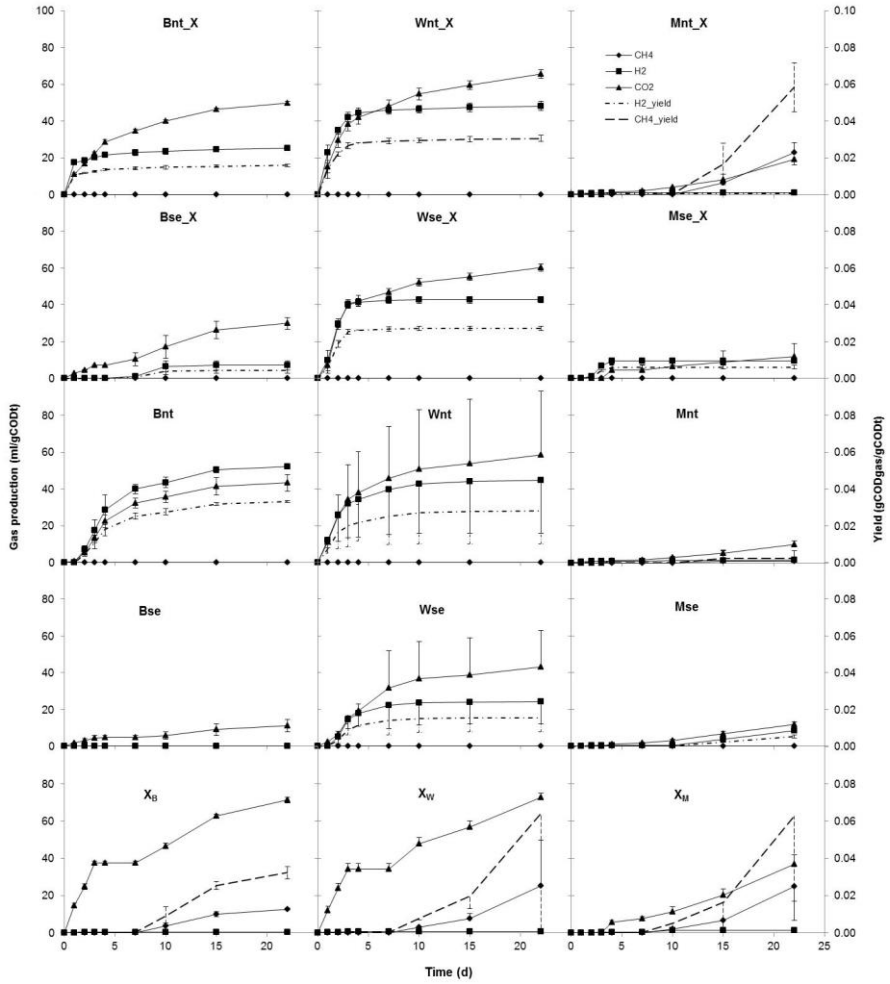

Figure 3-2 : Cumulated gas production during fermentation of investigated substrates

Legend symbols: CH_4 amount - full line/diamond, H_2 amount - full line/square, CO_2 amount - full line/triangle, CH_4 yield - dashed line, H_2 yield - dashed and dotted line
Left column: Beet pulp // middle column : Wheat bran // right column: Miscanthus; 1st & 2nd rows: inoculated substrates // 3rd & 4th rows: substrate controls // 5th row: inoculum controls; Solid lines refer to the left axis (ml/gCODt); Dashed lines refer to the right axis (gCODgas/gCODt); Error bars are occasionally presented only in one side for the sake of the figure's clarity; W: wheat bran; B: sugar beet pulp; M: miscanthus; X: inoculum; se: steam-exploded; nt: non-treated; CODt: total chemical oxygen demand;

3.3.4 Soluble metabolites

Table 3-2 provides an overview of the concentrations of soluble metabolites observed during fermentation of the investigated substrates. Apart from the

aggregated CODs concentration, we also measured the concentrations of individual VFA (i.e., acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, iso-valeric acid), ethanol and lactic acid as typical components of CODs during acidogenesis.

Table 3-2 : Maximum concentrations of soluble metabolites during fermentation of investigated substrates

W: wheat bran; B: sugar beet pulp; M: miscanthus; X: inoculum; se: steam-exploded; nt: non-treated; COD: chemical oxygen demand; CODs: soluble COD; ML: mixed liquor; tVFA: total volatile fatty acids; n.d.: not detected. All concentrations in g_{COD}/kg_{ML}

Series	Acetic acid	Butyric acid	tVFA	Lactic acid	Ethanol	CODs
Bnt_X	10.5 ± 0.6	7.1 ± 0.1	18.8 ± 0.6	0.9 ± 0.0	0.3 ± 0.1	22.2 ± 0.1
Bse_X	2.8 ± 0.3	1.7 ± 0.3	6.7 ± 0.5	0.6 ± 0.6	0.1 ± 0.0	13.7 ± 0.4
Bnt	2.0 ± 0.4	4.8 ± 0.4	6.8 ± 0.5	2.9 ± 0.2	0.1 ± 0.0	12.0 ± 1.6
Bse	0.5 ± 0.0	n.d.	0.8 ± 0.0	0.7 ± 0.1	0.1 ± 0.0	11.5 ± 0.7
Wnt_X	5.2 ± 0.7	7.5 ± 0.2	14.0 ± 0.7	0.4 ± 0.3	0.2 ± 0.0	21.6 ± 0.7
Wse_X	3.0 ± 0.2	5.7 ± 0.5	9.5 ± 0.5	1.7 ± 0.6	0.2 ± 0.0	17.5 ± 0.2
Wnt	1.7 ± 0.1	4.0 ± 1.0	5.8 ± 1.0	0.4 ± 0.4	1.8 ± 0.4	17.9 ± 0.7
Wse	1.0 ± 0.4	2.9 ± 1.8	4.1 ± 1.8	2.3 ± 0.1	0.2 ± 0.1	15.1 ± 0.5
Mnt_X	2.0 ± 0.1	0.4 ± 0.0	2.8 ± 0.1	0.2 ± 0.0	n.d.	3.6 ± 0.1
Mse_X	2.3 ± 0.0	3.3 ± 0.2	6.0 ± 0.2	0.3 ± 0.0	n.d.	7.3 ± 0.3
Mnt	0.6 ± 0.0	0.4 ± 0.0	1.0 ± 0.1	0.1 ± 0.0	n.d.	2.1 ± 0.1
Mse	0.8 ± 0.3	0.5 ± 0.17	1.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	6.5 ± 0.0
X_B	1.5 ± 0.1	0.2 ± 0.0	2.3 ± 0.8	n.d.	n.d.	3.1 ± 0.1
X_W	0.9 ± 0.1	0.3 ± 0.0	1.4 ± 0.5	n.d.	n.d.	2.4 ± 0.7
X_M	1.0 ± 0.1	0.2 ± 0.0	1.5 ± 0.1	0.2 ± 0.0	n.d.	1.9 ± 0.1

The evolution of CODs and the main individual metabolites is presented in Figure 3-3, in terms of yield of conversion of the initial CODt. In all cases, the percentage of CODt that was solubilised did not exceed 50%. For all substrates, the starting value of the CODs was higher after steam explosion (Figure 3-3). However, the increase of the CODs concentration with fermentation was generally lower for steam exploded substrates (Figure 3-3) and maximum concentrations were also lower, with the exception of miscanthus (Table 3-2). The addition of the inoculum improved the CODs concentrations in all cases. More specifically, the steam exploded series of beet pulp show no drastic solubilisation with time, while the CODs of the non-treated series either almost tripled (from 8 to 22 g_{CODs}/kg_{ML} for inoculated) or doubled (from 6 to 12 g_{CODs}/kg_{ML} for non-inoculated) (Figure 3-3, left). For wheat bran, both non-treated and steam-exploded series show a solubilisation with time (Figure 3-3, middle), but the non-treated series present the highest CODs concentrations. For miscanthus, steam explosion has almost tripled the initial CODs concentration to about 7.3 and 6.4 g_{CODs}/kg_{ML} for inoculated and non-inoculated reactors, respectively (Figure 3-3, right). However, there was

virtually no evolution of the CODs, independently of pre-treatment or inoculum presence.

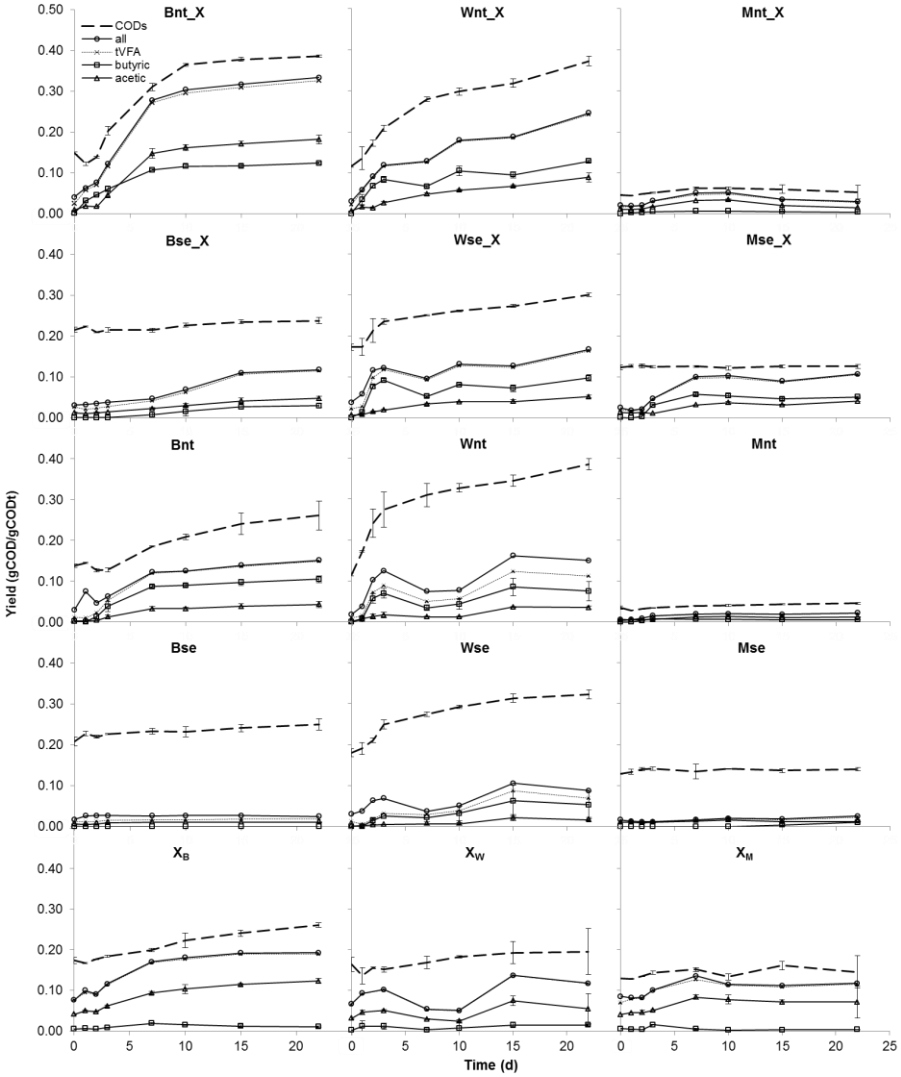

Figure 3-3 : Yield of conversion of the initial CODt into CODs and individual metabolites during fermentation of investigated substrates

Left column: Beet pulp // middle column : Wheat bran // right column: Miscanthus
1st & 2nd rows: inoculated substrates // 3rd & 4th rows: substrate controls // 5th row: inoculum controls
W: wheat bran; B: sugar beet pulp; M: miscanthus; X: inoculum; se: steam-exploded; nt: non-treated; COD: chemical oxygen demand; CODs: soluble COD; CODt: total COD; tVFA: total volatile fatty acids; all: tVFA + ethanol + lactic acid

The production of tVFA follows the same trend as CODs; the rate of increase and the final concentrations are lower for steam exploded beet pulp and wheat bran. On the contrary, steam-explosion has led to higher tVFA concentrations for miscanthus, particularly in the inoculated reactors (Table 3-2). In general, acetic and butyric acid were the predominant VFA in all cases and they also constitute the majority of the metabolites measured (represented by the line "all" in Figure 3-3); only in the case of non-inoculated wheat bran, the concentration of ethanol and lactic acid was not negligible. tVFA are not always the major part of the CODs, since there is a fraction of soluble organic matter that is not identified. Steam explosion has generally resulted in the increase of this undetermined part of CODs. All inoculated series have higher tVFA concentrations as compared to their corresponding non-inoculated ones. The highest concentrations and conversion yields are generally observed at the end of fermentation.

The inocula controls show a slight increase of the conversion yield of CODt into CODs and tVFA. Concentration of tVFA reaches on average approximately 70% of that of CODs.

3.4 Discussion

3.4.1 Influence of steam explosion

Influence on substrate composition

The residual solid materials left after steam explosion show higher relative percentage of lignin and cellulose as compared to the native substrates, while the relative percentage of soluble compounds like hemicellulose, starch and pectins (when relevant) strongly decreased. These results are in general accordance with literature concerning the decrease of hemicellulose and the solubilisation of the organic material by steam explosion (Guo *et al*., 2011; Han *et al*., 2010; Viola *et al*., 2008). Some of that soluble fraction might have remained in the solid residue, thus explaining the higher initial CODs value of all steam-exploded substrates. On the other side, reported results on lignin and cellulose percentage are often contradicting, probably due to the different severities corresponding to the steam explosion conditions (Chang *et al*., 2012; Estevez *et al*., 2012; Viola *et al*., 2008; Wang *et al*., 2010). Steam explosion has apparently also led to some degradation of the original substrate and the release of acidic components (e.g. acetic acid from deacetylation of hemicellulose, formic acid) as suggested by the lower initial pH value of all steam-exploded substrates (Ahring *et al*., 1996; Chang *et al*., 2012). This observation is consistent with the increased acidity of wheat straw after steam explosion (Han *et al*., 2010).

Influence on susceptibility to hydrolysis and fermentation

The majority of the soluble fraction was extracted from the substrate during steam explosion and did not take part later in the fermentation. The apparent loss of this hydrolysable material in the soluble phase after steam explosion had an effect on the susceptibility of the solid remainder to hydrolysis and fermentation. Observations are similar for both inoculated and non-inoculated substrates. Without steam explosion, substrates like wheat bran and beet pulp that have a lower content of recalcitrant lignin and cellulose and a higher content of digestible compounds (hemicellulose, starch, pectins) show an increase in their CODs concentration over time. This suggests that the microbial hydrolysis and solubilisation of the substrate is advancing (Chen *et al*., 2007; Rajagopal and Béline, 2011). The increase of the tVFA concentration and the biogas production observed also indicate their digestibility. Miscanthus on the other hand shows no significant evolution of the CODs and tVFA concentration. This can be attributed to the lignocellulosic structure that renders the material not easily accessible to the microorganisms (Zhao *et al*., 2012).

After steam-explosion, the remaining solid fraction has less hemicellulose (absolute reduction percentage of 53%, 85% and 59% for wheat bran, beet pulp and miscanthus, respectively) and an increased relative proportion of lignin and cellulose as compared to the reference material. The loss of hydrolysable material resulted in a less pronounced solubilisation and VFA production in the case of wheat bran. This decrease in hydrolytic and fermentative activity is more pronounced in the case of beet pulp. Miscanthus still shows no hydrolysis potential. Interestingly, we observe however a small increase in the digestibility of the inoculated miscanthus after steam explosion that is confirmed by an increase in the concentration of tVFA.

In general, the weakening and opening up of the recalcitrant structure of lignocellulosic material after steam explosion has been reported to improve the access to microorganisms and thus its biodigestibility. Guo *et al*. (2011) report an increase of VFA concentration after steam explosion of corn stalk. Estevez *et al*. (2012) report an increase up to 50% of the methane yield after steam explosion of Salix and Bruni *et al*. (2010) report an increased methane potential after steam pretreatment (not explosion) of digested biofibres. Wang *et al*. (2010) also report an increased anaerobic digestibility and an approximately 28% increase of methane production after steam explosion of bulrush.

This study refines this general statement by showing that the effect of steam explosion on the digestibility of a substrate is dependent on its original composition and its respective alteration. This conclusion is consistent with Viola *et al*. (2008) that reported that solubility and digestibility of the material after steam explosion depend on the lignocellulose matrix strengths. For

substrates like wheat bran and beet pulp with lower lignocellulose content, the loss of digestible material after steam explosion has a negative effect on the digestibility of the remaining material. This effect seems more important than the potential gain of digestibility offered through the weakening of the recalcitrant fraction, because this fraction already had a smaller contribution to the digestibility. On the contrary, for a highly recalcitrant substrate like miscanthus, where the loss of digestible material is less significant, the weakening of structure after steam explosion plays a more important role and results in a gain of digestibility.

3.4.2 Influence of inoculum

For all series, the addition of an external inoculum (besides the inherent microbial communities in the substrate) influenced the fermentation profile in a positive way: inoculated series exhibited higher concentrations in tVFA, higher yields of CODt conversion into tVFA, produced more biogas and in some cases showed an increased hydrolysis potential than the corresponding non-inoculated ones. This effect is most obvious in the case of sugar beet pulp, where the hydrolysis and fermentation advanced considerably better in the inoculated reactors. It is also visible, to a lesser extent, in the case of steam-exploded miscanthus, where the digestibility is increased in the presence of the inoculum. This positive effect is confirmed when comparing the difference of tVFA concentrations between non-inoculated and inoculated series with the tVFA concentration of the respective inocula (Table 3-2). The contribution of the inoculum is not limited to the fermentation of its proper organic matter but extends to the improved fermentation of the substrate as well.

Indeed, the addition of an external natural inoculum increases the microbial diversity in the system, reinforces the microbial activity and increases the chances of hydrolysing and converting the initial CODt into fermentation products (Temudo *et al*., 2008). The low water content of each substrate (10% of fresh matter (w/w); Table 3-1) and the upstream preparation of the substrate (e.g. thermal drying in the case of sugar beet pulp) may have contributed to the limited microbial population of the original material, thus leading to a more pronounced effect when adding an external source of microbial activity. The addition of the inoculum also led to a lower acidification of the fermentation liquor for all substrates. This is probably due to the high buffer capacity of the inoculum (activated sludge rich in bicarbonate) and is consistent with the pH increase observed in the inocula controls.

3.4.3 Metabolic profile

Acetic and butyric acid were the main fermentation products in all cases where fermentation occurred. This observation underlines the general reproducibility of metabolites produced by acidogenic fermentation despite the difference of

the substrate. This is consistent with results of other researchers working under similar conditions (Borja *et al*., 2005; Horiuchi *et al*., 2002; Parawira *et al*., 2004). Furthermore, the standard deviations of the three reactors within each condition (i.e. substrate inoculated or not) were in every case less than 5% of the corresponding value for CODs, acetic and butyric acid yields. This also indicates the reproducibility of the specific fermentation, despite the complexity of the liquor. Apparently, the microbial populations responsible for those acids are dominating the fermentation liquor. Beyond this general observation, the composition of the substrate influenced the specific metabolic profile of the fermentation. Butyrate appears to be predominant in all series involving wheat bran as substrate. A possible explanation is the high amount of proteins that are reported to lead to longer chain VFA when degraded (Chen *et al*., 2007). Also, wheat bran has the most reduced organic matter (highest COD/VS ratio, Table 3-1; comparable to 1.42 reported for proteinaceous material (Angelidaki *et al*., 2011)), which is likely to result in more reduced products like butyrate instead of acetate. Furthermore, a high starch percentage (as in the non-treated) is also linked to even-numbered acids from its degradation (Han and Shin, 2002).

Acetate is also present in all fermentation series. It is one of the main VFA associated with degradation of cellulose (Han and Shin, 2002). It is the main product in the beet pulp series. Deacetylation of pectins that are present in the substrate can possibly contribute to its production. Nevertheless, butyrate is the dominating VFA in the non-inoculated beet pulp. One possible explanation is that the heat and drying treatment of the pulp at the sugar factory selected some sporulating butyrate fermenter strains. These strains would be able to control the fermentation profile in the absence of the competing micro-organisms brought by the mixed inoculum.

In most cases where fermentation occurs (namely Bnt_X, Bnt, Wnt_X, Wse_X, Wnt, Wse), VFA production is faster during the first seven days and then continues at a slower rate. In most cases where hydrogen is produced, production is pronounced for the first three days; during this period, butyrate is the predominant metabolite and the butyrate/acetate ratio is elevated (Table 3-3). As Jones and Woods (1986) report, an elevated hydrogen partial pressure leads to a decreased activity of the NADH oxidoreductase & hydrogenase enzyme complex that no longer supports the conversion of reducing equivalents to hydrogen via the acetate pathway; to maintain redox balance, the excess of reducing equivalents needs to be consumed by an alternative pathway, such as the butyrate pathway, with a consequence that the butyrate/acetate ratio is elevated. Carbon dioxide production is also pronounced during this three-day period, probably due to the decarboxylation of pyruvate to acetyl-CoA. After this initial three-day phase, hydrogen production was in most cases negligible, but VFA (particularly acetic acid)

and carbon dioxide continued to be produced in a rather constant ratio (0.018 mol_{CO2}/g_{COD_tVFA} on average for days 10-22 for all series of Table 3-3, ranging from 0.007 to 0.03 mol_{CO2}/g_{COD_tVFA} for *Bnt_X* and *Wse*, respectively), suggesting an equilibration between the metabolic pathways of the two main acids.

Table 3-3: Butyrate/acetate ratio of hydrogen producing series

W: wheat bran; B: sugar beet pulp; X: inoculum; se: steam-exploded; nt: non-treated; COD: chemical oxygen demand;

Series	Butyrate/Acetate (g_{COD}/g_{COD})		
	day 0	day 3	day 22
Wnt_X	0.09	3.13	1.45
Wse_X	0.06	4.66	1.87
Wnt	0.00	3.98	2.13
Wse	0.00	5.23	3.18
Bnt_X	0.14	1.31	0.69
Bnt	0.00	1.19	1.04

Lactic acid and ethanol were in general only marginally produced. Their appearance was not following a specific trend. No correlation was found with the pretreatment of the material and the alteration of its composition. Lactic acid is considered a key intermediate that is subsequently quickly degraded (Batstone *et al*., 2002). Whenever observed in significant quantities (*Bnt*, *Wse*), this occurred during the first two days of fermentation and was consistent with a rapid decrease of pH and a delayed production of hydrogen or carbon dioxide for these series.

With the exception of non-treated inoculated miscanthus (*Mnt_X*), no methane was detected in the series where a substrate was present. Contrary to that, methane was detected in all inocula controls, despite the pre-treatment to inhibit methanogenesis. This observation points out that the pre-treatment of the inoculum is not sufficient to inhibit methanogenic activity. Indeed, the presence of the substrate and its suitability for fermentation contributed to the establishment of conditions unfavourable to methanogens: the pH stayed in a range below 6 and the conversion of CODs into VFA contributed to hold the pH in this area (Batstone and Jensen, 2011).

In the case of Mnt_X, the transition of the acidogenic fermentation to the methanogenic fermentation is evidenced by the methane formation, the increase of pH above 6 and by a decrease in the net yield of tVFA. The latter were probably consumed by the methanogens. The deviation between the pH value of the replicates at day 15 (represented by the error bar, Figure 3-1), highlights the complexity of the fermentation system. This can be attributed to the fact that during the transition to methanogenic activity, the associated

change in the microbial dynamics of the liquor is manifested in a different way between the replicates. At day 22, the deviation is smaller, signifying the wider establishment of methanogenic conditions between the replicates. The same observation is valid for the last days of fermentation for the inocula controls of wheat bran and miscanthus.

The metabolic profile of the inocula control is similar for the three inocula, with the inoculum of beet pulp showing a somewhat better solubilisation and acidogenesis. This resemblance is not unexpected since the origin of the inoculum is the same as is the pretreatment prior to fermentation. Due to the absence of an external substrate, only the organic matter of the inoculum itself is fermented and the concentrations of tVFA achieved are lower (Table 3-2).

3.4.4 Product concentrations & conversion yields for biorefining applications

We observe that in all cases the yield of solubilisation of organic matter is not exceeding 40% of the CODt introduced in the reactors. This means that hydrolysis of the material was not very extensive and this applies to all substrates tested, regardless of the effect of steam explosion. The recalcitrant structure of all substrates is most probably the main reason for the low hydrolysis potential. Moreover, maximum 4% of the CODt introduced in the reactor was converted to hydrogen and maximum 6% to methane (where relevant). A low yield of CODt conversion into gaseous products has also been reported by Bengtsson *et al*. (2008) (less than 4%) and Parawira *et al*. (2004). In our experiments, gaseous products are not significant in the balance of the reducing equivalents of the initial organic matter. However, they are important to monitor in order to interpret the metabolic profiles of the fermentation.

Table 3-4 presents an overview of highest product concentrations and corresponding conversion yields observed in the present and other acidogenic fermentation investigations (batch or continuous) of complex organic residues with high solids content.

Table 3-4 : Product concentrations and conversion yields of acidogenic fermentation of residual organic fractions

W: wheat bran; B: sugar beet pulp; M: miscanthus; X: inoculum; nt: non-treated; se: steam-exploded; COD: chemical oxygen demand; OFMSW: Organic fraction municipal solid waste; n.d.: not determined;

Substrate	**Concentration** (g_{COD_tVFA} /kg_{ML}*)	**Conversion yield** (g_{COD_tVFA}/g_{CODt})	**Acidification degree** (g_{COD_tVFA}/g_{CODs})	**Source**[#]
Bnt_X	18.8	0.33	0.88	This study
Wnt_X	14	0.25	0.65	This study
Vegetable and fruit	24	0.24	0.55	1

waste				
OFMSW	16	0.28	0.74	2
Sunflower cake	24	0.17	0.60	3
Potato waste	19	n.d.	1.00	4
Sugar beet waste mix	3.8	0.47	0.60	5
Mnt_X	2.8	0.05	0.79	This study
Bse_X	6.7	0.12	0.48	This study
Wse_X	9.5	0.17	0.55	This study
Mse_X	6.0	0.11	0.83	This study

** For comparison reasons it is assumed that $1kg_{ML}$ is equivalent to $1l_{ML}$*
[#]1: Traverso et al. (2000); 2: Doğan and Demirer (2009) ; 3 : De la Rubia et al. (2009) ; 4 : Parawira et al. (2004); 5 : Alkaya and Demirer (2011)

Despite the differences in composition, substrates with digestible components (e.g. sunflower cake with high protein content, potato waste with high starch content, wheat bran with significant proteins and starch content, vegetable/fruit waste with high sugar content, sugar beet pulp with high pectin content) exhibit comparable conversion yields. For product concentrations of the same order of magnitude, sugar beet pulp investigated in this study exhibits a relatively high conversion yield (0.33 g_{COD_tVFA}/g_{CODt}). An even higher conversion yield was observed again with a sugar beet pulp mix, with significantly lower, however, product concentrations as a result of dilution of pulp with sugar beet wastewater at equal amounts (Alkaya and Demirer, 2011). On the other hand, substrates with a high lignin and cellulose content (shown separately in Table 3-4) as in the present study, exhibit lower conversion yields. The ratio between tVFA and CODs (here defined as acidification degree) indicates the extent of the acidogenic conversion of the soluble organic matter and whether other soluble metabolites are present but resistant to acidogenesis (Bruni *et al*., 2010). Table 3-4 shows that it can vary considerably between substrates but remains in almost all cases above 50% (even reaching 100%), indicating that VFA constitute the majority of the soluble COD during acidogenic fermentation.

In view of real-life biorefining concepts, the subsequent utilisation of VFA will determine whether the achieved concentrations are sufficient. For applications that require high purity VFA (e.g. in chemical synthesis), concentrations in the magnitude of 20g/l as in our case (i.e., around 2% v/v) are rather low and the expenditure of separation of dilute VFA mixtures is added (Zacharof and Lovitt, 2013). For other applications however (e.g. biological transformations like polyhydroxyalkanoate production or biological nutrient removal) the purity of the product is not a critical factor and VFA can be directly used as mixtures. Furthermore, precise energy and

mass balances are required to prove the feasibility of such integrated cascading processes, in view of biorefining applications at larger scale.

Mixed acidogenic fermentation is envisaged as an advantageous pathway because of its potential to use readily available, zero-cost mixed microbial consortia, treat a variety of complex substrates, valorise the largest part of the substrate and produce an array of marketable metabolites vs. specific and costly enzyme cocktails and/or dedicated microbial strains for hydrolysis and fermentation that can exploit only part of the substrate (i.e. sugars) to yield the desired product (e.g. ethanol). The drawback of this approach is often lower overall conversion yields, also due to product inhibition. Indeed, ethanolic fermentation yields are in the area of 0.3-0.4 $g_{COD\_ethanol}/g_{CODt}$ for different lignocellulosic substrates and different process configurations (Paulova *et al*., 2015). These values are comparable but usually higher than mixed acidogenic fermentation (Table 3-4). Nevertheless, with research focusing now on downstream processing, mixed fermentations are expected to benefit from in-situ recovery processes that will drive the process further towards the product side and increase conversion yields (López-Garzón and Straathof, 2014).

3.5 Conclusions

Solid residues left after steam explosion extraction of valuable substances from lignocellulosic substrates can be used as substrate for acidogenic fermentation, in a concept of cascade biorefining. Steam exploded residues have higher relative percentage of lignin and cellulose and lower hemicellulose. The effect of steam explosion on the acidogenic fermentation profile is substrate-dependent. In general, the solid remainder after steam explosion didn't show a better digestibility than the reference starting material, most probably because most of the hydrolysable and digestible compounds were removed with the steam explosion soluble extract. Among the substrates investigated, beet pulp showed a different fermentation profile after steam explosion and a decreased digestibility. Wheat bran showed a similar fermentation profile after steam explosion and miscanthus showed a slight gain in digestibility. The presence of an external inoculum improved the acidogenic profile for all substrates as a result of the enrichment of the microbial activity. The maximum tVFA concentration was 19 g_{COD}/kg_{ML} and the maximum yield of CODt conversion into tVFA was 33% for the case of non-treated inoculated beet pulp. Inoculum pre-treatment is not sufficient for the prevention of methanogenesis; the fermentation of the substrate and the corresponding VFA production also contribute to that. The loss of COD in the form of gaseous metabolites was not significant. VFA were an important fraction of the CODs but not always the dominant one; acetic and butyric are the main VFA produced, with high reproducibility.

Future investigations will involve monitoring of the microbial community to investigate the precise link with the metabolic profile. The ultimate goal is to master mixed fermentation towards a specific VFA despite the complexity of both the substrate and the microbial consortium.

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3.7 References

Ahring, B.K., Jensen, K., Nielsen, P., Bjerre, A.B., Schmidt, A.S., 1996. Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria. Bioresource Technology 58, 107–113. https://doi.org/10.1016/S0960-8524(96)00090-9

Alkaya, E., Demirer, G.N., 2011. Anaerobic acidification of sugar-beet processing wastes: Effect of operational parameters. Biomass and Bioenergy 35, 32–39. https://doi.org/10.1016/j.biombioe.2010.08.002

Angelidaki, I., Karakashev, D., Batstone, D.J., Plugge, C.M., Stams, A.J.M., 2011. Biomethanation and its potential. Meth. Enzymol. 494, 327–351. https://doi.org/10.1016/B978-0-12-385112-3.00016-0

Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S., Pavlostathis, S., Rozzi, A., Sanders, W., Siegrist, H., Vavilin, V., 2002. The IWA Anaerobic Digestion Model No 1 (ADM1), By the IWA task group for mathematical modelling of anaerobic wastewater processes, IWA Publishing

Batstone, D.J., Jensen, P.D., 2011. Anaerobic Processes, in: Wilderer, P. (Ed.), Treatise on Water Science. Elsevier, Oxford, pp. 615–639. https://doi.org/10.1016/B978-0-444-53199-5.00097-X

Bengtsson, S., Hallquist, J., Werker, A., Welander, T., 2008. Acidogenic fermentation of industrial wastewaters: Effects of chemostat retention time and pH on volatile fatty acids production. Biochemical Engineering Journal 40, 492–499. https://doi.org/10.1016/j.bej.2008.02.004

Blakeney, A.B., Harris, P.J., Henry, R.J., Stone, B.A., 1983. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydrate Research 113, 291–299. https://doi.org/10.1016/0008-6215(83)88244-5

Borja, R., Sánchez, E., Rincón, B., Raposo, F., Martín, M.A., Martín, A., 2005. Study and optimisation of the anaerobic acidogenic fermentation of two-phase olive pomace. Process Biochemistry 40, 281–291. https://doi.org/10.1016/j.procbio.2004.01.002

Bruni, E., Jensen, A.P., Angelidaki, I., 2010. Steam treatment of digested biofibers for increasing biogas production. Bioresour. Technol. 101, 7668–7671. https://doi.org/10.1016/j.biortech.2010.04.064

Chang, J., Cheng, W., Yin, Q., Zuo, R., Song, A., Zheng, Q., Wang, P., Wang, X.,

Liu, J., 2012. Effect of steam explosion and microbial fermentation on cellulose and lignin degradation of corn stover. Bioresource Technology 104, 587–592. https://doi.org/10.1016/j.biortech.2011.10.070

Chen, Y., Jiang, S., Yuan, H., Zhou, Q., Gu, G., 2007. Hydrolysis and acidification of waste activated sludge at different pHs. Water Research 41, 683–689. https://doi.org/10.1016/j.watres.2006.07.030

Clescerl, L.S., Greenberg, A.E., Eaton, A.D. (Eds.), 1999. Standard Methods for Examination of Water & Wastewater, 20th edition. ed. Amer Public Health Assn, Washington, DC.

Combo, A.M.M., Aguedo, M., Quiévy, N., Danthine, S., Goffin, D., Jacquet, N., Blecker, C., Devaux, J., Paquot, M., 2013. Characterization of sugar beet pectic-derived oligosaccharides obtained by enzymatic hydrolysis. International Journal of Biological Macromolecules 52, 148–156. https://doi.org/10.1016/j.ijbiomac.2012.09.006

De la Rubia, M.A., Raposo, F., Rincón, B., Borja, R., 2009. Evaluation of the hydrolytic-acidogenic step of a two-stage mesophilic anaerobic digestion process of sunflower oil cake. Bioresour. Technol. 100, 4133–4138. https://doi.org/10.1016/j.biortech.2009.04.001

Doğan, E., Demirer, G.N., 2009. Volatile Fatty Acid Production from Organic Fraction of Municipal Solid Waste Through Anaerobic Acidogenic Digestion. Environmental Engineering Science 26, 1443–1450. https://doi.org/10.1089/ees.2009.0062

Escarnot, E., Agneessens, R., Wathelet, B., Paquot, M., 2010. Quantitative and qualitative study of spelt and wheat fibres in varying milling fractions. Food Chemistry 122, 857–863. https://doi.org/10.1016/j.foodchem.2010.02.047

Estevez, M.M., Linjordet, R., Morken, J., 2012. Effects of steam explosion and co-digestion in the methane production from Salix by mesophilic batch assays. Bioresource Technology 104, 749–756. https://doi.org/10.1016/j.biortech.2011.11.017

Federici, F., Fava, F., Kalogerakis, N., Mantzavinos, D., 2009. Valorisation of agro-industrial by-products, effluents and waste: concept, opportunities and the case of olive mill wastewaters. J. Chem. Technol. Biotechnol. 84, 895–900. https://doi.org/10.1002/jctb.2165

Guo, P., Mochidzuki, K., Cheng, W., Zhou, M., Gao, H., Zheng, D., Wang, X., Cui, Z., 2011. Effects of different pretreatment strategies on corn stalk acidogenic fermentation using a microbial consortium. Bioresour. Technol. 102, 7526–7531. https://doi.org/10.1016/j.biortech.2011.04.083

Han, S.-K., Shin, H.-S., 2002. Enhanced acidogenic fermentation of food waste in a continuous-flow reactor. Waste Manag Res 20, 110–118. https://doi.org/10.1177/0734242X0202000202

Han, G., Deng, J., Zhang, S., Bicho, P., Wu, Q., 2010. Effect of steam explosion treatment on characteristics of wheat straw. Industrial Crops and Products 31, 28–33. https://doi.org/10.1016/j.indcrop.2009.08.003

Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of

lignocellulosic biomass. Bioresource Technology 100, 10–18. https://doi.org/10.1016/j.biortech.2008.05.027

Horiuchi, J.-I., Shimizu, T., Tada, K., Kanno, T., Kobayashi, M., 2002. Selective production of organic acids in anaerobic acid reactor by pH control. Bioresource Technology 82, 209–213. https://doi.org/10.1016/S0960-8524(01)00195-X

Jones, D.T., Woods, D.R., 1986. Acetone-butanol fermentation revisited. Microbiol Rev 50, 484–524.

Kaparaju, P., Serrano, M., Thomsen, A.B., Kongjan, P., Angelidaki, I., 2009. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresour. Technol. 100, 2562–2568. https://doi.org/10.1016/j.biortech.2008.11.011

Kim, W., Hwang, K., Shin, S.G., Lee, S., Hwang, S., 2010. Effect of high temperature on bacterial community dynamics in anaerobic acidogenesis using mesophilic sludge inoculum. Bioresour. Technol. 101 Suppl 1, S17-22. https://doi.org/10.1016/j.biortech.2009.03.029

Kracher, D., Oros, D., Yao, W., Preims, M., Rezic, I., Haltrich, D., Rezic, T., Ludwig, R., 2014. Fungal secretomes enhance sugar beet pulp hydrolysis. Biotechnology Journal 9, 483–492. https://doi.org/10.1002/biot.201300214

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

Liu, H., Wang, J., Liu, X., Fu, B., Chen, J., Yu, H.-Q., 2012. Acidogenic fermentation of proteinaceous sewage sludge: Effect of pH. Water Research 46, 799–807. https://doi.org/10.1016/j.watres.2011.11.047

López-Garzón, C.S., Straathof, A.J.J., 2014. Recovery of carboxylic acids produced by fermentation. Biotechnology Advances 32, 873–904. https://doi.org/10.1016/j.biotechadv.2014.04.002

Mayer, F., Gerin, P.A., Noo, A., Lemaigre, S., Stilmant, D., Schmit, T., Leclech, N., Ruelle, L., Gennen, J., von Francken-Welz, H., Foucart, G., Flammang, J., Weyland, M., Delfosse, P., 2014. Assessment of energy crops alternative to maize for biogas production in the Greater Region. Bioresour. Technol. 166, 358–367. https://doi.org/10.1016/j.biortech.2014.05.054

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96, 673–686. https://doi.org/10.1016/j.biortech.2004.06.025

Parawira, W., Murto, M., Read, J.S., Mattiasson, B., 2004. Volatile fatty acid production during anaerobic mesophilic digestion of solid potato waste. J. Chem. Technol. Biotechnol. 79, 673–677. https://doi.org/10.1002/jctb.1012

Paulova, L., Patakova, P., Branska, B., Rychtera, M., Melzoch, K., 2015. Lignocellulosic ethanol: Technology design and its impact on process efficiency. Biotechnology Advances, BioTech 2014 and 6th Czech-Swiss Biotechnology Symposium 33, 1091–1107. https://doi.org/10.1016/j.biotechadv.2014.12.002

Rabetafika, H.N., Bchir, B., Blecker, C., Paquot, M., Wathelet, B., 2014. Comparative

study of alkaline extraction process of hemicelluloses from pear pomace. Biomass and Bioenergy 61, 254–264. https://doi.org/10.1016/j.biombioe.2013.12.022

Rajagopal, R., Béline, F., 2011. Anaerobic hydrolysis and acidification of organic substrates: determination of anaerobic hydrolytic potential. Bioresour. Technol. 102, 5653–5658. https://doi.org/10.1016/j.biortech.2011.02.068

Ramos, L.P., 2003. The chemistry involved in the steam treatment of lignocellulosic materials. Química Nova 26, 863–871. https://doi.org/10.1590/S0100-40422003000600015

Reisinger, M., Tirpanalan, Ö., Prückler, M., Huber, F., Kneifel, W., Novalin, S., 2013. Wheat bran biorefinery – A detailed investigation on hydrothermal and enzymatic treatment. Bioresource Technology 144, 179–185. https://doi.org/10.1016/j.biortech.2013.06.088

Sabiha-Hanim, S., Mohd Noor, M.A., Rosma, A., 2015. Fractionation of oil palm frond hemicelluloses by water or alkaline impregnation and steam explosion. Carbohydrate Polymers 115, 533–539. https://doi.org/10.1016/j.carbpol.2014.08.087

Schmidt, J.K., Riedele, C., Regestein, L., Rausenberger, J., Reichl, U., 2011. A novel concept combining experimental and mathematical analysis for the identification of unknown interspecies effects in a mixed culture. Biotechnol. Bioeng. 108, 1900–1911. https://doi.org/10.1002/bit.23126

Singhania, R.R., Patel, A.K., Christophe, G., Fontanille, P., Larroche, C., 2013. Biological upgrading of volatile fatty acids, key intermediates for the valorization of biowaste through dark anaerobic fermentation. Bioresour. Technol. 145, 166–174. https://doi.org/10.1016/j.biortech.2012.12.137

Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review. Int J Mol Sci 9, 1621–1651. https://doi.org/10.3390/ijms9091621

Temudo, M.F., Poldermans, R., Kleerebezem, R., van Loosdrecht, M.C.M., 2008. Glycerol fermentation by (open) mixed cultures: A chemostat study. Biotechnol. Bioeng. 100, 1088–1098. https://doi.org/10.1002/bit.21857

Traverso, P., Pavan, P., Bolzonella, D., Innocenti, L., Cecchi, F., Mata-Alvarez, J., 2000. Acidogenic fermentation of source separated mixtures of vegetables and fruits wasted from supermarkets. Biodegradation 11, 407–414.

Vanderghem, C., Brostaux, Y., Jacquet, N., Blecker, C., Paquot, M., 2012. Optimization of formic/acetic acid delignification of Miscanthus × giganteus for enzymatic hydrolysis using response surface methodology. Industrial Crops and Products 35, 280–286. https://doi.org/10.1016/j.indcrop.2011.07.014

Vignon, M.R., Garcia-Jaldon, C., Dupeyre, D., 1995. Steam explosion of woody hemp chènevotte. International Journal of Biological Macromolecules 17, 395–404. https://doi.org/10.1016/0141-8130(96)81852-6

Viola, E., Zimbardi, F., Cardinale, M., Cardinale, G., Braccio, G., Gambacorta, E., 2008. Processing cereal straws by steam explosion in a pilot plant to enhance digestibility in ruminants. Bioresource Technology 99, 681–689. https://doi.org/10.1016/j.biortech.2007.02.001

Wang, J., Yue, Z.-B., Chen, T.-H., Peng, S.-C., Yu, H.-Q., Chen, H.-Z., 2010. Anaerobic digestibility and fiber composition of bulrush in response to steam explosion. Bioresour. Technol. 101, 6610–6614. https://doi.org/10.1016/j.biortech.2010.03.086

Wu, M.M., Chang, K., Gregg, D.J., Boussaid, A., Beatson, R.P., Saddler, J.N., 1999. Optimization of steam explosion to enhance hemicellulose recovery and enzymatic hydrolysis of cellulose in softwoods. Appl Biochem Biotechnol 77, 47–54. https://doi.org/10.1385/ABAB:77:1-3:47

Zacharof, M.-P., Lovitt, R.W., 2013. Complex Effluent Streams as a Potential Source of Volatile Fatty Acids. Waste Biomass Valor 4, 557–581. https://doi.org/10.1007/s12649-013-9202-6

Zhao, X., Zhang, L., Liu, D., 2012. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. Biofuels, Bioprod. Bioref. 6, 465–482. https://doi.org/10.1002/bbb.1331

4 Mixed inoculum origin and lignocellulosic substrate type both influence the production of volatile fatty acids during acidogenic fermentation

Comment

Chronologically the first study of this thesis, the work conducted here aimed at providing the statistical basis to evaluate the reproducibility of a given fermentation system, by focusing on two major parameters, the substrate and the inoculum. The analysis conducted here was the reason why subsequent experiments (presented in the other chapters of this thesis) were not anymore designed to accommodate a potential statistical treatment, rather than practical implications of dealing with a large number of reactors (e.g. duplicates instead of triplicates). Substrates were "imposed" by the funding project and were tested as delivered. The pH selected was relatively high, approximately 6.5, considering that, in the absence of competition by methanogenic archaea, this pH area should be favourable also for acidogenic fermentation. The net decrease in VFA concentration, however, indicates the development of methanogenic activity, even though our experimental set-up did not allow measuring methane. In subsequent experiments, we decided to decrease the value of fermentation pH towards 5 (see Chapters 2 and 5). The outcome of this study, i.e. the uniqueness of every substrate-inoculum combination led to the realisation of the crucial role of the microbiota of the fermentation system and became the motivation for the microbial analyses in subsequent work (see Chapters 5, 6 and Annex 3).

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*Corresponding author

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Abstract

Volatile fatty acids (VFAs) are increasingly viewed as platform substances for the production of sustainable biofuels and green chemicals. Optimising the acidogenic fermentation requires determining which environmental parameters (temperature, pH, substrate, inoculum, etc) have the largest impact on the metabolite profile. The present study investigates the influence of substrate and mixed inoculum origin. Nine combinations of three complex lignocellulosic substrates and three different mixed inocula were monitored under batch conditions (35 °C; pH 6.5; triplicates, monitoring of pH, soluble COD and VFAs) during 30 days. All nine combinations led to a significantly different fermentation profile, and each combination represented a unique situation. The metabolite profiles were highly reproducible within each substrate-inoculum combination. The substrate COD conversion yield to VFAs was up to 64% (average 31%). VFAs represented most (between 61 and 98%) of the soluble COD. Acetic, propionic and butyric acids were always the major VFAs.

Keywords

Acidogenesis, volatile fatty acids, anaerobic processes, bioconversion, fermentation, acetic acid

4.1 Introduction

Anaerobic digestion (AD), i.e. the conversion of organic matter under anaerobic conditions, can be used to treat most liquid or solid organic wastes and residues from agricultural and agroindustrial origins (Pesta, 2007). The AD process takes place in four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Acidogenesis converts the hydrolysed substrates to short chain, volatile fatty acids (VFAs), that are usually further converted to methane. These VFAs intermediates are seen as potentially interesting platform substances in the biorefining of biowaste and residual biomass (e.g. lignocellulosic agroindustrial residues) to chemical building blocks with industrial relevance for the production of biofuels and green chemicals (Chang *et al*., 2010). The production of VFAs through biomass fermentation is of interest as it provides a more sustainable solution than the use of fast diminishing petrochemicals.

Overall, one can consider that three groups of microorganisms succeed each other in the AD process: acidogenic bacteria, followed by acetogenic bacteria and finally archaeal methanogens (Diamantis *et al*., 2007; Insam *et al*., 2010). The acidogenic bacteria are responsible for the fermentation of hydrolysis end products into VFAs, such as acetate, propionate, butyrate or iso-butyrate. These fermentation products, together with ethanol and lactate, are further oxidised to acetate, formate and CO_2 by the acetogenic bacteria, with concomitant transfer of electrons to produce H_2. These substances are the main substrates for the last phase methanogenesis, where methane is formed (Insam *et al*., 2010). A recent meta-analysis investigating the diversity of the microorganisms that are involved in AD shows the potential role of a large number of bacteria and archaea (Nelson *et al*., 2011). In the same study, it is estimated that so far nearly 60 % of the bacterial diversity and 90 % of the archaeal diversity involved in AD have been identified.

It has been shown that AD, including hydrolysis and acidogenesis, is influenced by many parameters. These parameters are among others temperature (Feng *et al*., 2009; Kim *et al*., 2002; Wang *et al*., 2005) pH (Bengtsson *et al*., 2008; Cheong and Hansen, 2007; Guo *et al*., 2010; Wang *et al*., 2005; Zoetemeyer *et al*., 1982), organic load (Bertin *et al*., 2010; Bouallagui *et al*., 2004; Lim *et al*., 2008), substrate composition (Akutsu *et al*., 2009; Lata *et al*., 2002; Prakasham *et al*., 2009; Temudo *et al*., 2008a, 2008b) and inoculum (Akutsu *et al*., 2008; Forster-Carneiro *et al*., 2007; Fournier *et al*., 1993). In order to optimise biomass fermentation at the industrial scale, it is necessary to determine which parameters have the largest influence.

Several studies reported the fermentation of simple substrates and with pure microbial strains. However, mixed cultures based on natural microbial

communities with a high diversity are currently increasingly used as inocula. Such mixed inocula enable the fermentation to proceed under non-sterile conditions without the risk of strain degeneration (Temudo *et al*., 2008a). Temudo *et al*. (2008a) compared the fermentation of several simple substrates by mixed cultures and they obtained the same catabolic products from xylose and glucose fermentation, whereas glycerol gave rise to another pattern of fermentation products. Another study by Akutsu *et al*. (2009) tested eight different mixed inocula and obtained similar fermentation patterns for a glycerol containing medium with all inocula. However, when they used a starchy medium, the inocula origin had a significant effect on the fermentation pattern. Moreover, another study using complex substrates such as sugar beet silage and grass/clover silage showed similar evolution of substrate hydrolysis when inoculated with one and the same mixed inoculum, whereas their microbial populations developed differently (Cirne *et al*., 2007). Their VFA profiles differed as well. This shows that substrate fermentation by mixed inocula is a complex topic.

The present study examined the impact of complex lignocellulosic substrate composition and mixed inoculum origin on the metabolic fermentation profile of the acidogenesis as well as the relative importance of those two parameters. It was hypothesized that if the type of substrate is the most important factor influencing the fermentation, the evolution of the VFAs over time would be reproducible regardless of the origin of the mixed inoculum. On the other hand, if the origin of the mixed inoculum is the most influencing factor, then a change of substrate would not greatly affect the fermentation profile. Special focus was put on hydrolysis efficiency and the conversion yield of the biomass substrate, in particular on the net production and consumption of VFAs. The identification of factors that mostly influence the fermentation of lignocellulosic substrates would greatly enhance the control of future biorefining processes at industrial scale.

4.2 Materials and Methods

4.2.1 Substrates and Inocula

Three complex lignocellulosic substrates with different lignocellulosic fibre content were used: dried sugar beet pulp (DSBP), wheat bran (WB) and distiller's dried grains with solubles (DDGS). DSBP was produced by Raffinerie Tirlemontoise after sugar extraction (Tienen, Belgium); it was mechanically pressed and then air-dried in a rotating drum drier at the sugar factory. WB was produced by Moulins de Statte (Huy, Belgium) and DDGS originated from the ethanolic fermentation of cereals produced by Alco Bio Fuel (Ghent, Belgium). Characterisation of the substrates (Table 4-1a) shows that they were similar in dry and volatile matter, with total solids (TS) of

approximately 0.9 kg kg_{FW}^{-1} and more than 90 % of the dry matter consisting of volatile solids (VS). The non-soluble dry and volatile matter (TS_{ns} and VS_{ns}, respectively) were higher for DSBP than for DDGS, whereas the total chemical oxygen demand (COD_t) was lower. The soluble COD (COD_s) ranged from 1/6th of the total COD (COD_t) for WB up to 1/4th of the COD_t for DDGS. Lignocellulosic fibres constitute more than 50% of the dry matter of all substrates, with DSBP being richer in cellulose and WB being richer in hemicellulose and lignin. With respect to the most digestible fractions, DDGS is richer in proteins, DBSP in pectins and WB in starch.

Table 4-1. Characteristics of the three substrates (a) and of the three inocula (b) that were used in the present study.

The values are the means (± standard deviation) of triplicates. FM= fresh matter, TS = total solids, VS = volatile solids, TSns = non-soluble dry matter, VSns = non-soluble volatile matter, CODt = total chemical oxygen demand and CODs = soluble COD, n.a. = not analysed

a) Substrates				
Parameter	Unit	DDGS	DSBP	WB
TS	kg kg_{FM}^{-1}	0.8826 (±0.0005)	0.901 (±0.003)	0.893 (±0.001)
VS	kg kg_{TS}^{-1}	0.9470 (±0.0003)	0.944 (±0.002)	0.933 (±0.001)
TS_{ns}	% TS	72.2 (±0.4)	89.6 (±0.8)	77 (±6)
VS_{ns}	% VS	74.9 (±0.3)	89.6 (±0.5)	78 (±6)
COD_t	kg kg_{TS}^{-1}	1.48 (±0.07)	1.17 (±0.06)	1.34 (±0.06)
COD_s	% COD_t	25 (±1)	20 (±1)	16.6 (±0.8)
Cellulose	kg kg_{TS}^{-1}	0.079 (±0.003)	0.206 (±0.002)	0.110 (±0.008)
Hemicellulose	kg kg_{TS}^{-1}	0.382 (±0.001)	0.321 (±0.009)	0.441 (±0.014)
Lignin	kg kg_{TS}^{-1}	0.044 (±0.004)	0.027 (±0.003)	0.078 (±0.001)
Protein	kg kg_{TS}^{-1}	0.355 (±0.007)	0.096 (±0.001)	0.178 (±0.001)
Starch	kg kg_{TS}^{-1}	n.a.	n.a.	0.154 (±0.007)
Pectins	kg kg_{TS}^{-1}	n.a.	0.112 (±0.001)	n.a.
Soluble carbohydrates	kg kg_{TS}^{-1}	0.000	0.001 (±0.001)	0.009 (±0.001)
b) Inocula				
Parameter	Unit	AFB	ASGS	WB
TS	g_{TS} L^{-1}	8.6 (±0.2)	16.6 (±0.2)	55.2 (±0.1)
VS	kg kg_{TS}^{-1}	0.54 (±0.01)	0.574 (±0.004)	0.815 (±0.007)
TS_{ns}	% TS	48.1 (±0.5)	89.9 (±0.8)	57 (±10)
VS_{ns}	% VS	63.4 (±0.4)	87 (±1)	55 (±11)
COD_t	kg kg_{TS}^{-1}	0.72 (±0.02)	0.67 (±0.05)	0.252 (±0.004)
COD_s	% COD_t	18.5 (±0.3)	11.6 (±0.9)	13.5 (±0.3)

Mixed inocula originated from anaerobic sludge (AS), granular sludge (GS) and acidogenic fermentation broth (AFB). The AS inoculum was produced in a semi-continuous laboratory-scale reactor maintained at 35°C and fed weekly through the addition of concentrated fresh activated sludge at a rate of 10 %

of the inoculum COD_t. Fresh activated sludge was collected in the recirculation loop, after the secondary clarifier, of a waste water treatment plant (Basse-Wavre, Belgium) and concentrated by decanting for 1 day in the dark at 4°C. The GS inoculum was obtained from a UASB reactor for the treatment of waste water from the brewery of Carlsberg (Celarevo, Serbia). The AFB inoculum was collected from the acidogenic reactor of the two-stage biomethanation plant of the farm l'Hoste (Basse-Wavre, Belgium) owned by Greenwatt S.A. (Louvain-la-Neuve, Belgium) and using maize silage as substrate. The inocula were stored in the dark at 4°C until use. Their composition is summarized in Table 4-1b. The inocula varied largely in TS and VS, which were lowest for AFB and highest for GS. The TS_{ns} and VS_{ns} were larger for AS than for the other two inocula, whereas the COD_t was lower for GS. The COD of the inoculum AFB was the most soluble and that of AS the least soluble.

4.2.2 Experimental set up and determination of the fermentation profiles

Fermentation was conducted over 30 days for the nine possible combinations of the three complex lignocellulosic substrates and the three mixed inocula. The fermentation was performed in batch bioreactors of 250 ml (Schott Duran GL 45). The bioreactor bottle was closed with a PBT screw-cap, containing a PTFE coated silicone seal and the lateral tube was closed with a conical rubber stopper. Each bioreactor was filled with 0.2 L of a mix consisting of substrate, inoculum and water according to the proportions indicated in Table 4-2. The total COD in the bioreactor varied between 12.5 and 21.7 g L^{-1}, though the COD ratio of substrate and inoculum was set to approximately 4 for all conditions (Table 4-2). The pH of this fermentation broth was decreased to 6.5 through the addition of a known volume of 1 M HCl. Before hermetic closure of the bioreactors, the headspace was flushed for two minutes with a constant flow of nitrogen gas in order to ensure the absence of oxygen in the bioreactors. All substrate-inoculum combinations were set up in triplicate.

Table 4-2: Composition of the fermentation broth at the start of the experimental period

Combination		Composition of fermentation broth			COD	
Substrate (S)	Inoculum (I)	Substrate (g)	Inoculum (mL)	Water (mL)	Total (g L^{-1})	ratio S/I
DDGS	AFB	1.92	100	100	15.59	4.04
	AS	2.49	70	130	20.14	4.18
	GS	3.07	70	160	21.62	4.12
DSBP	AFB	2.37	100	100	15.59	4.04
	AS	3.32	80	140	19.95	3.94

	GS	3.32	60	140	21.66	4.20
WB	AFB	2.09	100	100	12.50	4.04
	AS	2.72	70	130	20.14	4.18
	GS	2.82	60	135	21.59	4.05

The bioreactors were transferred to a 35°C incubation room and placed on a shaker orbiting at 120 rpm. The fermentation was monitored for 30 days and samples and measurements were taken on 14 occasions, i.e. at day 0, 2, 5, 7, 9, 12, 14, 16, 19, 21, 23, 26, 28 and 30, in order to determine the fermentation profile. The analysed parameters were: pH, COD_s, ethanol and volatile fatty acids (VFAs) concentration. At each sampling day, the fermentation broth was mixed with a magnetic bar and 9 mL were sampled from each bioreactor. The bioreactor pH was measured and adjusted to 6.5 with known volumes of 1 M HCl or 1 M NaOH. Sampling time was reduced as much as possible and the bioreactors headspace was flushed for two minutes with nitrogen gas before hermetic closure. The 9 mL samples were centrifuged (Beckmann Coulter TJ-25 Centrifuge) at 15,317 x g for 15 min and the supernatant was filtered through 0.3 µm filters (Millipore, cellulose ester membranes) and stored at -20 °C. This filtered supernatant was used to measure the COD_s and metabolites.

4.2.3 Analytical methods

TS content of the various substrates and inocula was determined by drying representative samples at 105 °C until a stable mass was obtained. VS was obtained from the same samples after burning the dry mass to ashes at 550 °C for 5 hours. The non-soluble total and volatile fractions (TS_{ns} and VS_{ns}, respectively) were determined after elimination of the soluble matter from the sample and represent the particle fraction of the substrates and inocula. These non-soluble fractions were obtained by suspending a known sample mass in a known volume of demineralised water. The suspension was vortexed for one min, centrifuged at 15,317 x g for 15 min and the supernatant was eliminated. The pellet was re-suspended in distilled water and the procedure was repeated three times. The final pellet was used to determine the total and volatile fraction in the same way as was done for TS and VS.

The COD analyses were performed with COD Cell Test method (Merck Spectroquant® kits 1.14541.0001 and 1.14555.0001) according to the provider's instructions and the COD values were measured on a photometer at 585 nm (Merck SQ200). Substrates used for COD_t measurements were ball milled (Mikro-Dismembrator II, B-Braun) and suspended in water for adequate dilution before analysis. For COD_s measurements, the substrates were suspended in water, vortexed and the supernatant was collected after 15 min centrifugation at 15,317 x g. COD_t of inocula were measured after

appropriate dilution of the inocula with distilled water. COD_s of inocula and of fermentation broth were measured on their respective supernatants after 15 min of centrifugation at 15,317 x g.

The concentrations of ethanol (EtOH) and five VFAs, i.e. acetic acid (Ac), propionic acid (Pr), isobutyric acid (iBut), butyric acid (But) and valeric acid (Val) present in the samples were analysed by gas chromatography with a flame ionization detector (GC-FID). Injection solutions for GC-FID analysis were prepared by mixing 500 µL of sample (thawed and filtered supernatant) with 600 µL of distilled water, 100 µL of a 50% w/v metaphosphoric acid aqueous solution (Fluka, ref 79613, 33.5-36.5 % metaphosphoric acid, with 57-63% $NaPO_3$) and 100 µL of a 0.92 g/l solution of 4-methyl valeric acid (4MV, 98%, Merck) as internal standard. One µL of this solution was injected in splitless mode at 250 ºC on a 2 m x 1.6 mm packed column, filled with 4 % Carbowax 20 M on Carbograph 1-DA 80/120 mesh (Alltech Part Nº17271). The oven was set at 180 ºC and helium was used as carrier gas at a constant flow of 15 mL min^{-1}. The detector was set at 250°C. Integration of the chromatograms was done manually with the Chrom-Card 2.0.0 software.

Calibration was performed for ethanol and each VFA by using the samples from day 14 as reference matrix for all the other samples originating from the same bioreactor. Calibration solutions were prepared following the standard addition method by mixing the sample from day 14, the standard addition solution (SA), the internal standard solution (4MV) and the metaphosphoric acid solution at proportions of 5:6:1:1 (v/v), respectively. Three concentration levels of SA were used by adding the SA stock solution at 0, 8.33 and 12.5 % (v/v), respectively, in distilled water. The SA stock solution was prepared by diluting at precisely known concentrations ethanol (Merck, 99.9%; diluted to 10 g/l), and each VFA (Fluka, 99.5% for VFA3, iVFA4, VFA4, 96% for VFA2, 95% for VFA5; diluted to 4 g/l).

Samples were analysed for fermentation days 0, 2, 5, 7, 9, 12, 14, 16, 21 and 30. All samples from one bioreactor were analysed as one series, using the same flame and the same calibration. All yield calculations ($COD_{metabolite}$ $COD_{initial\_substrate}^{-1}$) were corrected for the volumes removed (9 mL samples) and added (adjustment of pH) on the sampling dates.

4.2.4 Statistical analyses

Mean values and their standard deviation were calculated for all data obtained from the three bioreactors of each treatment, except for the treatment DDGS+AS where one bioreactor broke in the start of the experimental period. For this treatment, the duplicate data have been evaluated on the basis of the deviation to the mean.

The influence of substrate and inoculum were investigated by comparing the fermentation profiles. Since a comparison of the pH and yields did not enable detection of an influence of either the substrate or the inoculum on the fermentation, several parameters were defined that enabled a comparison of the fermentation profiles after analyses of variance (ANOVA). The ANOVAs were calculated with the use of the software Minitab (Minitab Inc., USA). Descriptive statistics were used to verify the normality and the homoscedasticity of the data. The parameters defined for the ANOVA, together with their relation to the fermentation profile, are specified in Table 4-3. Acetic, propionic and butyric acid were selected because these were the major fermentation products. Day 5 of the fermentation period was selected since, at that day, there was a maximum yield for most of the conditions. The fermentation profile was first analysed with ANOVA for interactive effects on the defined parameters between the substrate and the inoculum. If there was no such interactive effect (p-value > 0.05), the significance tests were performed on the full data set in order to analyse if there was an effect of the substrate or the inoculum on the fermentation parameter. If there was an interactive effect between the substrate and the inoculum, the data set was separated in order to analyse for each inoculum if there was an effect on the substrate and for each substrate if there was an effect on the inoculum.

Table 4-3: Selected parameters for the characterisation of the fermentation profiles

Parameter	Indication for
Ac	Total yield of acetic acid
Pr	Total yield of propionic acid
But	Total yield of butyric acid
tVFA	Total fermentation yield of VFAs and ethanol
$tVFA_{d5}$	Total fermentation yield of VFAs and ethanol at day 5
$[Ac/tVFA]_{d5}$	Proportion of acetic acid at day 5
$[Pr/tVFA]_{d5}$	Proportion of propionic acid at day 5
$[But/tVFA]_{d5}$	Proportion of butyric acid at day 5
$tVFA_{d7/d5}$	Ratio between the yield of tVFA at day 7 and day 5, indicating a production (>1), consumption (<1) or stabilisation (=1) of the analysed metabolites between days 7 and 5
$CODs_{d7/d5}$	Ratio between the solubilisation yields at day 7 and day 5, indicating a production (>1), consumption (<1) or stabilisation (=1) of the soluble matters between days 7 and 5
pH_{d7-d5}	Difference in pH between day 7 and day 5, indicating an alkalinisation (+_value), acidification (-_value) or stabilisation (0) between days 7 and 5

4.3 Results & Discussion

4.3.1 pH evolution

The pH of the fermentation broth was monitored over the full experimental period and adjusted at each sampling day to pH 6.5 (Fig. 1). This pH value was selected in the range that was indicated by previous works to be favourable to acidogens and their activities [19], [20], [25]. The pH evolution over time for each inoculum-substrate combination showed that overall the fermentation started with an acidification phase which varied in length and intensity (Figure 4-1). Based on this observation, one bioreactor of the treatment WB+GS (Figure 4-1i) was excluded for the calculation of the mean pH since this bioreactor did not show an acidification phase at the start of the fermentation period. Nevertheless, after the initial difference, the pH evolution of the excluded bioreactor was similar to that of the other treatment replicates. An alkalinisation phase followed the acidification phase. These two phases observed in the fermentation profiles probably reflect that the hydrolysis and acidogenic phases were progressively giving place to methanogenic activity (see section 4.3.6 below).

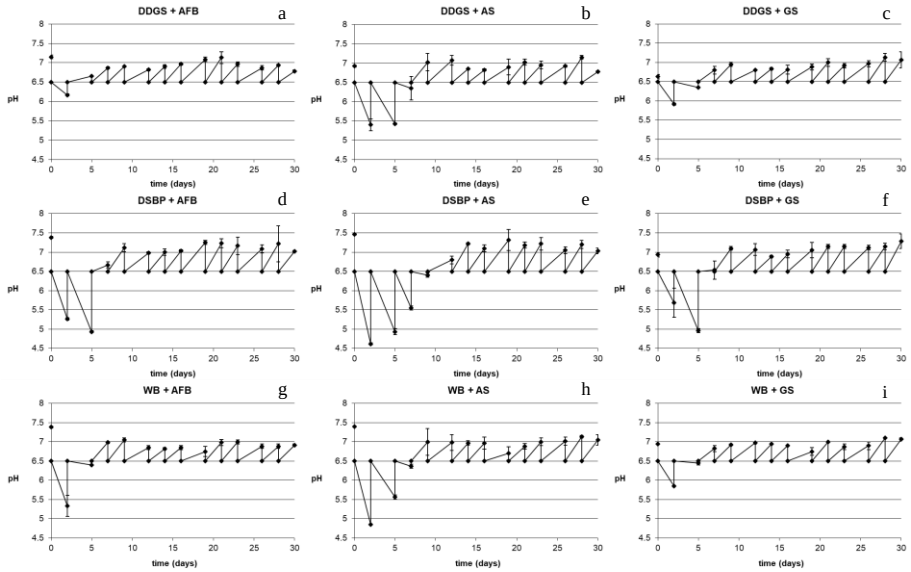

Figure 4-1: pH evolution in the bioreactors over the 30 days of the experimental period.

At each sampling day, the pH was adjusted to 6.5. The values are means ± standard deviations of 3 replicates for all inoculum-substrate combinations except for DDGS+AS (b) and WB+GS (i), where mean values ± deviation to the mean of 2 replicates are given. DDGS: distiller's dried grains with solubles, DSBP: dried sugar beet pulp, WB: wheat bran, AFB: acidogenic fermentation broth, AS: anaerobic sludge and GS: granular sludge.

The shortest and weakest acidification phase was obtained for DDGS+AFB, where the pH had a value superior to the designed value of 6.5 already at d5 (Figure 4-1a). The longest and strongest acidification phase was observed for DSBP+AS. In this treatment, the pH decreased to 4.6 and alkalinisation was observed only after d9 (Figure 4-1e). In nearly all inoculum-substrate combinations, the acidification was strongest at the start of the fermentation, i.e. during the first 2 days, owing to the conversion of the easily biodegradable fraction of the substrate into VFA. In general, the acidification of the DSBP fermentation broth was the strongest, increased up to d5 (Figure 4-1d-f) and was independent of the type of inoculum added (Figure 4-1). A justification of this stronger acidification of DSBP compared to the other substrates could be partly supported by the high concentration of readily biodegradable pectins in the substrate and the lower concentration in lignin that could render the cellulosic and hemicellulose fraction more accessible for conversion. Nevertheless, WB contains starch and DDGS contains proteins which are also potentially biodegradable fractions but have apparently contributed less to the acidification. From the inoculum viewpoint, the highest acidification achieved with AS, irrespective of the substrate used, could be explained by its higher microbial diversity (less adapted than GS and AFB) that would allow the conversion of a higher fraction of the substrate, but would on the other side also lead to the inability to avoid methanogenic activity (see chapter 3.6).

4.3.2 Net VFAs production and consumption

The rate of substrate conversion to ethanol and VFAs was analysed over time (Figure 4-2). The cumulated yield of all metabolites for each observation date showed that, although there was an initial phase of net production of metabolites, all fermentation conditions showed a second phase in which there was a net consumption of the different metabolites (Figure 4-2). A peak in net production was reached between d2 (Figure 4-2a, DDGS+AFB) and d12 (Figure 4-2d, DSBP+AFB).

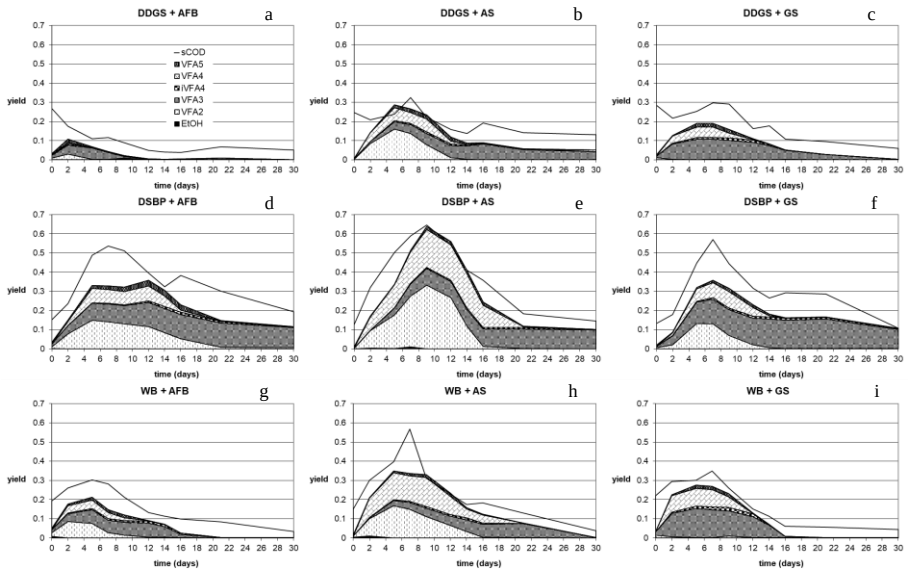

Figure 4-2: The evolution of substrate conversion yield to ethanol, volatile fatty acids (VFAs) and soluble COD in the bioreactors over the 30 days of the experimental period.

All metabolite values (grey areas) for each observation date are cumulated, except for the soluble COD (top line). The values are means of 3 replicates for all inoculum-substrate combinations except for DDGS+AS (b), for day 9 of DSBP+AFB (d), for day 7 of DSBP+GS (f) and for WB+GS (i), where mean values of 2 replicates are given. DDGS: distiller's dried grains with solubles, DSBP: dried sugar beet pulp, WB: wheat bran, AFB: acidogenic fermentation broth, AS: anaerobic sludge and GS: granular sludge. Metabolites (from bottom to top): ethanol (black, rarely visible), VFA2: acetic acid (vertical lines, large area), VFA3: propionic acid (shaded grey, intermediate area), iso-VFA4= iso-butyric (white and black squares, small area), VFA4: butyric acid (white bricks, large area), VFA5: valeric (black with white points, tiny area), CODs (top independent line).

Ethanol was generally absent in all inoculum-substrate combinations. The metabolites were largely or fully consumed during the end of the fermentation period, and acetic acid was the first VFA being consumed. Propionic acid seemed more resistant to degradation and was repeatedly observed until d30. The acetogenesis of propionic acid is known to be experimentally and thermodynamically more limited than the conversion of acetic and butyric acids and (Batstone and Jensen, 2011; Diamantis *et al*., 2007; Lee *et al*., 2008). Overall, the major metabolites produced were acetic, propionic and butyric acids, and their highest average concentrations obtained during the fermentation period were 4.5 ± 0.92, 2.2 ± 0.27 and 2.7 ± 0.58 g L^{-1}, respectively. However, the GS inoculum combined with the substrates DDGS (Figure 4-2c) and with WB (Figure 4-2i) led to low concentrations of acetic acid.

4.3.3 Biomass substrate hydrolysis and conversion

The hydrolysis of the substrates present in the fermentation broth was investigated through the evolution of the COD solubilisation yield (Figure 4-2). In general, the yield of CODs was higher but generally followed the evolution of the cumulated metabolites, with an initial net solubilisation period of 5-9 days upon which a period followed with a net consumption of solubilised substrates. This pattern was observed for nearly all combinations, with the exception of DDGS+AFB (Figure 4-2a), which lacked the net solubilisation period. In general, this pattern was less pronounced for all DDGS series, irrespective of the different inocula used and the hydrolytic capacity of the corresponding microbial communities. This is possibly due to the increased content of proteins that are more slowly hydrolysed compared to other digestible compounds like carbohydrates (Fang and Yu, 2002). Alternatively, thermal degradation of proteins and polysaccharides and Maillard reactions during DDGS distillation might also explain their lower sensitivity to microbial hydrolysis.

For the DDGS and WB substrates, the CODs yield was lower at the end of the experimental period than at the start. However, for the DSBP substrate, after an initial increase, the CODs yield had returned to approximately the value observed at the start of the fermentation period (Figure 4-2). In the cases of DDGS and WB, the total VFA yield decreased to almost 0 and was in general lower than that at the start, whereas in the case of DSBP, the yield at the end had increased to approximately 0.1 g_{COD} $g_{COD\_initial\_substrate}^{-1}$ and mainly consisted of VFA3 (Figure 4-2d-f). These results were independent of the type of inoculum applied.

The lowest conversion yield was obtained with DDGS+AFB (Figure 4-2a, yield of 0.11 ± 0.01 $g_{COD\_metabolite}$ $g_{COD\_initial\_substrate}^{-1}$ at d2) and the highest conversion yield was obtained for DSBP+AS (Figure 4-2e, yield of 0.6 ± 0.14 $g_{COD\_metabolite}$ $g_{COD\_initial\_substrate}^{-1}$ at d9). When making an overall comparison of the fermentation profiles obtained for each substrate with the different inocula, the DSBP substrate always resulted in the highest conversion yield, independently of the inoculum used, whereas the DDGS substrate resulted in the lowest conversion yield. Given that the fibre content (cellulose, hemicellulose, lignin) of those two substrates is apparently similar (50,5% and 55.4% of the dry matter, respectively), this difference could be due to the higher content of easily hydrolysable pectin in DSBP as compared to the higher content of proteins in DDGS (Table 4-1a). Again, thermal degradation of proteins and polysaccharides and Maillard reactions during DDGS distillation might explain their lower sensitivity to microbial hydrolysis. The AS inoculum always resulted in the highest substrate conversion yield, which could indicate that AS is “richer” in terms of hydrolytic and metabolic activity

than the other inocula, possibly as a result of its regular feeding and maintenance prior to the experiments. Most of the soluble COD consisted of VFAs, with a lowest proportion of 62 % for the DDGS+AFB treatment and a highest proportion of 99 % for the DSBP+AS treatment.

4.3.4 Impact of substrate and inoculum on the metabolic fermentation profile

The effect of inoculum and substrate as well as their interaction on various aspects of the metabolic fermentation profile were analysed in detail with a statistical approach using ANOVA, by use of 11 selected parameters which best characterised the fermentation profile (Table 4-3). The obtained p-values are summarised in Table 4-4.

Table 4-4: P-values for the ANOVA performed on the parameters defined for the characterisation of the fermentation profiles[a]

	Ac	Pr	But	tVFA	$tVFA_{d5}$	$[Ac/tVFA]_{d5}$	$[Pr/tVFA]_{d5}$	$[But/tVFA]_{d5}$	$tVFA_{d7/d5}$	$CODs_{d7/d5}$	pH_{d7-d5}
Interaction between substrate and inoculum											
	0.003[b]	0.060	***0.001***	0.163	0.070	***0.000***	***0.000***	***0.000***	***0.032***	0.201	***0.000***
Effect of substrate											
		0.000		***0.000***	0.000					0.739	
AFB	***0.000***		***0.000***			***0.000***	***0.000***	***0.000***	***0.029***		***0.000***
AS	***0.001***		***0.004***			0.227	0.055	***0.015***	***0.037***		0.352
GS	***0.001***		0.462			***0.000***	0.115	***0.001***	0.807		***0.000***
Effect of inoculum											
		0.041		***0.000***	0.008					***0.019***	
DDGS	***0.000***		0.055			***0.000***	***0.001***	***0.002***	***0.001***		***0.041***
DSBP	***0.000***		***0.000***			***0.003***	***0.000***	***0.000***	***0.058***		***0.000***
WB	***0.000***		***0.004***			***0.000***	***0.012***	***0.000***	***0.042***		***0.004***

[a]*The relation of the various parameters to the fermentation profile are detailed in Table 4-3*
[b]*The values in bold italics indicate a significant interaction or a significant effect of the substrate or the inoculum on the parameter*

In general, the studied parameters were dependent on the combination of both substrate and inoculum, and only four parameters (Pr, tVFA, $tVFA_{d5}$ and $CODs_{d7/d5}$) did not show a statistically significant interaction effect between substrate and inoculum. Globally, based on the number of p-values lower than 0.05, the effect of the substrate was less marked than that of the inoculum, given that the effect of the inoculum was significant for nearly all of the parameters studied. The AFB inoculum was the inoculum the most influenced by the substrate, since only the parameter $CODs_{d7/d5}$ was not affected by the substrate. Similarly, Forster-Carneiro *et al*. (2007) observed that different inocula introduced in biowaste lead to different acidogenic profiles. On the other hand, Temudo *et al*. (2008a) observed that their single inoculum evolved to the dominance of different microbial communities depending on the

substrate used.

4.3.5 Reproducible fermentation profiles controlled by both substrate and inoculum

Most variation coefficients between replicates were between 10 and 17% of the mean for metabolites with conversion yields of at least 0.05 $g_{COD\_metabolite} \cdot g_{COD\_substrate}^{-1}$. These quite weak (for biological processes) mean variation coefficients indicate a good reproducibility of the fermentation profiles within the different substrate – inoculum combinations, despite the non-axenic conditions and the use of complex substrates as well as mixed microbial communities. Each replicate within a specific inoculum-substrate combination gave a similar fermentation profile.

The statistical analysis of the experimental results showed that between the different combinations of inoculum and substrate, each combination represented a statistically unique condition. All of the nine tested combinations led to a distinctive fermentation profile and this is consistent with the increased reproducibility among reactors of the same condition. Statistical analyses confirmed that in general both the substrate and the inoculum had an effect on the fermentation profile. These results are consistent with the observation of Akutsu *et al*. (2009) that both inocula and substrates were of importance for fermentation patterns when eight different mixed inocula were used to ferment various non-lignocellulosic substrates.

The origin of the mixed inoculum had a stronger effect on the metabolic fermentation profile than the complex lignocellulosic substrate.

4.3.6 Fermentation profiles

For all substrate-inoculum combinations, the initial acidification (Fig. 1) and increase of the soluble COD and VFAs (Figure 4-2) in the fermentation broth was followed by a gradual alkalinisation and decrease of soluble COD and VFAs over time. Importantly, this decrease of CODs coincided with the decrease in total VFAs. Some production of gas was detected, though the used experimental set-up did not enable the quantification or characterisation of this gas. The experimental conditions would justify an initial production of hydrogen (in addition to CO_2) followed with the production of methane, as has been observed previously (Forster-Carneiro *et al*., 2007). Such a production of hydrogen and/or methane could explain the decrease of CODs and VFAs concentrations in the fermentation broth. As mentioned in section 4.3.1, the observed two phases of the fermentation profiles probably reflect that the hydrolysis and acidogenic phases were progressively replaced by acetogenesis and methanogenesis. This assumption could further be supported by the observation that DSBP substrate, which always showed the strongest

initial acidification, also resulted in the highest net substrate conversion yields to CODs and VFAs between days 5 to 10, independently of the inoculum used. The same was the case for the AS inoculum that also led to the strongest acidification, and also resulted in the highest net substrate conversion yields to CODs and VFAs, independently of the substrate. Good acidification would have limited the methanogenic consumption of VFAs and CODs as a result of acidic conditions unfavourable for methanogenic populations, resulting in the highest net VFAs and CODs yields. Other studies have shown the start of methanogenesis within a similar frame of time (Forster-Carneiro *et al*., 2007; Lee *et al*., 2008). The experimental conditions tested were thus not able to enhance acidogenesis while excluding methanogenesis. Several factors could be responsible for this, such as (i) a pH regulated at 6.5 which was not acidic enough to completely inhibit the methanogenic activity (Batstone and Jensen, 2011), (ii) a relatively low organic load and (iii) the use of inocula that were too rich in methanogens (AS, GS) and not pre-treated against their activity.

Only the first days of fermentation can thus be considered as representative of the hydrolysis and acidogenic phases. The strength and the length of the acidification phase (Figure 4-1) in the start seem to be correlated to the substrate conversion yield to VFAs (Figure 4-2). A long and strong acidification phase resulted in a high net substrate to VFAs conversion yield, whereas a short and weak acidification phase resulted in a low conversion yield. This is in accordance with the fact that a decrease in pH can probably be related to the production of VFAs (Wang *et al*., 2009). In a study of the effect of pH on the hydrolysis of organic solid waste, Veeken *et al*. (2000) showed that the pH is an essential factor for hydrolysis rate and that its lowering below neutral values leads to a clearly increased hydrolysis rate. Such an enhanced hydrolysis could subsequently lead to an increased substrate conversion yield.

4.3.7 Production of VFAs

The best conversion yield observed was around 64% (Figure 4-2, DSBP + AS) before VFAs started to be consumed. The average substrate conversion yield was 31 %, which is equivalent to the yields obtained from AD of wastewaters rich in organic matter (Demirel and Yenigun, 2004; Guerrero *et al*., 1999). The conversion yields and concentrations can possibly be improved for certain conditions, though it is known that lignocellulose structure can be a limiting factor for AD (Mata-Alvarez *et al*., 2000). It should be kept in mind that the substrates used in the present work were not subjected to any kind of pre-treatment to improve digestibility.

A good selectivity for the conversion of the substrate to VFAs was obtained, with approximately 66% up to nearly all (99%) of the soluble COD as VFAs.

The soluble COD concentration was always larger than the sum of the analysed metabolites, which means that some minor part of the soluble matter was not identified with the methods employed in this study.

For all substrate-inoculum combinations, the same three VFAs were the major metabolites, namely acetic acid (maximum 4.5 $g \cdot L^{-1}$), propionic acid (maximum 2.2 $g \cdot L^{-1}$) and butyric acid (maximum 2.7 $g \cdot L^{-1}$), albeit with some variations in their relative proportions depending on substrate and inoculum. This was previously observed for the fermentation of, for example, proteins (Weimer *et al*., 2009), primary sludge (Wu *et al*., 2009), olive mill waste water (Bertin *et al*., 2010) and sucrose-rich wastewater (Wang *et al*., 2005). Acetic acid was often the dominant VFA in the start of the experimental period. Such dominance of acetic acid has already been observed in several studies (Burrell *et al*., 2004; Feng *et al*., 2009; Wu *et al*., 2009; Zhang *et al*., 2009). Though, the consecutive decrease of acetic acid indicated that this VFA was easily consumed and converted to other metabolites (possibly CH_4). Propionic acid showed the largest resistance to degradation.

The industrial production and recovery of biomass-derived VFAs would require higher VFAs concentration. Pre-treatments to improve the digestibility of the poorly degradable lignocellulosic biomass, increased available substrate concentration (maximum 17 $g_{COD}\ l^{-1}$ tested in the present laboratory study), specific engineering of the mixed inoculum to reduce the presence of methanogens, as well as fermentation under conditions favourable for acidogenesis but unfavourable for methanogenesis, are approaches that could increase the amount of VFAs produced. Nevertheless, the good reproducibility of the fermentation profiles observed in the present study meets one condition identified by Chang *et al*. (2010) to enable VFAs as platform substances in the biorefining of biomass to biofuels and biochemicals, i.e. stable fermentation methods.

4.4 Conclusion

In this work, a set of parameters was defined, that allowed to statistically compare the fermentation profiles of several combinations of complex lignocellulosic substrates with mixed inocula. Based on these parameters, the fermentation profiles were highly reproducible within each substrate-inoculum combination, but significantly influenced by both the mixed inoculum origin and the lignocellulosic substrate type. Nevertheless, under the nine experimental combinations tested, the same three VFAs were always dominating the fermentation products, i.e. acetate, butyrate and propionate. Together with the high reproducibility, this is an unequivocal advantage for industrialisation of a biorefining process and opens a pathway towards a biomass-derived VFA platform.

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4.6 References

Akutsu, Y., Li, Y.-Y., Tandukar, M., Kubota, K., Harada, H., 2008. Effects of seed sludge on fermentative characteristics and microbial community structures in thermophilic hydrogen fermentation of starch. International Journal of Hydrogen Energy 33, 6541–6548. https://doi.org/10.1016/j.ijhydene.2008.08.038

Akutsu, Y., Lee, D.-Y., Li, Y.-Y., Noike, T., 2009. Hydrogen production potentials and fermentative characteristics of various substrates with different heat-pretreated natural microflora. International Journal of Hydrogen Energy 34, 5365–5372. https://doi.org/10.1016/j.ijhydene.2009.04.052

Batstone, D.J., Jensen, P.D., 2011. Anaerobic Processes, in: Wilderer, P. (Ed.), Treatise on Water Science. Elsevier, Oxford, pp. 615–639. https://doi.org/10.1016/B978-0-444-53199-5.00097-X

Bengtsson, S., Hallquist, J., Werker, A., Welander, T., 2008. Acidogenic fermentation of industrial wastewaters: Effects of chemostat retention time and pH on volatile fatty acids production. Biochemical Engineering Journal 40, 492–499. https://doi.org/10.1016/j.bej.2008.02.004

Bertin, L., Lampis, S., Todaro, D., Scoma, A., Vallini, G., Marchetti, L., Majone, M., Fava, F., 2010. Anaerobic acidogenic digestion of olive mill wastewaters in biofilm reactors packed with ceramic filters or granular activated carbon. Water Res. 44, 4537–4549. https://doi.org/10.1016/j.watres.2010.06.025

Bouallagui, H., Torrijos, M., Godon, J.J., Moletta, R., Ben Cheikh, R., Touhami, Y., Delgenes, J.P., Hamdi, M., 2004. Two-phases anaerobic digestion of fruit and vegetable wastes: bioreactors performance. Biochemical Engineering Journal 21, 193–197. https://doi.org/10.1016/j.bej.2004.05.001

Burrell, P.C., O'Sullivan, C., Song, H., Clarke, W.P., Blackall, L.L., 2004. Identification, Detection, and Spatial Resolution of Clostridium Populations Responsible for Cellulose Degradation in a Methanogenic Landfill Leachate Bioreactor. Appl Environ Microbiol 70, 2414–2419. https://doi.org/10.1128/AEM.70.4.2414-2419.2004

Chang, H.N., Kim, N.-J., Kang, J., Jeong, C.M., 2010. Biomass-derived volatile fatty acid platform for fuels and chemicals. Biotechnol Bioproc E 15, 1–10. https://doi.org/10.1007/s12257-009-3070-8

Cheong, D.-Y., Hansen, C.L., 2007. Feasibility of hydrogen production in

thermophilic mixed fermentation by natural anaerobes. Bioresource Technology 98, 2229–2239. https://doi.org/10.1016/j.biortech.2006.09.039

Cirne, D.G., Lehtomäki, A., Björnsson, L., Blackall, L.L., 2007. Hydrolysis and microbial community analyses in two-stage anaerobic digestion of energy crops. J. Appl. Microbiol. 103, 516–527. https://doi.org/10.1111/j.1365-2672.2006.03270.x

Demirel, B., Yenigun, O., 2004. Anaerobic acidogenesis of dairy wastewater: the effects of variations in hydraulic retention time with no pH control. Journal of Chemical Technology & Biotechnology 79, 755–760. https://doi.org/10.1002/jctb.1052

Diamantis, V.I., Vaiopoulou, E., Aivasidis, A., 2007. Fundamentals and Applications of Anaerobic Digestion for Sustainable Treatment of Food Industry Wastewater, in: Oreopoulou, V., Russ, W. (Eds.), Utilization of By-Products and Treatment of Waste in the Food Industry. Springer US, pp. 73–97.

Fang, H.H.P., Yu, H., 2002. Mesophilic acidification of gelatinaceous wastewater. Journal of Biotechnology 93, 99–108. https://doi.org/10.1016/S0168-1656(01)00397-2

Feng, L., Chen, Y., Zheng, X., 2009. Enhancement of waste activated sludge protein conversion and volatile fatty acids accumulation during waste activated sludge anaerobic fermentation by carbohydrate substrate addition: the effect of pH. Environ. Sci. Technol. 43, 4373–4380.

Forster-Carneiro, T., Pérez, M., Romero, L.I., Sales, D., 2007. Dry-thermophilic anaerobic digestion of organic fraction of the municipal solid waste: focusing on the inoculum sources. Bioresour. Technol. 98, 3195–3203. https://doi.org/10.1016/j.biortech.2006.07.008

Fournier, D., Schwitzguébel, J.P., Péringer, P., 1993. Effect of different heterogeneous inocula in acidogenic fermentation of whey permeate. Biotechnol Lett 15, 627–632. https://doi.org/10.1007/BF00138553

Guerrero, L., Omil, F., Méndez, R., Lema, J.M., 1999. Anaerobic hydrolysis and acidogenesis of wastewaters from food industries with high content of organic solids and protein. Water Research 33, 3281–3290. https://doi.org/10.1016/S0043-1354(99)00041-X

Guo, X.M., Trably, E., Latrille, E., Carrère, H., Steyer, J.-P., 2010. Hydrogen production from agricultural waste by dark fermentation: A review. International Journal of Hydrogen Energy 35, 10660–10673. https://doi.org/10.1016/j.ijhydene.2010.03.008

Insam, H., Franke-Whittle, I., Goberna, M., 2010. Microbes in Aerobic and Anaerobic Waste Treatment, in: Insam, H., Franke-Whittle, I., Goberna, M. (Eds.), Microbes at Work. Springer Berlin Heidelberg, pp. 1–34.

Kim, M., Ahn, Y.-H., Speece, R.E., 2002. Comparative process stability and efficiency of anaerobic digestion; mesophilic vs. thermophilic. Water Research 36, 4369–4385. https://doi.org/10.1016/S0043-1354(02)00147-1

Lata, K., Rajeshwari, K.V., Pant, D.C., Kishore, V.V.N., 2002. Volatile fatty acid production during anaerobic mesophilic digestion of tea and vegetable market

wastes. World Journal of Microbiology and Biotechnology 18, 589–592. https://doi.org/10.1023/A:1016314903817

Lee, C., Kim, J., Shin, S.G., Hwang, S., 2008. Monitoring bacterial and archaeal community shifts in a mesophilic anaerobic batch reactor treating a high-strength organic wastewater. FEMS Microbiol. Ecol. 65, 544–554. https://doi.org/10.1111/j.1574-6941.2008.00530.x

Lim, S.-J., Kim, B.J., Jeong, C.-M., Choi, J., Ahn, Y.H., Chang, H.N., 2008. Anaerobic organic acid production of food waste in once-a-day feeding and drawing-off bioreactor. Bioresour. Technol. 99, 7866–7874. https://doi.org/10.1016/j.biortech.2007.06.028

Mata-Alvarez, J., Macé, S., Llabrés, P., 2000. Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. Bioresource Technology 74, 3–16. https://doi.org/10.1016/S0960-8524(00)00023-7

Nelson, M.C., Morrison, M., Yu, Z., 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. 102, 3730–3739. https://doi.org/10.1016/j.biortech.2010.11.119

Pesta, G., 2007. Anaerobic Digestion of Organic Residues and Wastes, in: Oreopoulou, V., Russ, W. (Eds.), Utilization of By-Products and Treatment of Waste in the Food Industry. Springer US, pp. 53–72.

Prakasham, R.S., Brahmaiah, P., Sathish, T., Sambasiva Rao, K.R.S., 2009. Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. International Journal of Hydrogen Energy 34, 9354–9361. https://doi.org/10.1016/j.ijhydene.2009.09.104

Temudo, M.F., Muyzer, G., Kleerebezem, R., van Loosdrecht, M.C.M., 2008a. Diversity of microbial communities in open mixed culture fermentations: impact of the pH and carbon source. Appl. Microbiol. Biotechnol. 80, 1121–1130. https://doi.org/10.1007/s00253-008-1669-x

Temudo, M.F., Poldermans, R., Kleerebezem, R., van Loosdrecht, M.C.M., 2008b. Glycerol fermentation by (open) mixed cultures: A chemostat study. Biotechnol. Bioeng. 100, 1088–1098. https://doi.org/10.1002/bit.21857

Veeken, A., Kalyuzhnyi, S., Scharff, H., Hamelers, B., 2000. Effect of pH and VFA on Hydrolysis of Organic Solid Waste. Journal of Environmental Engineering 126, 1076–1081. https://doi.org/10.1061/(ASCE)0733-9372(2000)126:12(1076)

Wang, G., Mu, Y., Yu, H.-Q., 2005. Response surface analysis to evaluate the influence of pH, temperature and substrate concentration on the acidogenesis of sucrose-rich wastewater. Biochemical Engineering Journal 23, 175–184. https://doi.org/10.1016/j.bej.2005.01.002

Wang, Y., Zhang, Y., Meng, L., Wang, J., Zhang, W., 2009. Hydrogen–methane production from swine manure: Effect of pretreatment and VFAs accumulation on gas yield. Biomass and Bioenergy 33, 1131–1138. https://doi.org/10.1016/j.biombioe.2009.04.004

Weimer, P.J., Russell, J.B., Muck, R.E., 2009. Lessons from the cow: what the ruminant animal can teach us about consolidated bioprocessing of cellulosic biomass. Bioresour. Technol. 100, 5323–5331.

https://doi.org/10.1016/j.biortech.2009.04.075

Wu, H., Yang, D., Zhou, Q., Song, Z., 2009. The effect of pH on anaerobic fermentation of primary sludge at room temperature. Journal of Hazardous Materials 172, 196–201. https://doi.org/10.1016/j.jhazmat.2009.06.146

Zhang, P., Chen, Y., Zhou, Q., 2009. Waste activated sludge hydrolysis and short-chain fatty acids accumulation under mesophilic and thermophilic conditions: effect of pH. Water Res. 43, 3735–3742. https://doi.org/10.1016/j.watres.2009.05.036

Zoetemeyer, R.J., van den Heuvel, J.C., Cohen, A., 1982. pH influence on acidogenic dissimilation of glucose in an anaerobic digestor. Water Research 16, 303–311. https://doi.org/10.1016/0043-1354(82)90190-7

5 Metabolic and microbial profile of mixed acidogenic fermentation of apple pulp by ruminal fluid and pretreated activated sludge

Comment

This study was initiated after a remark on the analogy between an acidogenic bioreactor and the rumen of a cow, during an internal seminar where partial results of Chapter 2 were presented. It was at the same time that we were convinced of the need to further deepen on the microbiome of the fermentation system as an explanation of the uniqueness of the fermentation profile in Chapter 3. With this study, we combined these two elements: examining the performance of rumen inoculum next to the standard inoculum used so far and focusing on the microbial analysis for more insight on the microbiota evolution. The goal was not to replicate the rumen rather than to examine how an untreated rumen inoculum would respond in laboratory (or more industry-related) conditions. Apple pulp (after juice extraction and hoy water washing) was used as a substrate because (i) it was available at the time of the experiment, (ii) it was a good "compromise" substrate (sufficient but not very high lignocellulosic content, sufficient but not too high solids content, sufficient digestible components) and (iii) it had already been tested under acidogenic conditions in Chapter 2. The pH was adjusted at the area of 5-5.5, continuing the strategy of Chapter 2. This was the first study were microbial analysis was combined with metabolic analysis, a methodology that was decided following the results of Chapter 4 and further adopted in Chapter 6 and Annex 3.

Chapter prepared for submission:

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*Corresponding author

Specific AP contribution: experimental work, data analysis, data interpretation, article compilation

Abstract

Apple pulp is an attractive substrate for valorisation through bioconversion. In this study, the production of volatile fatty acids (VFA) by acidogenic fermentation was examined, by inoculating apple pulp with either pre-treated activated sludge or untreated rumen fluid. The dynamics of bacterial communities were assessed by PCR/DGGE. Fermentation with pre-treated activated sludge led to higher concentrations of total VFA (tVFA) in the mixed liquor (ML) reaching 11.5 g_{COD_tVFA}/kg_{ML}, corresponding to a conversion yield of 0.21 g_{COD_tVFA}/g_{CODt}, after 15 fermentation days. Pre-treatment of activated sludge led to the selection of a reduced but better performing consortium, containing mainly members of the *Clostridium* group, which coincided with an increase in tVFA concentration. Inoculation with rumen fluid only marginally improved the performance of non-inoculated apple pulp. Other dominating microorganisms identified in the fermentations belonged to the *Selenomonas*, *Prevotella*, *Aeromonas*, *Bacillus* and *Ruminococcus* genera.

Keywords

Apple pulp, mixed acidogenic fermentation, microbial communities, volatile fatty acids, carboxylate

5.1 Introduction

Mixed acidogenic fermentation of organic residues has gained increasing interest during the last years, due to the potential of valorising waste fractions for the production of base chemicals of industrial interest, notably volatile fatty acids (VFA), considered as valuable carboxylate platform molecules (Singhania *et al.*, 2013). Food processing by-products are particularly interesting due to the importance their production volumes, although the lignocellulosic structure of some remains challenging in terms of valorisation (Ravindran and Jaiswal, 2016). One example is apple pulp, the end-residue of one of the most consumed fruit in the world, with particular valorisation interest in many regions (Shalini and Gupta, 2010). Its composition includes not only recalcitrant substances like lignin and cellulose, but also a number of more readily digestible components such as free sugars, pectins and hemicellulose (Aguedo *et al.*, 2012), which makes it an attractive substrate for bioconversion. It has been the subject of previous studies related to its utilisation in anaerobic conversion for biogas and hydrogen production (Kafle and Kim, 2013; Llaneza Coalla *et al.*, 2009), but to the best of our knowledge, not for the direct production of VFA.

One of the main challenges towards upscaling mixed acidogenic fermentation is the control of its complexity, which results from the use of composite substrates, like agroindustrial residues with mixed microbial consortia of natural origin (Kleerebezem and van Loosdrecht, 2007). The enrichment of the substrate inherent flora with an external mixed inoculum enables a more efficient conversion of the substrate (Forrest *et al.*, 2012). Activated sludge and ruminal fluid are inocula commonly used for anaerobic conversions.

Activated sludge is readily available and contains a diverse microbiota, but often requires additional pre-treatment to favour acidogenic populations (Akutsu *et al.*, 2009). Infantes *et al.* (2012) used activated sludge to examine the effect of pH, temperature and undissociated acid concentration in the acidogenic fermentation of synthetic wastewater, while Wang *et al.*, 2014 compared activated and anaerobic sludge for the acidogenic fermentation of food waste. Activated sludge, itself a waste with large availability, has also been used as a fermentation (co-)substrate (Yuan *et al.*, 2015; Zhang *et al.*, 2016).

The interest of rumen fluid lies in its established microbial community, already adapted to acidogenic conditions due to rumen physiology (Morgavi *et al.*, 2010; Weimer *et al.*, 2015). Total VFA (tVFA) concentrations in the rumen have been reported in the range of 70-180 mM (Weimer and Kohn, 2016). The application of rumen microorganisms to acidogenic conversions of complex substrates is attractive due to the potential diversity of rumen consortia (Zhao *et al.*, 2016) that offer a higher hydrolytic and acidogenic

capacity than other conventional inocula and allow their direct use without additional pre-treatment (Hernández-García *et al.,* 2015; for a recent review see also Yue *et al.* (2013).

A better understanding and more targeted control of mixed fermentation relies on the analysis of the structure and dynamics of microbial communities along the fermentation (Briones and Raskin, 2003). Depending on the source and treatment of the inoculum, the endogenous microbiota of the substrate and the fermentation conditions, the microbial communities may develop in different ways leading to different product distribution and concentration (Ye *et al.*, 2007). Yet, for a given set of operation conditions, fermentation performance could also be very similar even if the microbial communities are very different (Forrest *et al.*, 2012). Acknowledging the necessity of monitoring microbial communities, various authors are now using molecular biology techniques to decipher the microbial composition and dynamics in acidogenic fermentation (Cheng *et al*., 2014; Kim *et al*., 2011; Park *et al*., 2015; Ye *et al*., 2007; Yuan *et al*., 2015, among others). While these studies are focusing on different fermentation parameters, they still converge to the existence of a number of core families that dominate the microbial structure of acidogenic fermentation: *Clostridiaceae*, *Ruminococcaceae*, *Lactobacillaceae* and *Bacillaceae* (*Firmicutes*), *Bacteroidaceae* (*Bacteroidetes*) and *Pseudomonadaceae* (*Proteobacteria*).

The present study examines how an untreated acidogenic rumen inoculum performs in an external environment relevant to bioprocessing applications and how this performance compares to (i) a pretreated activated sludge, a commonly used inoculum and (ii) the inherent microbiota of the substrate without external inoculum addition. Furthermore, the dynamics of the microbiota during fermentation were investigated via culture-independent molecular techniques. To the best of our knowledge such comparisons have not been conducted so far with apple pulp. The long-term aim is to identify robust correlations between the metabolic and microbial profile of acidogenic fermentation, in the scope of directing the process towards a target product at continuous operation mode.

5.2 Materials and methods

5.2.1 Substrate and inocula

Apple pulp was collected from a syrup producing industry (Liège, Belgium) after fruit pressing and hot water recovery of the residual juice. Two types of external inoculum were tested. The first was based on activated sludge collected from a municipal wastewater treatment plant (Mont-Saint-Guibert, Belgium). After settling for two days at 4°C and supernatant removal, the

concentrated sludge was incubated under anaerobic conditions at 35°C for five days. The concentrated sludge was then acidified at pH 5.5 with HCl 1M, incubated under agitation for 1h at 70°C, cooled down at room temperature and readjusted at pH 5.5 before its use as acidogenic inoculum (hereafter referred to as "sludge"). The pretreatment served to select spore-forming acidogenic populations and to eliminate methanogenic populations (Arslan *et al*., 2016). The second inoculum was rumen fluid collected from a cow (Centre Alphonse de Marbaix, Louvain-la-Neuve, Belgium). The fluid was used directly, without any pre-treatment, as it was considered already adapted to acidogenic conditions in the rumen of the cow. Table 5-1 presents the properties of the substrate and inocula.

Table 5-1: Substrate and inocula characteristics

Mean values and standard deviation of n repetitions, where n refers to the number in brackets. In the case of COD of apple pulp, deviation from the mean was used instead of standard deviation. For more information on the apple pulp composition, the reader is referred to Aguedo et al. (2012). TS: Total Solids; FM: Fresh Matter; VS: Volatile solids; COD: Chemical Oxygen Demand.

	TS (g_{TS}/g_{FM})	**VS (g_{VS}/g_{TS})**	**COD (g_{COD}/g_{VS})**
Apple pulp	0.221 ± 0.003 (4)	0.991 ± 0.000 (4)	1.644 ± 0.004 (2)
Activated sludge	0.023 ± 0.003 (3)	0.430 ± 0.003 (3)	1.921 ± 0.003 (3)
Rumen fluid	0.039 ± 0.000 (3)	0.737 ± 0.003 (3)	1.426 ± 0.001 (3)

5.2.2 Experimental procedure

Acidogenic fermentation was performed in 250 ml reactors (Scott Duran GL 45) equipped with a lateral glass tube for gas sampling. Five series were tested in duplicate: apple pulp inoculated with activated sludge (*code AS*), apple pulp inoculated with rumen fluid (code *AR*), apple pulp control without external inoculum (*code A*), activated sludge control without addition of substrate (*code S*) and rumen fluid control without addition of substrate (*code R*). In the mixed series (*i.e.* presence of both substrate and inoculum), the substrate and the inoculum were introduced at a ratio of about 4 $g_{COD\_substrate}/g_{COD\_inoculum}$ to achieve initial total COD (CODt) concentration in the mixed liquor (ML) of about 54 g_{CODt}/kg_{ML}. The corresponding initial concentrations for inocula controls and substrate control were 12 and 43 g_{CODt}/kg_{ML}, respectively. Water was added to the reactors up to a mixed liquor volume of 100 ml. Before hermetic closure, the bioreactor headspace was flushed for 1 minute with nitrogen. The bioreactors were incubated at 35°C in the dark under batch conditions. The fermentation was monitored for 15 days. The pH was measured at days 0, 1, 2, 3, 7, 10 and 15; for series AS, AR and A the pH was adjusted at 5.0-5.5 with 1M HCl or NaOH. Sampling of mixed liquor took place at days 0, 1, 2, 3, 7, 10 and 15 and flushing with nitrogen took place every time the reactors were opened. The measurement of the pressure in the

reactors was performed with a manometer and the sampling of the gas phase was achieved through the lateral glass tube of the reactors. The monitored parameters were: pH, soluble COD (CODs), amount and composition of biogas (*i.e.* H_2, CO_2 and CH_4) and concentration of metabolites (*i.e.* acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids). Mixed liquor samples were centrifuged (Beckmann Coulter TJ-25 centrifuge) at 15,300 g for 10 min. The supernatant was stored at -20 °C for metabolic analyses. The solid phase was stored at -20 °C for the analysis of microbial communities.

5.2.3 Analytical methods

5.2.3.1 Substrate and inoculum characterisation

Total solids (TS), volatile solids (VS) and total COD (CODt) were measured according to Standard Methods (APHA, 1998). Soluble COD (CODs) was measured with the COD Cell Test method (Spectroquant® ThermoReactor 620, Photometer SQ200, Merck Germany) according to the manufacturer's protocol.

5.2.3.2 Biogas

The composition of the biogas was analysed using a gas chromatograph (Compact GC, Interscience) with thermal conductivity detectors (GC-TCD). The chromatograph combined two channels. The front channel was equipped with a Rt-QBond column (10 m x 0.32 mm), which separated CO_2 from the other gases. The back channel included two columns in series: one similar to the front channel (Rt-QBond, 2 m x 0.32 mm) which retained CO_2, followed by a Molsieve 5A column (7 m x 0.32 mm) that separated the other gases. A valve between the two columns, allowed to back-flush CO_2 to the injection port so that it did not enter in the Molsieve column. The elution was performed under isotherm conditions at 60 °C, with helium as carrier gas at 20 ml/min and a pressure of 80 kPa for the front channel, and 70 °C with argon as carrier gas at 10 ml/min and a pressure of 70 kPa for the back channel. The detectors were heated at 90 °C and the filaments at 170 °C.

5.2.3.3 Soluble metabolites

VFA were analysed using a gas chromatograph (Trace, Thermo Finnigan) with a flame ionisation detector (GC-FID). The column (2m x 1/8') was packed with 4% Carbowax 20M on Carbograph 1-DA, 80/120 mesh (Alltech). The temperature and pressure of the injector was 205 °C and 250 kPa, respectively. The temperature of the detector was 250 °C. The sequence for the temperature of the oven started at 160 °C at injection, rising to 200 °C at 25 °C/min and maintained until the end of the elution. The carrier gas was N_2 at 1.5 ml/min. Samples were acidified with oxalic acid while pivalic acid was

used as internal standard.

5.2.3.4 Microbial communities

The solid phase of the mixed liquor samples was thawed, centrifuged at 10,000 rpm for 15 min and the solid phase was homogenised with a Dismembrator (B-Braun, Germany) for 2 min. Then, 500 µl of sterile Ringer solution (Oxoid, UK) and 500 µl of sterile glycerol (Merck, Germany) were added to the homogenised sample for preservation at -20 °C.

Analysis of microbial communities was conducted by DNA extraction, amplification by Polymerase Chain Reaction (PCR), separation of the amplicons by Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing. Extraction of DNA was performed using PowerSoil® DNA isolation kit according to the specifications of the manufacturer. The extracted DNA was amplified with a "touch-down" PCR, which consisted of an initial denaturation at 94 °C for 5 min, followed by 16 cycles of 1 min at 94 °C, 0.5 min at a temperature decreasing by 1 °C/cycle (starting from 68 °C) and 1 min at 72°C, then 15 cycles of 1 min at 94 °C, 0.5 min at 52 °C and 1 min at 72°C, and a final elongation at 72 °C for 3 min. The PCR was performed with 0.25 µM of the forward primer for universal bacteria S-D-Bact-0517-a-S-17 (5'-GCCAGCAGCCGCGGTAA-3') (Wang and Qian, 2009) fused with a 40-bp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') GC clamp *(*Muyzer *et al.*, 1993) and 0.25 µM of the reverse primer for universal bacteria S-D-Bact-1061-a-A-19 (5'-CACGACACGAGCTGACGAC-3') (Degnan and Ochman, 2012). The amplification covered the V4 to V6 hypervariable regions of 16S rRNA gene. The amplified DNA was visualised under UV light after electrophoresis (30 min at 100 V) on a 0.8% agarose gel stained with 0.5 µg/ml ethidium bromide.

Separation of the DNA fragments was achieved by DGGE. The denaturing gel was composed of 7% acrylamide/bis-acrylamide 37.5:1 (BioRad, CA, USA) in 0.5x TAE pH 7.8. The denaturing gradient (35-65%) was obtained by an instrument (Variomag, FL, USA) connecting two solutions at 35% and 65% denaturing concentrations (100% corresponding to 7 mol/L urea and 40 % v/v formamide). DNA loading was performed at 12 V to limit the diffusion of DNA out of the well. Electrophoresis was conducted on an INGENYphorU-2x2 (Ingeny International BV, Goes, Pays-Bas) for 16h at 60 °C and 120 V. The gel was removed and placed for 1h in a SYBRGold solution prepared with 400 ml ultrapure water, 4 ml TAE 50x and 40 µl SYBR gold 10,000x (Invitrogen, NM, USA). The gel was further placed on a transilluminator (Clare Chemical Research, CO, USA) permitting to visualise DNA and excise the bands using sterile scalpel blades. The DNA bands were then placed in ultrapure water at 4 °C during 48h to let DNA diffuse. DNA-containing water

was diluted 3200-fold to increase the signal-to-noise ratio which can be low with excised bands, and reamplified by PCR under the same conditions as described above without the GC clamp. A second electrophoresis on an agarose gel was performed to confirm the amplification efficiency and amplicons were sequenced (Macrogen Inc., Amsterdam, the Netherlands).

Statistical analysis and treatment of the DGGE gels was conducted with the Gel Compare software (Applied Maths NV, Sint-Martens-Latem, Belgium). The Pearson product-moment correlation coefficient (PPMCC) (Pearson, 1926) was used as similarity coefficient to perform the cluster analysis of DGGE profiles based on their densitometric curves. For measuring reproducibility, the DGGE similarity coefficients were calculated using the Dice coefficient (Dice, 1945) to reduce the bias induced by variable loading volumes in the gel.

5.3 Results & Discussion

5.3.1 Profiles of soluble and gaseous metabolites

The profiles of the soluble metabolites observed in the different fermentations are shown in Figure 5-1. Inoculation with pre-treated activated sludge (AS series) led to slightly higher tVFA concentrations than with rumen (AR series), at the level of 11.5 $g_{COD\_tVFA}/kg_{ML,}$ compared to 9.6 g_{COD_tVFA}/kg_{ML}, respectively. The corresponding conversion yields were 0.21 and 0.18 g_{COD_tVFA}/g_{CODt}, respectively. Interestingly, the non-inoculated pulp reached a concentration of 8.9 g_{COD_tVFA}/kg_{ML} (corresponding to a 0.21 g_{COD_tVFA}/g_{CODt} yield), very close to that of the AR series.

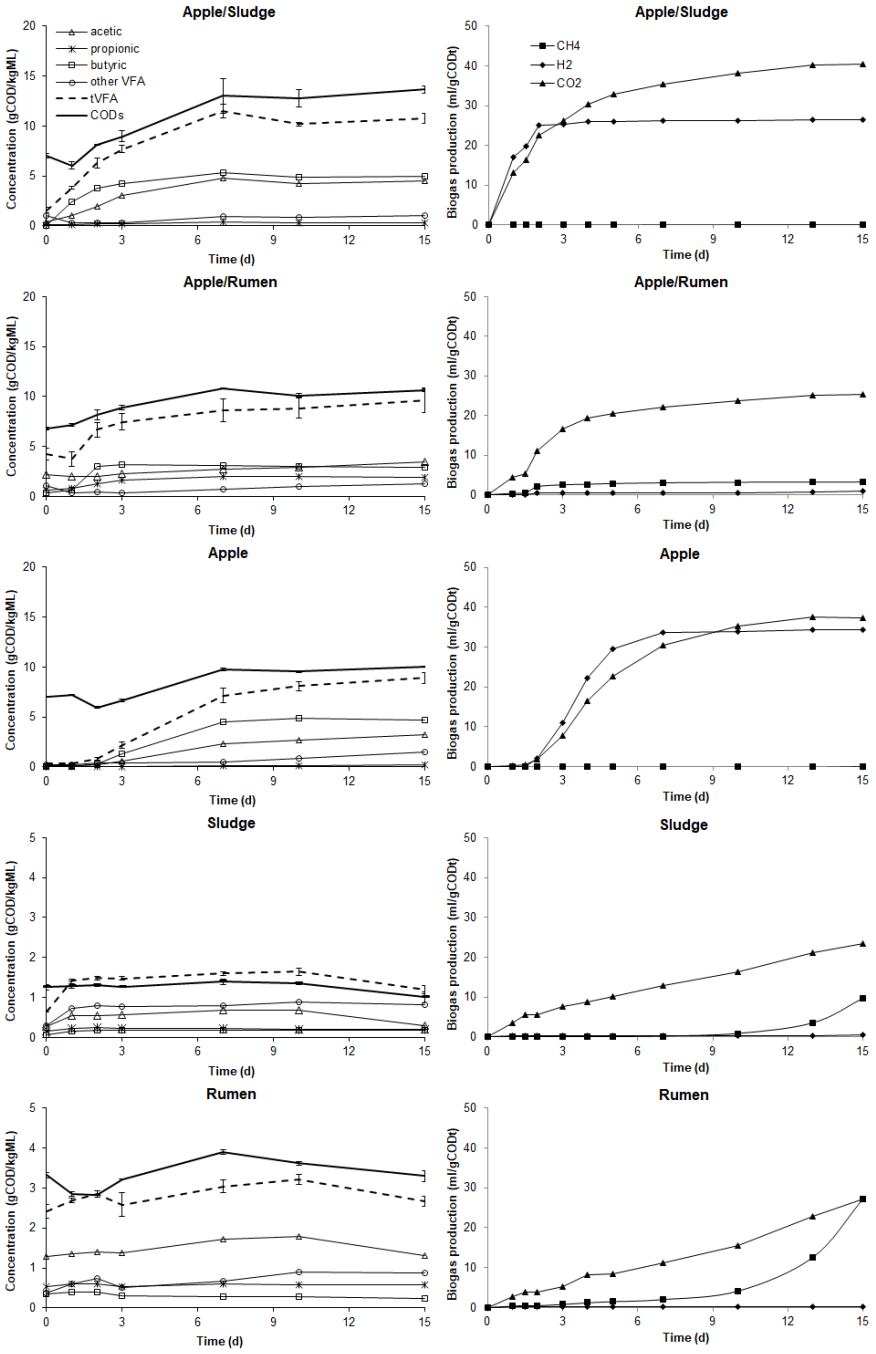

Figure 5-1: Concentration of soluble metabolites (left) and cumulated yield of gaseous metabolites (relative to initial COD) (right) in fermentation of AS, AR, A, S and R series (from top to bottom).

The initial concentrations of tVFA in the AS and AR reactors were 1.6 and 4.2 g_{COD_tVFA}/kg_{ML}, respectively. This difference is due to the presence of tVFA in the cow rumen. There is, therefore, a 7-fold net increase of the tVFA concentration in the AS series, compared to a 2-fold increase in the AR series. The VFA rapidly constituted the majority of the CODs fraction in both mixed series indicating that acidogenesis was the main fermentation taking place. Acetic and butyric acids were the dominant acids produced, as previously reported for mixed acidogenic fermentation using complex substrates (Arslan *et al.*, 2016). Interestingly, the AR series also induced the production of propionic acid, up to a concentration of 2.0 g_{COD}/kg_{ML}. The presence of propionic acid in the AR could be correlated to the absence of H_2, since the stoichiometry of propionic acid production from glucose, for instance, requires the consumption of H_2. This is in line with the absence of propionic acid in the AS series and the corresponding presence of H_2 (about 2% of the initial CODt).

No methane was detected in the AS series and minute amounts (corresponding to less than 1% of the initial CODt) were observed in the AR series. Methane formation in the activated sludge controls, although not significant in terms of COD (about 7% of the initial CODt), indicates that the acid and thermal pre-treatment alone was not sufficient to avoid methanogenesis. Instead, a combination of factors, namely the pre-treatment of inoculum, the maintenance of pH in the acidic range and the conversion of the substrate itself to yield VFA, achieved the desired result, as illustrated by the absence of methane in the AS series. In addition, little methane was formed in the control reactors of the rumen fluid (about 3% of the initial CODt), where neither pre-treatment nor pH adaptation took place. Weimer *et al.*, 2009 reported that hydrogenotrophic methanogenesis (i.e. reduction of CO_2 by H_2) is the primary methane formation pathway in the rumen, however Figure 5-1 shows no hydrogen production for the rumen control. It can be assumed that the little methane produced was either produced via the aceticlastic pathway, that is not dominant in the rumen because of short feed retention times, or that hydrogen produced is immediately consumed by methanogens and does not appear in the Figure 5-1.

As shown in Table 5-2, the VFA yields recorded in this study are comparable to those previously observed in similar acidogenic fermentations of agroindustrial waste. Moreover, specifically for the AR series, the level of maximum VFA concentration achieved was approximately 93 mM, within the range of typical VFA concentration in the rumen, *i.e.* 70-180 mM (Weimer and Kohn, 2016).

Table 5-2: VFA yield for batch acidogenic conversion of agroindustrial waste
RF: Rumen fluid; AS: activated sludge; T: temperature

Inoculum	Substrate	Conditions		Substrate Concentration ($g_{VS\_substrate}/l$)	VFA yield ($g_{COD\_tVFA}/g_{VS\_substrate}$)	Source
		pH	T (°C)			
RF	Apple pulp	5-5.5	35	26.3	0.37	This study
RF	Corn stover	6	35	15	0.43	1
RF	Wheat straw	6.15	30	15.4*	0.55*	2
AS	Apple pulp	5-5.5	35	26.3	0.44	This study
AS	Food waste	6	30	53.40	0.58	3

**calculated based on the information in the reference*
1: Hu and Yu (2005); 2: Lazuka et al. (2015); 3: Wang et al. (2014)

5.3.2 Profiles of bacterial communities

The bacterial communities were characterised by PCR-DGGE for all fermentation series at days 0, 1, 2, 3, 7, 10 and 15 (Figure 5-2). The species identified from the DGGE profiles are presented in Table 5-3.

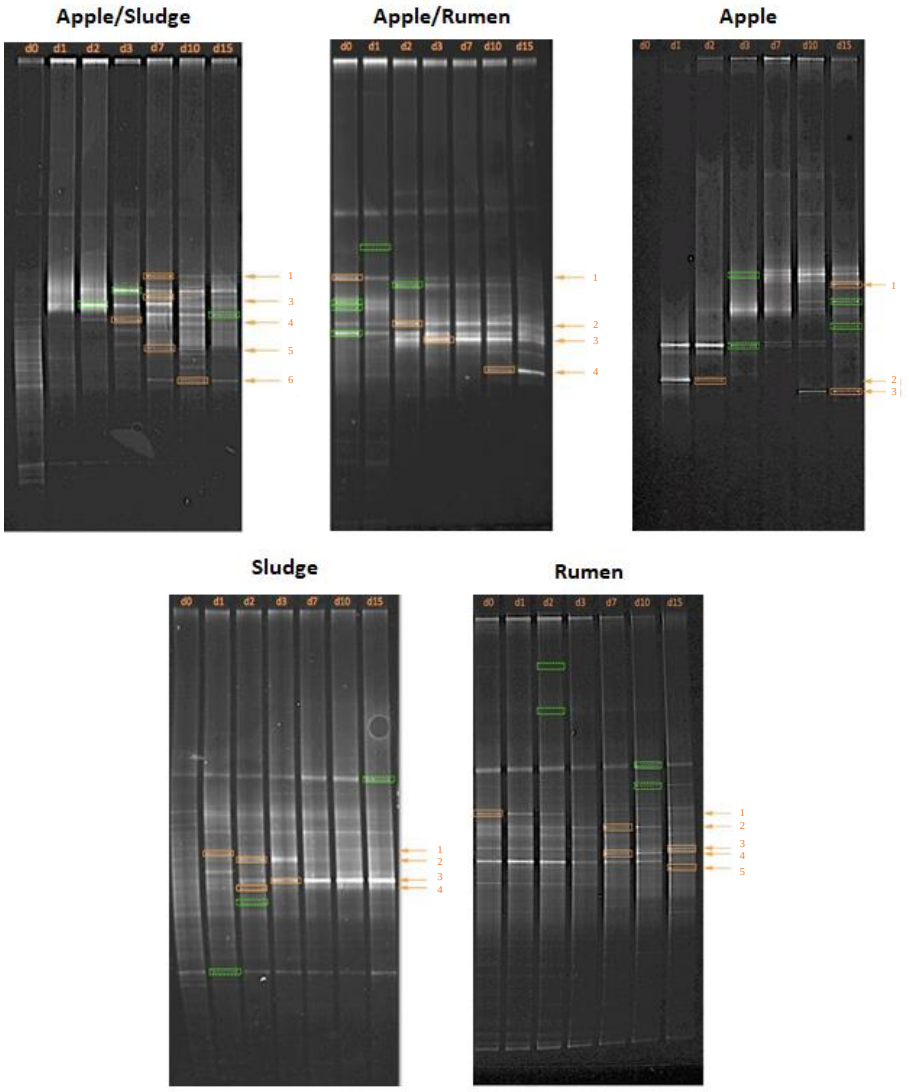

Figure 5-2: DGGE profiles of acidogenic fermentation series per sampling day.
The orange frames and the adjacent numbers correspond to the sequenced bands, as shown in Table 5-3. The green dashed frames correspond to species whose sequences were not exploitable. The profile of one reactor from each series is shown (see reproducibility discussion in section 3.4 and Fig. SM1); d: day

In the AS series, a large number of weak bands were present at day 0 (d0). These bands were similar to those observed in the sludge inoculum (similarity coefficient of 63%). At d1 and d3, the number of bands decreased and the intensity of the bands became more contrasted, suggesting the preferential

development of a reduced number of bacteria. Band #4, corresponding to a bacterium of the *Ruminococcaceae* family, seemed to be persistent between d0 and d3, but then disappeared. The number of bands increased again at d7 and remained relatively stable thereafter. New bands observed on d7 were notably affiliated to *Clostridium straminisolvens* (band #1), a spore-forming species isolated by Kato *et al.* (2004) from a cellulose-degrading bacterial community, *Clostridium thermosuccinogenes* (band #3), a thermophilic bacterium able to ferment carbohydrates into succinate, acetate, formate, lactate and ethanol (Sridhar and Eiteman, 2001), *Clostridium* sp. (band #5) and *Ethanoligenens harbinense* (band #6), an ethanol-based H_2-producing bacterium isolated by Ren *et al.* (2007) from an anaerobic reactor treating beet molasses.

In the AR series, the microbial consortium seemed much more stable throughout the whole experiment, the most important evolution being the increased intensity of a limited number of bands, notably those affiliated to *Prevotella bryantii* (band #3) from d2, a common amylolytic bacterium accounting for over 19% of total bacteria counts in the rumen (Lou *et al.*, 1997), and *Selenomonas ruminantium* (band #2), a propionate-producing rumen microorganism (Koike *et al.*, 2007), the dominance of which could be correlated to the production of propionic acid in the AR reactors. Conversely, the intensity of the band related to a member of the *Acinetobacter* genus (band #1), also reported by Jung *et al.* (2015) during acidogenic fermentation of macroalgae, was maximal on d0 but steadily decreased thereafter.

The DGGE profile of the non-inoculated pulp in Figure 5-2 revealed the absence of visible bands at d0, which suggested that the inherent microorganisms have not yet started to develop in the pulp. This is probably due to the very low pH of the substrate (approximately 3.5) and the low bacterial load resulting from the substrate treatment prior to our experiments (hot water extraction for residual juice recovery). A reduced number of bacteria developed on d1, including a member of the *Bacillus subtilis* or *Bacillus amyloliquefaciens* groups (band #2). Bacteria of these groups are often associated with the degradation of poly- and oligosaccharides (Ouoba *et al.*, 2007; Zhu *et al.*, 2013). These bacteria had little to no effect on VFA production and could not be maintained in the reactor; from d3, the diversity switched towards a different consortium, which coincided with the increase in VFA production (Figure 3-1). Members of the second consortium were also observed in the AS series, notably *C. straminisolvens* and *E. harbinense*. Based on (i) their late appearance in the A series and the absence of notable effect on VFA production in the meantime (from d7 to d15), and (ii) their appearance in AS series only at d7, when most of the VFA were already produced, it could be postulated that these bacteria did not play a major role in VFA production.

In the sludge series, a high number of weak bands were present on d0. Most of these bands remained present throughout the whole experiment, but a few bacteria seemed particularly favoured by the conditions, in particular *Azospira* sp. (band #3) and *Comamonas* sp. (band #4) from d2, both genera identified by Zhang *et al*. (2016) during acidogenesis of waste activated sludge. By contrast, *Dechloromonas* sp. (band #2), reported by Yuan *et al.* (2015) also during acidogenesis of waste activated sludge, was impacted by the reactor conditions since its abundance decreased from d3 to undetectable level at the end of the experiment.

A limited evolution was observed for the rumen fluid control reactors. Some bands remained present throughout the whole experiment, e.g. *Ruminococcus flavefaciens* (band #2), a rumen fibrolytic bacterium able to produce acetate, lactate, succinate, formate and CO_2 (Sawanon and Kobayashi, 2006). On the other hand, some abundant bacteria observed at d0 were impacted and the intensity of the associated bands decreased, notably *Acinetobacter haemolyticus* from d2 (band #1), a lipolytic species used by Kumar *et al.* (2014) to enhance biowaste conversion to H_2 and CH_4. New bacteria were also detected, including *Comamonas denitrificans* or *Comamonas testosteroni* (band #6), both being reported as denitrifying bacteria in wastewater treatment (Gumaelius *et al.*, 2001).

Table 5-3: Microorganisms identified from the PCR-DGGE gels
Band numbers correspond to the ones in Figure 5-2; F: Firmicutes; B: Bacteroidetes; P: Proteobacteria.

Series	Band	Affiliation	Accession number of closest relative	Nucleotide Identity (%)
AS	1	*Clostridium straminisolvens* (F)	NR024829	431/433 (99%)
AS	3	*Clostridium thermosuccinogenes* (F)	LN881583	139/141 (99%)
AS	4	*Ruminococcaceae bacterium* (F)	JQ607885	475/486 (98%)
AS	5	*Clostridium sp.* (F)	AY949859	472/480 (98%)
AS	6	*Ethanoligenens harbinense* (F)	NR074333	441/458 (96%)
AR	1	*Acinetobacter sp.* (P)	MF872590	350/351 (99%)
AR	2	*Selenomonas ruminantium* (F)	LC037209	455/468 (97%)
AR	3	*Prevotella bryantii* (B)	JQ674698	455/456 (99%)
AR	4	*Prevotella sp.* (B)	DQ278861	486/489 (99%)
A	1	*Clostridium straminisolvens* (F)	NR024829	420/445 (94%)
A	2	*Bacillus subtilis* (F)	JQ308560	440/444 (99%)
		Bacillus amyloliquefaciens (F)	JQ936682	
A	3	*Ethanoligenens harbinense* (F)	NR074333	407/431 (94%)
S	1	*Rummeliibacillus pycnus* (F)	LC000684	213/227 (94%)
S	2	*Dechloromonas sp.* (P)	KX622737	479/491 (98%)
S	3	*Azospira sp.* (P)	KC247691	492/492 (100%)

		Dechlorosoma sp. (P)	LC097211	
S	4	*Comamonas sp.* (P)	KX826980	476/501 (95%)
R	1	*Acinetobacter haemolyticus* (P)	KJ563239	431/436 (99%)
R	2	*Ruminococcus flavefaciens* (F)	KX155563	162/166 (97%)
R	3	*Prevotella sp.* (B)	DQ278861	484/487 (99%)
R	4	*Azonexus sp.* (P)	DQ833391	412/422 (98%)
		Dechloromonas sp. (P)	AB795533	
R	6	*Comamonas denitrificans* (P)	KU991392	214/218 (98%)
		Comamonas testosteroni (P)	KY048436	

Interestingly, from the species detected by DGGE in the present study, none has been reported before by another acidogenic study using the same technique. In fact, an examination of previous works that performed DGGE for acidogenic fermentations at varying conditions (Cho *et al*., 2015; Han *et al*., 2016; Kim *et al*., 2011, 2013; Seon *et al*., 2014; Ye *et al*., 2007) revealed that, out of approximately 90 entries at the species level (excluding uncultured bacteria), only 3 (*Lactobacilus acidophilus, Pseudomonas fluorescens and Bifidobacterium thermacidophilum*) have been reported more than once. This observation indicates the richness of acidogenic microorganisms present in bioreactors and raises the question of whether a core acidogenic consortium exists that is ubiquitous under different experimental conditions. At phylum level, 48% of the species identified with DGGE belong to *Firmicutes*, followed by *Proteobacteria* (40%) and *Bacteroidetes* (12%).

5.3.3 Effect of inoculum on acidogenic performance and microbial dynamics

The rumen inoculum is known to maintain an acidogenic microbiota (Weimer *et al.*, 2015) and already contained VFA, as observed on d0 for both metabolic profiles of series R and AR (Figure 5-1). The VFA concentration (approximately 2.5 g_{COD_tVFA}/kg_{ML}) is consistent with the values reported by Hernández-García *et al.* (2015). However, the final performance of the rumen inoculum was lower than that of the pre-treated activated sludge. Shin *et al.* (2011) argued that this low performance could be attributed to the transfer of the rumen microorganisms from their regular environment to a foreign one and the associated acclimation difficulties (e.g. decreased buffering capacity due to the absence of saliva (bicarbonate buffer), VFA accumulation due to absence of VFA extraction by absorption through the rumen wall). Furthermore, a pH value lower than 6 (as in our experiments) may hinder the hydrolytic capacity of ruminal bacteria due to impaired adhesion to the substrate, thus leading to a poor acidogenic performance (Hernández-García *et al.*, 2015). The hypothesis that the low VFA production was caused by impaired metabolic capacities, is reinforced by the observation that the microbial profile of AR series remained relatively stable over the experimental

period, as confirmed by the similarity indices between DGGE profiles from successive days (Figure 5-3). This suggests that a majority of bacteria were present all along the experiment but did not contribute significantly to acidogenesis. Similarly, the rumen control series did not undergo a sharp remodelling of the microbial consortia (Figure 5-4) and the presence of typical rumen bacteria (e.g. *R. flavefaciens*) remained stable over time while little VFA were produced.

Compared to the non-inoculated pulp, the addition of the rumen fluid offered no substantial gain in final tVFA concentration (only 7%) while this increase reached almost 30% in the case of activated sludge. This highlights the role of the inherent acidogenic flora of agroindustrial residues and the necessity to carefully select an external inoculum that will lead to a gain in acidogenic performance compared to the inherent microbiota of the substrate (Golub *et al.*, 2013).

Figure 5-3: Similarity matrix of DGGE profiles of reactors from AS and AR series.

The name of the series and the day of fermentation are indicated above and at the left of the matrix. Numbers refer to the coefficient of similarity (in %) between different fermentation days. One reactor of each series is considered.

The high number of weak bands at d0 of AS series can probably be explained by the sludge pre-treatment which reduced the microbial load. Following that, the medium was re-colonised by a reduced number of acidogenic species, probably originating from spores. The spore-forming genus *Clostridium* was indeed particularly represented, with *C. straminisolvens* (band #1), *C. thermosuccinogenes* (band #3) and *Clostridium* sp. (band #5). This impact of thermal treatment was reflected not only in the lowest similarity percentage between d0 and d1 to 3 (12.8 to 18.4%, see Figure 5-3), but also in the metabolic profile (Figure 5-1) that showed a steep increase of the tVFA concentration between d0 and d7, and a production of hydrogen which indicated that the acidogenic fermentation was starting.

A high degree of similarity in the AS series (above 80% within the period d3

to d15) reflected the stability of the microbial consortia established after d3. This is consistent with the VFA profile (Figure 5-1) that showed a peak of VFA production at d7 and stabilisation of VFA concentration until the end of fermentation. This microbial stability characterized also the AR series with a similarity index even higher (above 90% within the period d2 to d10) and again a rather stable VFA profile.

Furthermore, d0 in the S series showed a similar DGGE fingerprint as d0 in the AS series (large number of weak bands). This was expected, since the same inoculum was used in both series and the effect of the substrate addition was not yet marked. This effect was more visible during the following days where the similarity of the profiles of the two series was much lower (i.e. similarity coefficient of 14% and 15% between the reactors of the two series for d10 and d15, respectively).

5.4 Conclusion

Pre-treated activated sludge performed better than untreated ruminal fluid in VFA concentration and conversion yield. Inoculum pre-treatment led to a selection of a reduced but better performing consortium, containing mainly members of the *Clostridium* group, which coincided with a marked increase in tVFA concentration. The presence of propionic acid, the absence of hydrogen and the presence of *S. ruminantium* were simultaneously observed in reactors inoculated with ruminal fluid and are probably related. The reactors with non-inoculated apple pulp performed almost as well as reactors with rumen inoculated apple pulp, highlighting the importance of the inherent acidogenic microorganisms.

5.5 Acknowledgements

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5.6 References

Aguedo, M., Kohnen, S., Rabetafika, N., Vanden Bossche, S., Sterckx, J., Blecker, C., Beauve, C., Paquot, M., 2012. Composition of by-products from cooked fruit processing and potential use in food products. Journal of Food Composition and Analysis 27, 61–69. https://doi.org/10.1016/j.jfca.2012.04.005

Akutsu, Y., Lee, D.-Y., Li, Y.-Y., Noike, T., 2009. Hydrogen production potentials and fermentative characteristics of various substrates with different heat-pretreated natural microflora. International Journal of Hydrogen Energy 34, 5365–5372. https://doi.org/10.1016/j.ijhydene.2009.04.052

Arslan, D., Steinbusch, K.J.J., Diels, L., Hamelers, H.V.M., Strik, D.P.B.T.B.,

Buisman, C.J.N., De Wever, H., 2016. Selective short-chain carboxylates production: A review of control mechanisms to direct mixed culture fermentations. Critical Reviews in Environmental Science and Technology 46, 592–634. https://doi.org/10.1080/10643389.2016.1145959

Briones, A., Raskin, L., 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Current Opinion in Biotechnology 14, 270–276. https://doi.org/10.1016/S0958-1669(03)00065-X

Cho, H.U., Kim, Y.M., Choi, Y.-N., Kim, H.G., Park, J.M., 2015. Influence of temperature on volatile fatty acid production and microbial community structure during anaerobic fermentation of microalgae. Bioresource Technology 191, 475–480. https://doi.org/10.1016/j.biortech.2015.03.009

Degnan, P.H., Ochman, H., 2012. Illumina-based analysis of microbial community diversity. ISME J 6, 183–194. https://doi.org/10.1038/ismej.2011.74

Dice, L.R., 1945. Measures of the Amount of Ecologic Association Between Species. Ecology 26, 297–302. https://doi.org/10.2307/1932409

Forrest, A.K., Hollister, E.B., Gentry, T.J., Wilkinson, H.H., Holtzapple, M.T., 2012. Comparison of mixed-acid fermentations inoculated with six different mixed cultures. Bioresource Technology 118, 343–349. https://doi.org/10.1016/j.biortech.2012.05.043

Golub, K.W., Forrest, A.K., Wales, M.E., Hammett, A.J.M., Cope, J.L., Wilkinson, H.H., Holtzapple, M.T., 2013. Comparison of three screening methods to select mixed-microbial inoculum for mixed-acid fermentations. Bioresource Technology 130, 739–749. https://doi.org/10.1016/j.biortech.2012.10.010

Gumaelius, L., Magnusson, G., Pettersson, B., Dalhammar, G., 2001. Comamonas denitrificans sp. nov., an efficient denitrifying bacterium isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 51, 999–1006. https://doi.org/10.1099/00207713-51-3-999

Han, G., Shin, S.G., Lee, J., Lee, C., Jo, M., Hwang, S., 2016. Mesophilic Acidogenesis of Food Waste-Recycling Wastewater: Effects of Hydraulic Retention Time, pH, and Temperature. Applied Biochemistry and Biotechnology 180, 980–999. https://doi.org/10.1007/s12010-016-2147-z

Hernández-García, H., Olguín, E.J., Sánchez-Galván, G., Monroy-Hermosillo, O., 2015. Production of Volatile Fatty Acids during the Hydrolysis and Acidogenesis of Pistia stratiotes Using Ruminal Fluid. Water, Air, & Soil Pollution 226. https://doi.org/10.1007/s11270-015-2494-3

Hu, Z.-H., Yu, H.-Q., 2005. Application of rumen microorganisms for enhanced anaerobic fermentation of corn stover. Process Biochemistry 40, 2371–2377. https://doi.org/10.1016/j.procbio.2004.09.021

Infantes, D., González del Campo, A., Villaseñor, J., Fernández, F.J., 2012. Kinetic model and study of the influence of pH, temperature and undissociated acids on acidogenic fermentation. Biochemical Engineering Journal 66, 66–72. https://doi.org/10.1016/j.bej.2012.04.017

Jung, K., Kim, W., Park, G.W., Seo, C., Chang, H.N., Kim, Y.-C., 2015. Optimization

of volatile fatty acids and hydrogen production from Saccharina japonica: acidogenesis and molecular analysis of the resulting microbial communities. Applied Microbiology and Biotechnology 99, 3327–3337. https://doi.org/10.1007/s00253-015-6419-2

Kafle, G.K., Kim, S.H., 2013. Anaerobic treatment of apple waste with swine manure for biogas production: Batch and continuous operation. Applied Energy 103, 61–72. https://doi.org/10.1016/j.apenergy.2012.10.018

Kato, S., Haruta, S., Cui, Z.J., Ishii, M., Yokota, A., Igarashi, Y., 2004. Clostridium straminisolvens sp. nov., a moderately thermophilic, aerotolerant and cellulolytic bacterium isolated from a cellulose-degrading bacterial community. Int. J. Syst. Evol. Microbiol. 54, 2043–2047. https://doi.org/10.1099/ijs.0.63148-0

Kim, J., Shin, S.G., Han, G., O'Flaherty, V., Lee, C., Hwang, S., 2011. Common key acidogen populations in anaerobic reactors treating different wastewaters: Molecular identification and quantitative monitoring. Water Research 45, 2539–2549. https://doi.org/10.1016/j.watres.2011.02.004

Kim, W., Shin, S.G., Lim, J., Hwang, S., 2013. Effect of temperature and hydraulic retention time on volatile fatty acid production based on bacterial community structure in anaerobic acidogenesis using swine wastewater. Bioprocess and Biosystems Engineering 36, 791–798. https://doi.org/10.1007/s00449-013-0905-7

Kleerebezem, R., van Loosdrecht, M.C., 2007. Mixed culture biotechnology for bioenergy production. Current Opinion in Biotechnology, Energy biotechnology / Environmental biotechnology 18, 207–212. https://doi.org/10.1016/j.copbio.2007.05.001

Koike, S., Yabuki, H., Kobayashi, Y., 2007. Validation and application of real-time polymerase chain reaction assays for representative rumen bacteria. Animal Science Journal 78, 135–141. https://doi.org/10.1111/j.1740-0929.2007.00417.x

Kumar, P., Pant, D.C., Mehariya, S., Sharma, R., Kansal, A., Kalia, V.C., 2014. Ecobiotechnological Strategy to Enhance Efficiency of Bioconversion of Wastes into Hydrogen and Methane. Indian Journal of Microbiology 54, 262–267. https://doi.org/10.1007/s12088-014-0467-7

Lazuka, A., Auer, L., Bozonnet, S., Morgavi, D.P., O'Donohue, M., Hernandez-Raquet, G., 2015. Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium. Bioresource Technology 196, 241–249. https://doi.org/10.1016/j.biortech.2015.07.084

Llaneza Coalla, H., Blanco Fernández, J.M., Morís Morán, M.A., López Bobo, M.R., 2009. Biogas generation apple pulp. Bioresource Technology 100, 3843–3847. https://doi.org/10.1016/j.biortech.2009.03.012

Lou, J., Dawson, K.A., Strobel, H.J., 1997. Glycogen biosynthesis via UDP-glucose in the ruminal bacterium Prevotella bryantii B1(4). Appl. Environ. Microbiol. 63, 4355–4359.

Morgavi, D.P., Forano, E., Martin, C., Newbold, C.J., 2010. Microbial ecosystem and methanogenesis in ruminants. Animal 4, 1024–1036. https://doi.org/10.1017/S1751731110000546

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59, 695–700.

Ouoba, L.I.I., Diawara, B., Christensen, T., Mikkelsen, J.D., Jakobsen, M., 2007. Degradation of polysaccharides and non-digestible oligosaccharides by Bacillus subtilis and Bacillus pumilus isolated from Soumbala, a fermented African locust bean (Parkia biglobosa) food Condiment. Eur Food Res Technol 224, 689–694. https://doi.org/10.1007/s00217-006-0359-0

Park, G.W., Seo, C., Jung, K., Chang, H.N., Kim, W., Kim, Y.-C., 2015. A comprehensive study on volatile fatty acids production from rice straw coupled with microbial community analysis. Bioprocess and Biosystems Engineering 38, 1157–1166. https://doi.org/10.1007/s00449-015-1357-z

Pearson, K., 1926. On the coefficient of racial likeness. Biometrika 18, 105–117. https://doi.org/10.1093/biomet/18.1-2.105

Ravindran, R., Jaiswal, A.K., 2016. Exploitation of Food Industry Waste for High-Value Products. Trends in Biotechnology 34, 58–69. https://doi.org/10.1016/j.tibtech.2015.10.008

Ren, N., Xing, D., Rittmann, B.E., Zhao, L., Xie, T., Zhao, X., 2007. Microbial community structure of ethanol type fermentation in bio-hydrogen production. Environ. Microbiol. 9, 1112–1125. https://doi.org/10.1111/j.1462-2920.2006.01234.x

Sawanon, S., Kobayashi, Y., 2006. Synergistic fibrolysis in the rumen by cellulolytic Ruminococcus flavefaciens and non-cellulolytic Selenomonas ruminantium: Evidence in defined cultures. Animal Science Journal 77, 208–214. https://doi.org/10.1111/j.1740-0929.2006.00339.x

Seon, J., Lee, T., Lee, S.C., Pham, H.D., Woo, H.C., Song, M., 2014. Bacterial community structure in maximum volatile fatty acids production from alginate in acidogenesis. Bioresource Technology 157, 22–27. https://doi.org/10.1016/j.biortech.2014.01.072

Shalini, R., Gupta, D.K., 2010. Utilization of pomace from apple processing industries: a review. J Food Sci Technol 47, 365–371. https://doi.org/10.1007/s13197-010-0061-x

Shin, S.G., Yoo, S., Hwang, K., Song, M., Kim, W., Han, G., Hwang, S., 2011. Dynamics of transitional acidogenic community along with methanogenic population during anaerobic digestion of swine wastewater. Process Biochemistry 46, 1607–1613. https://doi.org/10.1016/j.procbio.2011.05.001

Singhania, R.R., Patel, A.K., Christophe, G., Fontanille, P., Larroche, C., 2013. Biological upgrading of volatile fatty acids, key intermediates for the valorization of biowaste through dark anaerobic fermentation. Bioresour. Technol. 145, 166–174. https://doi.org/10.1016/j.biortech.2012.12.137

Sridhar, J., Eiteman, M.A., 2001. Metabolic Flux Analysis of Clostridium thermosuccinogenes. Applied Biochemistry and Biotechnology 94, 51–70. https://doi.org/10.1385/ABAB:94:1:51

Wang, Y., Qian, P.-Y., 2009. Conservative Fragments in Bacterial 16S rRNA Genes and Primer Design for 16S Ribosomal DNA Amplicons in Metagenomic Studies. PLoS ONE 4, e7401. https://doi.org/10.1371/journal.pone.0007401

Wang, H., Wang, J., Fang, Z., Wang, X., Bu, H., 2010. Enhanced bio-hydrogen production by anaerobic fermentation of apple pomace with enzyme hydrolysis. International Journal of Hydrogen Energy 35, 8303–8309. https://doi.org/10.1016/j.ijhydene.2009.12.012

Wang, K., Yin, J., Shen, D., Li, N., 2014. Anaerobic digestion of food waste for volatile fatty acids (VFAs) production with different types of inoculum: Effect of pH. Bioresource Technology 161, 395–401. https://doi.org/10.1016/j.biortech.2014.03.088

Weimer, P.J., Russell, J.B., Muck, R.E., 2009. Lessons from the cow: what the ruminant animal can teach us about consolidated bioprocessing of cellulosic biomass. Bioresour. Technol. 100, 5323–5331. https://doi.org/10.1016/j.biortech.2009.04.075

Weimer, P.J., Nerdahl, M., Brandl, D.J., 2015. Production of medium-chain volatile fatty acids by mixed ruminal microorganisms is enhanced by ethanol in co-culture with Clostridium kluyveri. Bioresource Technology 175, 97–101. https://doi.org/10.1016/j.biortech.2014.10.054

Weimer, P.J., Kohn, R.A., 2016. Impacts of ruminal microorganisms on the production of fuels: how can we intercede from the outside? Applied Microbiology and Biotechnology 100, 3389–3398. https://doi.org/10.1007/s00253-016-7358-2

Ye, N.-F., Lü, F., Shao, L.-M., Godon, J.-J., He, P.-J., 2007. Bacterial community dynamics and product distribution during pH-adjusted fermentation of vegetable wastes. J. Appl. Microbiol. 103, 1055–1065. https://doi.org/10.1111/j.1365-2672.2007.03321.x

Yuan, Y., Wang, S., Liu, Y., Li, B., Wang, B., Peng, Y., 2015. Long-term effect of pH on short-chain fatty acids accumulation and microbial community in sludge fermentation systems. Bioresource Technology 197, 56–63. https://doi.org/10.1016/j.biortech.2015.08.025

Yue, Z.-B., Li, W.-W., Yu, H.-Q., 2013. Application of rumen microorganisms for anaerobic bioconversion of lignocellulosic biomass. Bioresource Technology 128, 738–744. https://doi.org/10.1016/j.biortech.2012.11.073

Zhang, D., Fu, X., Dai, X., Chen, Y., Dai, L., 2016. A new biological process for short-chain fatty acid generation from waste activated sludge improved by Clostridiales enhancement. Environ Sci Pollut Res 23, 23972–23982. https://doi.org/10.1007/s11356-016-7579-z

Zhao, B., Liu, J., Frear, C., Holtzapple, M., Chen, S., 2016. Consolidated bioprocessing of microalgal biomass to carboxylates by a mixed culture of cow rumen bacteria using anaerobic sequencing batch reactor (ASBR). Bioresource Technology 222, 517–522. https://doi.org/10.1016/j.biortech.2016.09.120

Zhu, Z., Zhang, F., Wei, Z., Ran, W., Shen, Q., 2013. The usage of rice straw as a major substrate for the production of surfactin by Bacillus amyloliquefaciens XZ-

173 in solid-state fermentation. Journal of Environmental Management 127, 96–102. https://doi.org/10.1016/j.jenvman.2013.04.017

5.7 Supplementary material

5.7.1 Standard bacterial cultures

In addition to the molecular techniques, standard bacterial cultures from the fermentation samples were developed for comparison purpose. The reference medium used was solid Lysogeny Broth (LB) medium, composed of 5 g/l of NaCl, 5 g/l of yeast extract (Oxoid), 10 g/l of tryptone (Oxoid) and 15 g/l of agar (BioRad). 10 mg of the solid phase of the fermentation sample were diluted 10 times, plated onto Petri dishes and incubated in anaerobic jar for 48 h at 30 °C. Isolated colonies were streaked on solid LB medium and incubated for another 48h. Identification of isolates was achieved by partial 16S rRNA gene sequencing. Briefly, a fresh colony was picked and placed in 200 µl of ultrapure water. After a vigorous mix, DNA-containing water was used for a subsequent PCR using bacterial universal primers S-D-Bact-0338-a-S-17 (5'-ACTCCTACGGGAGGCAG-3') (Yu *et al*., 2005) and S-D-Bact-1383-a-A-20 (5'-GGCGTGTGTACAAGGCCCGG-3') (Timmery *et al*., 2011). These primers were chosen because they generate a longer amplicon, which can ease the taxonomic assignment. The PCR mix was prepared with 0.25 µM forward and reverse primers, 1x colourless GoTaq® reaction buffer (Promega, USA), 0.2 mM of each deoxynucleotide, 1.5 mM MgCl2 and 0.03 u/µl GoTaq® DNA polymerase (Promega). The thermal profile was as follows: 94 °C for 5 min, followed by 30 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C and 1 min at 72°C, and a final elongation at 72 °C for 10 min. PCR amplification products were confirmed by electrophoresis at 100 V for 30 min on 0.8% agarose gels stained with 0.5 µg/ml ethidium bromide. Sequencing was realized on ABI 3730 XL DNA Analyzer (Life Technologies, Carlsbad, USA) at Macrogen Europe (Amsterdam, The Netherlands). The taxonomic assignment was performed using the online BlastN at NCBI (http://blast.ncbi.nlm.nih.gov) and the online RDPII tool Seqmatch release 11.1 (http://rdp.cme.msu.edu/seqmatch/). Long-term storage of bacterial isolates was achieved by suspending a fresh colony into a sterile mix of 1 ml of liquid LB and 0.5 ml of glycerol, stored at -80 °C. Bacteria isolated on solid LB medium and sequenced are presented in Table 5-4.

Table 5-4: Microorganisms isolated and sequenced from bacterial cultures

Affiliation (phylum)	Day	Series	Nucleotide identity %	Reference of similar sequence detection
Clostridium thiosulfatireducens (F)	0 3	AS AS	569/570 (99%) 536/537 (99%)	Hernández-Eugenio *et al*., 2002

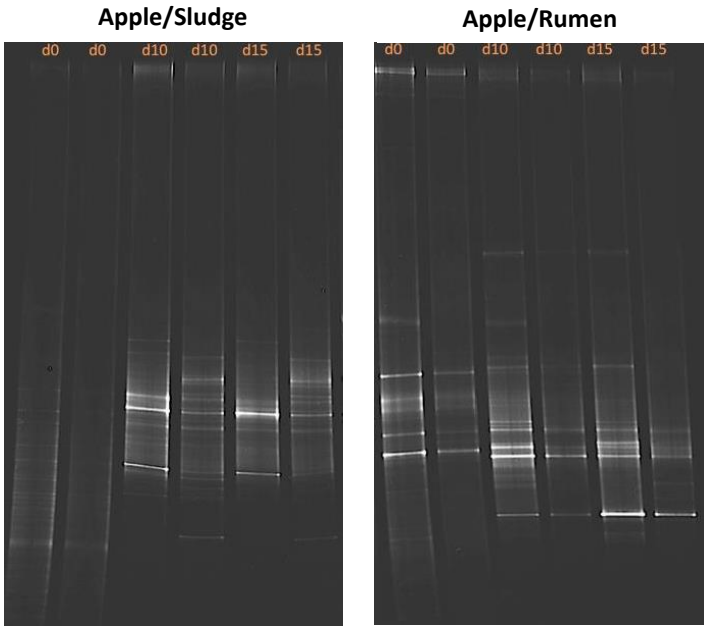
	15	AS	369/369 (99%)	
Clostridium argentinense (F)	0 0	AS S	594/594 (100%) 503/503 (100%)	-
Bacillus licheniformis (F)	0 3 0 15	AS AS S R	336/343 (98%) 204/211 (98%) 278/279 (99%) 961/962 (99%)	Perego *et al*., 2003
Bacillus amyloliquefaciens (F)	0	AS	336/343 (98%)	Yong *et al*., 2013
Clostridium bifermentans (F)	3 3 3 3 15	AS AR S R S	525/527 (99%) 623/623 (100%) 179/181 (99%) 832/832 (100%) 632/632 (100%)	Myszka *et al*., 2012
Clostridium difficile (F)	3 3 3 15 0	AS AR S S R	525/527 (99%) 623/623 (100%) 528/531 (99%) 427/436 (98%) 832/832 (100%)	-
Clostridium botulinum (F)	3 15 0 15	AS AS S R	427/429 (99%) 468/472 (99%) 503/503 (100%) 771/771 (100%)	-
Clostridium sporogenes (F)	15 0 15	AS S R	468/472 (99%) 152/152 (100%) 771/771 (100%)	Gottumukkala *et al*., 2013
Clostridium butyricum (F)	15 15 3 15	AS AR A R	386/391 (99%) 933/937 (99%) 889/890 (99%) 959/960 (99%)	Wang *et al*., 2008
Enterococcus faecalis (F)	0	AR	832/832 (100%)	Wee *et al*., 2004
Clostridium glycolicum (F)	0	AR	684/684 (100%)	Hunger *et al*., 2011
Clostridium sticklandii (F)	0 3	AR R	935/939 (99%) 942/947 (99%)	Fonknechten *et al*., 2010
Clostridium beijerinckii (F)	3 3 15	AR A A	138/141 (98%) 842/842 (100%) 828/834 (99%)	Lin *et al*., 2007
Lactobacillus mucosae (F)	3	AR	742/743 (99%)	Mamuad *et al*., 2017
Clostridium scatologenes (F)	15	AR	854/854 (100%)	Zhu *et al*., 2015
Clostridium perfringens (F)	0	S	506/512 (99%)	Wang *et al*., 2011
Clostridium hungatei (F)	3 15	S S	587/587(100%) 410/417 (98%)	Monserrate *et al*., 2001
Clostridium lituseburense (F)	15	S	665/669 (99%)	Song *et al*., 2011
Clostridium metallolevans (F)	0	R	735/735 (100%)	Ellis *et al*., 2014
Actinomyces nasicola (A)	3	R	814/824 (99%)	Nyonyo *et al*., 2014

5.7.2 Reproducibility of metabolic performance and DGGE profiles

Between the two reactors of the AS series, the similarity indices of the DGGE profiles for d0, d10 and d15 were 55%, 67% and 45%, respectively (DGGE

profiles are shown in Figure 5-4). The corresponding values between the two AR reactors were 50%, 40% and 52%, respectively. We can, therefore, conclude that the reproducibility of the microbial profiles of the series containing apple pulp and an inoculum is rather low. This, nevertheless, is not reflected on the metabolic side since the average deviation between the two reactors in the tVFA concentration after d2 is not exceeding 8 and 13% in the AS and AR series, respectively. This difference between DGGE and metabolic reproducibility could be explained in two ways. First, it is likely that different microorganisms could lead to the same metabolic products, thus exhibiting similar metabolic but different microbial profiles (Forrest *et al.*, 2012). Second, it could be possible that only a few bacteria would be responsible for the majority of the VFA production. As a consequence, the common presence of these few players in both reactors could be sufficient to generate the metabolic profile, even if the rest of the microbial population would considerably differ.

Contrary to the AR series, the R series showed a remarkable similarity between the two reactors of above 84% for the pairwise comparisons of any given day (DGGE profiles are shown in Figure 5-4). This suggests that the differences between the two AR reactors explained above may be due to the addition of the apple pulp and its inherent microbial flora.



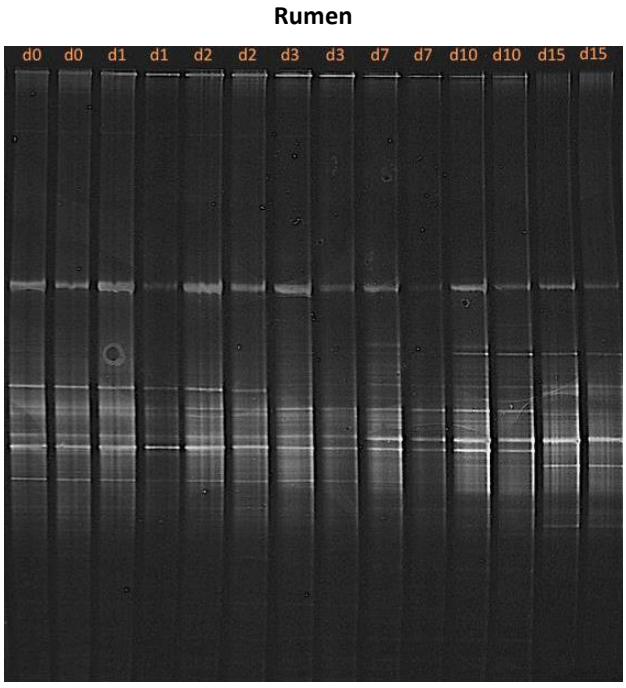


Figure 5-4: DGGE profiles of AS, AR and R duplicates.

5.7.3 References

Ellis, J.T., Sims, R.C., Miller, C.D., 2014. Microbial bioproducts from cheese whey through fermentation with wastewater sludge Clostridium isolates. Canadian Journal of Microbiology 60, 431–435. https://doi.org/10.1139/cjm-2013-0803

Fonknechten, N., Chaussonnerie, S., Tricot, S., Lajus, A., Andreesen, J.R., Perchat, N., Pelletier, E., Gouyvenoux, M., Barbe, V., Salanoubat, M., Le Paslier, D., Weissenbach, J., Cohen, G.N., Kreimeyer, A., 2010. Clostridium sticklandii, a specialist in amino acid degradation: revisiting its metabolism through its genome sequence. BMC Genomics 11, 555. https://doi.org/10.1186/1471-2164-11-555

Forrest, A.K., Hollister, E.B., Gentry, T.J., Wilkinson, H.H., Holtzapple, M.T., 2012. Comparison of mixed-acid fermentations inoculated with six different mixed cultures. Bioresource Technology 118, 343–349. https://doi.org/10.1016/j.biortech.2012.05.043

Gottumukkala, L.D., Parameswaran, B., Valappil, S.K., Mathiyazhakan, K., Pandey, A., Sukumaran, R.K., 2013. Biobutanol production from rice straw by a non-acetone producing Clostridium sporogenes BE01. Bioresource Technology 145, 182–187. https://doi.org/10.1016/j.biortech.2013.01.046

Hernández-Eugenio, G., Fardeau, M.-L., Cayol, J.-L., Patel, B.K.C., Thomas, P., Macarie, H., Garcia, J.-L., Ollivier, B., 2002. Clostridium thiosulfatireducens sp. nov., a proteolytic, thiosulfate- and sulfur-reducing bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor. Int. J. Syst. Evol. Microbiol. 52, 1461–1468. https://doi.org/10.1099/00207713-52-5-1461

Hunger, S., Gößner, A.S., Drake, H.L., 2011. Trophic Links between the Acetogen Clostridium glycolicum KHa and the Fermentative Anaerobe Bacteroides xylanolyticus KHb, Isolated from Hawaiian Forest Soil. Appl. Environ. Microbiol. 77, 6281–6285. https://doi.org/10.1128/AEM.05519-11

Lin, P.-Y., Whang, L.-M., Wu, Y.-R., Ren, W.-J., Hsiao, C.-J., Li, S.-L., Chang, J.-S., 2007. Biological hydrogen production of the genus Clostridium: Metabolic study and mathematical model simulation. International Journal of Hydrogen Energy 32, 1728–1735. https://doi.org/10.1016/j.ijhydene.2006.12.009

Mamuad, L. l., Kim, S. h., Choi, Y. j., Soriano, A. p., Cho, K. k., Lee, K., Bae, G. s., Lee, S. s., 2017. Increased propionate concentration in Lactobacillus mucosae–fermented wet brewers grains and during in vitro rumen fermentation. J Appl Microbiol 123, 29–40. https://doi.org/10.1111/jam.13475

Monserrate, E., Leschine, S.B., Canale-Parola, E., 2001. Clostridium hungatei sp. nov., a mesophilic, N2-fixing cellulolytic bacterium isolated from soil. Int. J. Syst. Evol. Microbiol. 51, 123–132. https://doi.org/10.1099/00207713-51-1-123

Myszka, K., Leja, K., Olejnik-Schmidt, A.K., Czaczyk, K., 2012. Isolation process of industrially useful Clostridium bifermentans from natural samples. Journal of Bioscience and Bioengineering 113, 631–633. https://doi.org/10.1016/j.jbiosc.2012.01.003

Nyonyo, T., Shinkai, T., Mitsumori, M., 2014. Improved culturability of cellulolytic rumen bacteria and phylogenetic diversity of culturable cellulolytic and xylanolytic bacteria newly isolated from the bovine rumen. FEMS Microbiol.

Ecol. 88, 528–537. https://doi.org/10.1111/1574-6941.12318

Perego, P., Converti, A., Del Borghi, M., 2003. Effects of temperature, inoculum size and starch hydrolyzate concentration on butanediol production by Bacillus licheniformis. Bioresour. Technol. 89, 125–131.

Song, J., An, D., Ren, N., Zhang, Y., Chen, Y., 2011. Effects of pH and ORP on microbial ecology and kinetics for hydrogen production in continuously dark fermentation. Bioresource Technology 102, 10875–10880. https://doi.org/10.1016/j.biortech.2011.09.024

Timmery, S., Hu, X., Mahillon, J., 2011. Characterization of Bacilli Isolated from the Confined Environments of the Antarctic Concordia Station and the International Space Station. Astrobiology 11, 323–334. https://doi.org/10.1089/ast.2010.0573

Wang, M.-Y., Tsai, Y.-L., Olson, B.H., Chang, J.-S., 2008. Monitoring dark hydrogen fermentation performance of indigenous Clostridium butyricum by hydrogenase gene expression using RT-PCR and qPCR. International Journal of Hydrogen Energy 33, 4730–4738. https://doi.org/10.1016/j.ijhydene.2008.06.048

Wang, R., Zong, W., Qian, C., Wei, Y., Yu, R., Zhou, Z., 2011. Isolation of Clostridium perfringens strain W11 and optimization of its biohydrogen production by genetic modification. International Journal of Hydrogen Energy 36, 12159–12167. https://doi.org/10.1016/j.ijhydene.2011.06.105

Wee, Y.-J., Kim, J.-N., Yun, J.-S., Ryu, H.-W., 2004. Utilization of sugar molasses for economical l(+)-lactic acid production by batch fermentation of Enterococcus faecalis. Enzyme and Microbial Technology 35, 568–573. https://doi.org/10.1016/j.enzmictec.2004.08.008

Yong, X., Zhang, R., Zhang, N., Chen, Y., Huang, X., Zhao, J., Shen, Q., 2013. Development of a specific real-time PCR assay targeting the poly-γ-glutamic acid synthesis gene, pgsB, for the quantification of Bacillus amyloliquefaciens in solid-state fermentation. Bioresource Technology 129, 477–484. https://doi.org/10.1016/j.biortech.2012.11.092

Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnology and Bioengineering 89, 670–679. https://doi.org/10.1002/bit.20347

Zhu, Z., Guo, T., Zheng, H., Song, T., Ouyang, P., Xie, J., 2015. Complete genome sequence of a malodorant-producing acetogen, Clostridium scatologenes ATCC 25775T. Journal of Biotechnology 212, 19–20. https://doi.org/10.1016/j.jbiotec.2015.07.013

6 Contribution of natural microbial consortia to the production of lactic acid from beer hot trub

Comment

This study is deviating slightly from the general scheme of acidogenic fermentation to VFA followed in this thesis, as lactic acid fermentation is a different metabolic pathway. Nevertheless, it fits very well into the general frame of mixed fermentation for carboxylic acids and is therefore an integral part of this thesis. This study was realised after the interesting results from the fermentation of trub under different conditions (i.e. with/without inoculum, with/without pH adjustment). As explained further below, the presence of lactic acid was rather unexpected due to the existence of hop acids, substances with bacteriostatic effect. This was all the more surprising since partners of the WALAID tried unsuccessfully to produce lactic acid from trub through pure culture fermentation with hop resistant strains. This study added, therefore, a further potential advantage to the discussion on the use of mixed consortia; that of degrading unwanted substances to achieve targeted carboxylate production.

Chapter submitted for publication:

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*Corresponding author:

Specific AP contribution: experimental work, data analysis, data interpretation, article compilation

Abstract

Hop α-acids, iso-α-acids and β-acids are known to inhibit lactic acid bacteria. Despite this inhibitory effect, the application of mixed microbial fermentation was investigated for the production of lactic acid, a biobased acid with many chemical applications, from hot trub, a residue from beer brewing. Lactic acid concentrations as high as about 20 g/l were obtained. A microbial analysis by PCR/DGGE and culture dependent techniques identified dominant microorganisms that could be responsible for this result, but none of them has been reported to be resistant to hop substances. Hop acids were detected at the beginning of the experiments but were not present anymore at the days of highest lactic acid concentrations. The presence of lactic acid is thus attributed to the collective metabolic activity of the mixed cultures, through which hop substances were degraded and lactic acid bacteria were able to develop.

Keywords

Hot trub, lactic acid, hop resistance, mixed fermentation, brewery residues

6.1 Introduction

Brewery residues are considered substrates with a considerable potential for biotechnological applications (Brito *et al*., 2007; Mathias *et al*., 2014; Mussatto, 2009; Xiros and Christakopoulos, 2012). Hot trub is an important waste fraction in breweries and is generated during wort boiling and hop addition by the coagulation and precipitation of high mass proteins, phenolic compounds, hop substances, and polysaccharides not hydrolysed during mashing (Mathias *et al*., 2015).

Trub can be valorised in cattle feeding but needs to be stabilised for storage (Mathias *et al*., 2014). Direct lactic acid fermentation in the trub could be a sustainable way of stabilising trub, without the need of adding external acids. Alternatively, lactic acid is a base chemical already produced at industrial scale through pure microbial fermentation of sugar monomers and with a market potential to reach $3.8 bn by 2020 (Focus on Catalysts, 2016). Production from trub could be an additional, more sustainable way to feed green chemistry (Castillo Martinez *et al*., 2013; Li and Cui, 2010).

The majority of lactic acid bacteria are known to be inhibited by the presence of α-acids, iso-α-acids and β-acids released by hop in the beer broth and in the trub (Fernandez and Simpson, 1993; Haas and Barsoumian, 1994; Sakamoto and Konings, 2003; Yansanjav *et al*., 2004 and more recently Garcia-Garcia *et al*., 2017; Garofalo *et al*., 2015; Mathias *et al*., 2015). Nevertheless, there exist hop resistant species that are responsible for the so called "beer spoilage": increase of beer turbidity and unpleasant sensory changes (for beer spoilage bacteria and hop resistance mechanisms see reviews of Suzuki (2011) and Sakamoto and Konings (2003)).

Briefly, tolerance to hop acids is commonly attributed to the genes *horA*, *horC and hitA* coding for the HorA, HorC and HitA proteins, respectively. HorA and HorC have been proposed to actively transport hop acids out of the cells. HitA is linked to the disruption of the ionophoric activity of hop acids: hop acids cause, in the presence of Mn^{+2}, dissipation of the proton motive force of the cell, thus leading to its death. The HitA protein has been reported to compete with hop acids for the Mn^{+2}, thus preventing their detrimental effect. (Hayashi *et al.*, 2001; Haakensen *et al*., 2009; Suzuki, 2011)

In a previous study, we identified hot trub as a promising substrate for mixed acidogenic fermentation (Perimenis *et al*., 2017). Its conversion resulted in the highest VFA concentration in the mixed liquor (ML) and the highest substrate-to-VFA conversion yield among other substrates tested. In that study we presented results of two series of trub fermentation: (i) trub inoculated with pretreated (acidification at pH 5.5, heating at 70 °C for 1 hour) activated sludge and pH adjustment at 5.5 and (ii) trub non-inoculated with pH

adjustment at 5.5. A third, unpublished, combination was also tested, namely (iii) trub inoculated with pretreated activated sludge without pH adjustment. Interestingly, lactic acid was found to be the main product of condition (iii) reaching concentrations of 11.4 g_{COD}/kg_{ML} for an initial total COD concentration of 59.3 g_{CODt}/kg_{ML}. This interesting result raised the question of whether this was due to the presence of hop resistant species or a result of the collective action of the mixed consortium that degraded inhibitory hop substances, thus allowing lactic bacteria to develop.

Adding to the motivation of this study, partners of the Wal-Aid project have attempted without success to grow lactic acid bacteria as pure cultures in the presence of trub. *Lactobacillus plantarum* et *Pediococcus pentosaceus* were used as species, both of which have been reported to be present in beer, the first having even been reported to exhibit weak hop resistance (Haakensen *et al*., 2009; Suzuki, 2011; Yansanjav *et al*., 2004). So, the question why mixed fermentation succeeds in producing lactic acid from trub when pure fermentations fail is pertinent.

The present study examines the potential of producing lactic acid by mixed fermentation with trub as substrate. It also aims at investigating whether specific species from a mixed consortium could be responsible for the lactic acid production in the presence of hop. A microbial analysis using culture-dependent and culture-independent techniques (i.e. DNA extraction, amplification through Polymerase Chain Reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing) was used. The use of these two techniques has been suggested to be a useful tool for the detection of hop resistant lactic acid bacteria (Garofalo *et al*., 2015). To the best of our knowledge, this is the first study investigating the potential of using mixed fermentation specifically for lactic acid production from hot trub.

6.2 Materials and methods

6.2.1 Experimental procedure

The trub and the inoculum (concentrated activated sludge) were the same as described in Perimenis *et al*. (2017). The inoculum used in the present study was not pretreated, in order to maintain the maximum microbial diversity and activity. Batch fermentations were performed in duplicates in 250 ml reactors (Scott Duran GL 45) equipped with a lateral glass tube for gas sampling. Working volume was 100 ml. Table 6-1 presents the initial concentrations of the different series tested

Table 6-1: Initial substrate concentration in lactic acid fermentation of trub

Code	Series	Reactor	Trub (g)	Inoculum (g)	Substrate concentration (g_{COD}/kg_{ML})
Trub_as	Trub inoculated with activated sludge	R1 R2	50.7 50.8	48.2 46.8	149 149
Trub_as_ pellet	Trub inoculated with the solid phase of centrifuged activated sludge	R3 R4	92.7 93.7	7* 7*	273 275

** solid phase from centrifugation of 50g activated sludge*

The batch incubation was conducted at 35°C. After 23 days, a fraction of the mixed liquor was transferred to new reactors and fed with fresh trub as Figure 6-1 shows. The new series and their respective reactors deriving from the transfer were *Trub_as_transf (R1b & R2b)* and *Trub_as_pellet_transf (R3b & R4b).* Samples of the gas phase and the mixed liquor were taken at regular intervals for analysis. The sampling of the gas phase and the measurement of the pressure in the reactors with a manometer was achieved through the lateral glass tube of the reactors. Flushing with nitrogen took place each time the reactors were opened. The parameters monitored were: pH, biogas production and composition and the concentration of metabolites (e.g. VFA, ethanol, lactic acid). The samples of the mixed liquor were first centrifuged at 15,300 x g for 10 min. The supernatant was separated and stored at -20°C for the metabolic analysis. The solid remainder was also stored at -20°C for the microbial analysis.

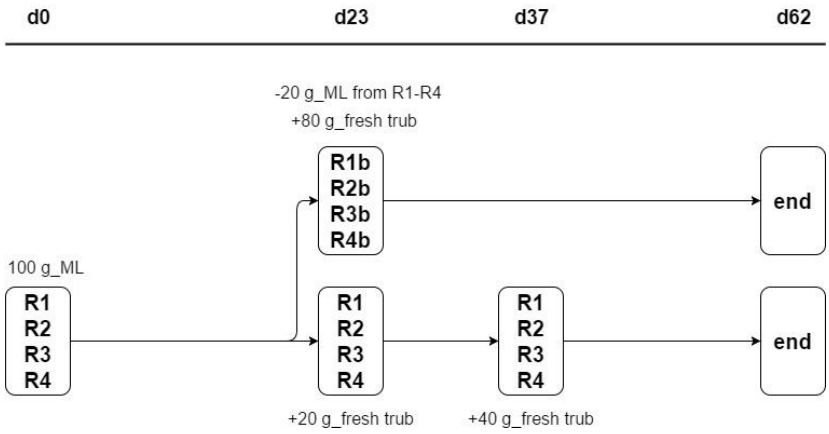

Figure 6-1: Fermentation plan

Note: addition of 40g of fresh trub in R1-R4 at day 37 was necessary to ensure sufficient volume for sampling.

6.2.2 Analytical methods

6.2.2.1 Gaseous and soluble metabolites

The composition of the biogas was analysed using a gas chromatograph (Compact GC, Interscience) with thermal conductivity detectors (GC-TCD) as described in Perimenis *et al*. (2017). Ethanol concentration was analysed using a gas chromatograph (Trace, Thermo Finnigan) with a flame ionisation detector (GC-FID). The column (2m x 1/8') was packed with 4% Carbowax 20M on Carbograph 1-DA, 80/120 mesh (Alltech). The temperature and pressure of the injector was 205 °C and 250 kPa, respectively. The temperature of the detector was 250 °C. The sequence for the temperature of the oven started at 160 °C at injection, rising to 200 °C at 25 °C/min and maintained until the end of the elution. The carrier gas was N_2 at 1.5 ml/min. Samples were acidified with oxalic acid while 4-methyl-valeric acid was used as internal standard. VFA and lactic acid were analysed by HPLC (Agilent 1200 Series) with a Photo Diode-Array Detector at 210 nm. The eluent was a solution of 95% H_2SO_4 0.005M and 5% acetonitrile at a flowrate of 0.6 ml/min. The column was a Bio Rad Aminex HPX 87H at 40 °C and the sample volume injected was 5 μl.

Hop acids (co-humulone, ad-humulone, n-humulone and their isomers) were detected by HPLC (Waters 996 series) with a Photo Diode-Array Detector at 314 nm for α-acids and 270 nm for iso-α-acids, on the basis of the protocol of Analytica 7.8. Two Agilent Zorbax XDB C8 columns 4.6 x 150mm were used in series at 35 °C. A mix of two eluent solutions at 1 ml/min flowrate was used: solution A was composed of 950 ml methanol, 40 ml mQ H_2O, 10 ml H_2PO_4 85% and 37.5 mg Na_2EDTA; solution B was composed of 750 ml methanol, 240 ml mQ H_2O, 10 ml H_2PO_4 85% and 37.5 mg Na_2EDTA. Injection volume was 10 μl and the analysis last 1 h. The eluent programme was as follows: 37% A/63% B (0-20th min), 37% A/63% B (20th to 30th min), 100% A (30th to 40th min), 100% A (40th to 50th min), 37% A/63% B (50th to 60th min).

6.2.2.2 PCR/DGGE

Extraction of DNA was performed using PowerSoil® DNA isolation kit according to the specifications of the manufacturer. The extracted DNA was amplified with a "touch-down" PCR with the use of universal primers for bacteria and specific primers for lactic acid bacteria.

The amplification with universal primers consisted of an initial denaturation at 94 °C for 5 min, followed by 16 cycles of 1 min at 94 °C, 0.5 min at a temperature decreasing by 1 °C/cycle (starting from 67 °C) and 45 s at 72 °C; then 15 cycles of 1 min at 94 °C, 0.5 min at 52 °C and 45 s at 72°C and a final

elongation for 8 min at 72 °C. The forward primer was S-D-Bact-0517-a-S-17 (5'-GCCAGCAGCCGCGGTAA-3') (Wang and Qian, 2009) fused with a 40-bp GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') (Muyzer *et al*., 1993) and the reverse primer S-D-Bact-1061-a-A-19 (5'-CACGACACGAGCTGACGAC-3') (Degnan and Ochman, 2012). The amplification with lactic acid bacteria primers consisted of an initial denaturation at 94 °C for 2 min, followed by 20 cycles of 1 min at 94 °C, 1 min at a temperature decreasing by 1 °C/cycle (starting from 65 °C) and 1 min at 72 °C; then 15 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72°C and a final elongation for 10 min at 72 °C. The forward primer was S-*-Lac-0353-a-S-19 (5'-AGCAGTAGGGAATCTTCCA-3') with the same GC clamp and the reverse primer S-*-Lac-0680-a-A-18 (5'-ATTYCACCGCTACACATG-3') (Walter *et al*., 2001). The amplified DNA was visualised under UV light after electrophoresis (28 min at 100 V) on a 0.8% agarose gel stained with 0.5 µg/ml ethidium bromide.

Separation of the DNA fragments was achieved by DGGE. Two stock solutions at 80% and 0% of the denaturing gel were prepared. The 80% stock solution contained 1 ml TAE 50x buffer, 20 ml acrylamide/bis-acrylamide 37.5:1 (Bio-Rad), 33.6g urea 7M and 32ml formamide 40% v/v and was brought to 100 ml with ultrapure H_2O. The 0% stock solution contained 1 ml TAE 50x buffer, 20 ml acrylamide/bis-acrylamide 37.5:1 and 79 ml ultrapure H_2O. The denaturing gradient (30-70%) was obtained by an instrument (Variomag, FL, USA) connecting two solutions at 30% and 70% denaturing concentrations, obtained from the original stock solutions. DNA loading was performed at 12 V to limit the diffusion of DNA out of the well. Electrophoresis was conducted on an INGENYphorU-2x2 (Ingeny International BV, Goes, Pays-Bas) for 16h at 60 °C and 120 V. The gel was removed and placed for 1h in a SYBRGold solution prepared with 400 ml ultrapure water, 4 ml TAE 50x and 40 µl SYBR gold 10,000x (Invitrogen, NM, USA). The gel was then placed on a transilluminator (Clare Chemical Research, CO, USA) permitting to visualise DNA and excise the bands using sterile scalpel blades. The DNA bands were then placed in ultrapure water at 4 °C during 48h to let DNA diffuse. DNA-containing water was diluted 100-fold to increase the signal-to-noise ratio which can be low with excised bands, and reamplified by PCR under the same conditions as described above without the GC clamp. A second electrophoresis on an agarose gel was performed to confirm the amplification efficiency and amplicons were sequenced (Macrogen Inc., Amsterdam, the Netherlands).

6.2.2.3 Standard bacterial culture

Standard bacterial cultures from the fermentation samples were developed for

comparison. 5 mg of the centrifuged solid phase of the sample were initially diluted 10, 100 and 1000 times with sterile Ringer solution (Oxoid, UK), plated onto Petri dishes and incubated in anaerobic jar for 36 h at 30 °C. The reference medium used was solid MRS medium (BD Difco). Isolated colonies were picked and placed in 100 µl of ultrapure water. The DNA-containing water was used for a subsequent PCR using bacterial universal primers S-D-Bact-0338-a-S-17 (5'-ACTCCTACGGGAGGCAG-3') (Yu *et al*., 2005) and S-D-Bact-1383-a-A-20 (5'-GGCGTGTGTACAAGGCCCGG-3') (Timmery *et al*., 2011). The thermal profile of the amplification was as follows: 94 °C for 5 min, followed by 30 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C and 1 min at 72°C, and a final elongation at 72 °C for 10 min. Verification electrophoresis and sequencing was conducted as described above.

6.3 Results & Discussion

6.3.1 pH and metabolites

The pH evolution is shown in Figure 6-2. pH drops as low as 3.5 in series *Trub_as* and *Trub_as_pellet*. The evolution of pH is compatible to lactic acid accumulation that is known to result in pH dropping to values below 4 (Itoh *et al*., 2012; Liang *et al*., 2016). Indeed, lactic acid accumulation takes place when the substrate has high concentrations of easily degradable substances and is a preferred pathway because it enables rapid disposal of reducing equivalents (Agler *et al*., 2011). Hot trub is such a substrate, due to the high content in sugars, approximately 20 % dry matter (Mathias *et al*., 2015). Furthermore, with every reinoculation we observe an increase of the pH which is compatible with the fact that the mixed liquor is diluted with fresh trub whose original pH is close to 5.

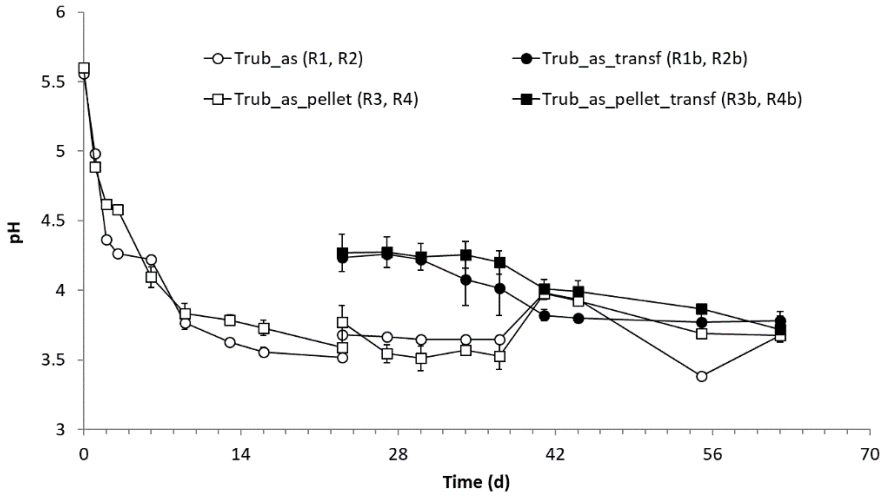

Figure 6-2: pH evolution of fermentation series during lactic acid fermentation of trub

The initial reactor series (R1 to R4) were refed with fresh trub on day 23 and day 37.

Figure 6-3 presents the evolution of lactic acid concentration in the different series tested. Lactic acid concentration continuously increased for reactors R1-R4 until d23, when a portion of the mixed liquor was removed to serve as inoculum for R1b-R4b and was replaced by fresh trub. After d23, the series inoculated with the activated sludge pellet (*Trub_as_pellet*) showed an increase in lactic acid concentration with a peak of approximately 18.6 g_{COD}/kg_{ML} at d27 and then a drop to finally 9.3 g_{COD}/kg_{ML}. On the contrary, the series with the inoculum without centrifugation (*Trub_as*) followed an inverse path: a moderate drop between d23 and d44 and a steep increase to reach a maximum of 20 g_{COD}/kg_{ML}. The series after transfer and feeding showed a gradual increase in lactic acid concentration but did not exceed maximum concentration reached in the original series. This slower trend in the new series could mean that the ratio mixed liquor:fresh trub during transfer was low (i.e. 1:4, w:w).

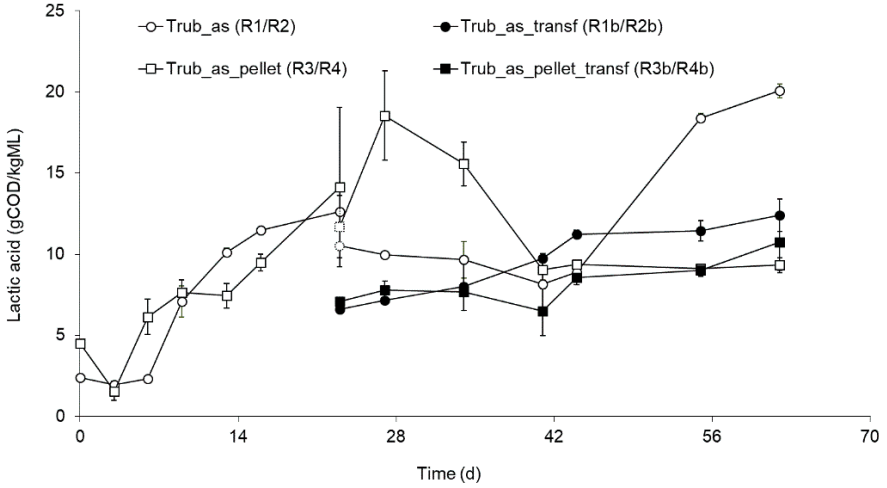

Figure 6-3 Lactic acid concentration in the fermentation series
The initial reactor series (R1 to R4) were refed with fresh trub on day 23 and day 37.

Other metabolites measured in the fermentation series of Figure 6-3 are presented in Table 6-2. It is interesting to note that metabolic pathways sometimes coincide, leading to the simultaneous production of different metabolic products and suggesting that no microorganism group has established clear dominance. In the series of interest for the production of lactic acid (i.e. *Trub_as* and *Trub_as_pellet*), VFA concentration is high mainly at the beginning of fermentation and decreasing afterwards, while lactic acid increases mostly towards the end. Ethanol is linked to a large variance in *Trub_as_transf*, as the two reactors did not respond in the same way to the reinoculation.

Table 6-2: Predominance of metabolic products of fermentation series and day of highest concentration in brackets.

Series	tVFA (g_{COD}/kg_{ML})	Ethanol (g_{COD}/kg_{ML})	Lactic acid (g_{COD}/kg_{ML})
Trub_as	15.3 ± 3.4 (d9)	1.0 ± 0.1 (d27)	20.1 ± 0.4 (d62)
Trub_as_transf	5.14 ± 0.4 (d44)	9.9 ± 9.4 (d41)	12.4 ± 1.0 (d62)
Trub_as_pellet	11.6 ± 2.1 (d3)	40.4 ± 0.9 (d6)	18.6 ± 2.7 (d27)
Trub_as_pellet_transf	17.1 ± 1.1 (d55)	18.8 ± 1.5 (d62)	10.8 ± 1.8 (d62)

Mixed fermentation of trub has led to the production of lactic acid at concentrations of up to 20 g_{COD}/kg_{ML}. Similar lactic acid concentrations have been reported by Wu *et al*. (2015) during continuous fermentation of simulated fruit and vegetable waste (approximately 15-20 g/l), by Cirne *et al*. (2007) in the acidogenic stage of a two-stage anaerobic digestion of sugar beet

(approximately 20 g/l) and by Probst *et al*. (2015) during fermentation of municipal solid waste (approximately 25 g/l). Indicative of the spectrum of lactic acid concentrations reported, Liang *et al*. (2016) reported approximately 6.5 g/l from mixed batch fermentation of potato peel waste, while RedCorn and Engelberth (2016) reported concentrations of approximately 60 g/l from batch mixed fermentation of food waste.

For the series leading to the highest concentrations in lactic acid, samples at d0 and d62 (*Trub_as*, R1) and at d0 and d27 (*Trub_as_pellet*, R3) were analysed for a qualitative detection of a-acids, iso-α-acids and β-acids. While α-acids and β-acids were not detected in any of the samples, iso-α-acids were present at the beginning, but not detected at the day of highest lactic acid concentration (see Figure 6-5 and Figure 6-6 in section 6.7). This result is consistent with the hypothesis that hop acids were degraded by other microorganisms of the mixed consortium (Huszcza and Bartmańska, 2008), thus allowing lactic acid bacteria to develop.

6.3.2 Microbial analysis

Following the results of the metabolic profile, samples from d62 for reactors R1 and R2 of *Trub_as* and from d27 for reactors R3 and R4 of *Trub_as_pellet* were used for PCR-DGGE and standard bacterial cultures. The DGGE profiles with both sets of primers revealed a small number of bands, i.e. a weak bacterial diversity, which is an indication that trub is a highly selective substrate (Figure 6-4). Bands U1, U2, L1 and L2 could be excised, but only L1 and L2, corresponding to R4-d27 and R3-d27, respectively, gave consistent sequences. Two species were identified as closest relatives to the L1 sequence with a 100% nucleotide identity: *Lactobacillus amylovorus* (AY944408), a starch-degrading microorganism reported by Nakamura (1981) and *Lactobacillus ultunensis* (AY253660), isolated by Roos *et al*. (2005) from human stomach mucosa. Results of sequencing were identical for L1 and L2, which, given the significant distance between the two bands, is most probably due to an artefact of the technique (Neilson *et al*., 2013).

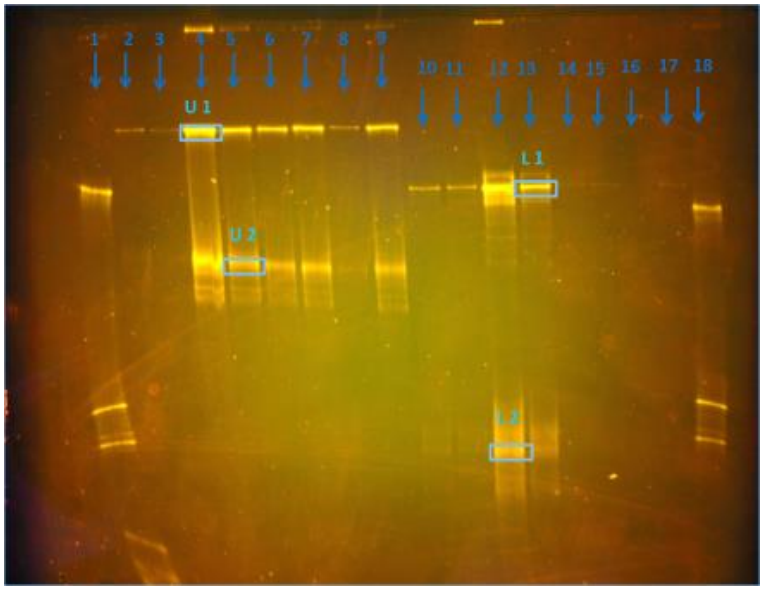

Figure 6-4: DGGE profile from lactic acid fermentation of hot trub
1-9: universal primers (U); 1: ladder; 2: R1-d62; 3: R2-d62; 4: R3-d27; 5: R4-d27; 6-9: other reactors
10-18: lactic acid bacteria (L) primers; 10: R1-d62; 11: R2-d62; 12: R3-d27; 13: R4-d27; 14-17: other reactors; 18: ladder

From the bacterial cultures, two colonies were isolated and identified by 16S RNA sequencing. The first colony was isolated from R4 at d27 and corresponded to 99.8% to *Acetobacter pomorum* (AJ419835), a microorganism reported in industrial vinegar fermentations (Sokollek *et al*., 1998). The second colony was isolated from R2 at d62 and corresponded to *Rummeliibacillus pycnus* (AB271739), a reclassification of *Bacillus pycnus* (Vaishampayan *et al*., 2009) and recently used for the production of polyhydroxyalkanoates (PHA) from palm oil mill effluent (Junpadit *et al*., 2017).

None of the identified and sequenced species from both techniques has been reported to be hop resistant before (Sakamoto and Konings, 2003; Suzuki, 2011). This does not exclude the possibility that they have indeed developed a certain hop resistance; for that, further experiments should be conducted with the isolated species growing as pure cultures in the presence of trub in their growth medium. It is interesting to note that Haakensen *et al*. (2009) also suggested a possible positive correlation between the presence of ethanol and increased hop resistance; although this claim should be further investigated, we did observe high concentrations of ethanol in one of the series that resulted in high lactic acid concentrations (*Trub_as_pellet*, see Table 6-2). A metagenomic approach using next generation sequencing could be helpful in

providing a more complete view of present species for the detection of potentially hop resistant and hop degrading microorganisms (Liang *et al*., 2016)

6.4 Conclusions

Lactic acid at concentrations of up to 20g/l was produced during mixed fermentation of hot trub. This result was rather unexpected due to the bacteriostatic effect of hop substances in the trub. The microbial analysis revealed microorganisms that were involved in the fermentation, but none of them has been reported to be hop resistant. Hop acids present at the beginning of the experiments were not detected anymore at the days of the highest lactic acid concentration. The presence of lactic acid is, therefore, more plausibly explained by the microbial activity in the mixed consortia that led to degradation of hop substances, allowing the development of lactic acid bacteria rather than the direct development of some hop resistant lactic acid species. This is a promising result for the valorisation of trub, a common brewery residue, through the production of a bio-based monomer of industrial interest. It is also an important finding highlighting the advantage of a mixed consortium that can be used not only as a producer of a target metabolite but also as a simultaneous degrader of inhibitory substances.

6.5 Acknowledgements

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6.6 References

Agler, M.T., Wrenn, B.A., Zinder, S.H., Angenent, L.T., 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends Biotechnol. 29, 70–78. https://doi.org/10.1016/j.tibtech.2010.11.006

Brito, A.G., Peixoto, J., Oliveira, J.M., Oliveira, J.A., Costa, C., Nogueira, R., Rodrigues, A., 2007. Brewery and Winery Wastewater Treatment: Some Focal Points of Design and Operation, in: Utilization of By-Products and Treatment of Waste in the Food Industry. Springer, Boston, MA, pp. 109–131. https://doi.org/10.1007/978-0-387-35766-9_7

Castillo Martinez, F.A., Balciunas, E.M., Salgado, J.M., Domínguez González, J.M., Converti, A., Oliveira, R.P. de S., 2013. Lactic acid properties, applications and production: A review. Trends in Food Science & Technology 30, 70–83. https://doi.org/10.1016/j.tifs.2012.11.007

Cheng, W., Chen, H., Yan, S., Su, J., 2014. Illumina sequencing-based analyses of bacterial communities during short-chain fatty-acid production from food waste and sewage sludge fermentation at different pH values. World Journal of Microbiology and Biotechnology 30, 2387–2395. https://doi.org/10.1007/s11274-014-1664-6

Cirne, D.G., Lehtomäki, A., Björnsson, L., Blackall, L.L., 2007. Hydrolysis and microbial community analyses in two-stage anaerobic digestion of energy crops. J. Appl. Microbiol. 103, 516–527. https://doi.org/10.1111/j.1365-2672.2006.03270.x

Degnan, P.H., Ochman, H., 2012. Illumina-based analysis of microbial community diversity. ISME J 6, 183–194. https://doi.org/10.1038/ismej.2011.74

Fernandez, J.L., Simpson, W.J., 1993. Aspects of the resistance of lactic acid bacteria to hop bitter acids. Journal of Applied Bacteriology 75, 315–319. https://doi.org/10.1111/j.1365-2672.1993.tb02782.x

Focus on Catalysts, 2016. Lactic acid market to reach $3.82 bn by 2020, 2016. Focus on Catalysts 2016, 2. https://doi.org/10.1016/j.focat.2016.01.048

Garcia-Garcia, J.H., Damas-Buenrostro, L.C., Cabada-Amaya, J.C., Elias-Santos, M., Pereyra-Alférez, B., 2017. Pediococcus damnosus strains isolated from a brewery environment carry the horA gene. J. Inst. Brew. 123, 77–80. https://doi.org/10.1002/jib.397

Garofalo, C., Osimani, A., Milanović, V., Taccari, M., Aquilanti, L., Clementi, F., 2015. The Occurrence of Beer Spoilage Lactic Acid Bacteria in Craft Beer Production: Beer spoilage lactic acid bacteria…. Journal of Food Science 80, M2845–M2852. https://doi.org/10.1111/1750-3841.13112

Haakensen, M., Schubert, A., Ziola, B., 2009. Broth and agar hop-gradient plates used to evaluate the beer-spoilage potential of Lactobacillus and Pediococcus isolates. International Journal of Food Microbiology 130, 56–60. https://doi.org/10.1016/j.ijfoodmicro.2009.01.001

Haas, G.J., Barsoumian, R., 1994. Antimicrobial Activity of Hop Resins. Journal of Food Protection 57, 59–61. https://doi.org/10.4315/0362-028X-57.1.59

Hayashi, N., Ito, M., Horiike, S., Taguchi, H., 2001. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. Appl Microbiol Biotechnol 55, 596–603. https://doi.org/10.1007/s002530100600

Huszcza, E., Bartmańska, A., 2008. The implication of yeast in debittering of spent hops. Enzyme and Microbial Technology 42, 421–425. https://doi.org/10.1016/j.enzmictec.2008.01.004

Itoh, Y., Tada, K., Kanno, T., Horiuchi, J.-I., 2012. Selective production of lactic acid in continuous anaerobic acidogenesis by extremely low pH operation. J. Biosci. Bioeng. 114, 537–539. https://doi.org/10.1016/j.jbiosc.2012.05.020

Junpadit, P., Suksaroj, T.T., Boonsawang, P., 2017. Transformation of Palm Oil Mill Effluent to Terpolymer Polyhydroxyalkanoate and Biodiesel Using Rummeliibacillus pycnus Strain TS8. Waste Biomass Valor 8, 1247–1256. https://doi.org/10.1007/s12649-016-9711-1

Li, Y., Cui, F., 2010. Microbial Lactic Acid Production from Renewable Resources, in: Singh, O.V., Harvey, S.P., (Eds), Sustainable Biotechnology. Springer, Dordrecht, pp. 211–228. https://doi.org/10.1007/978-90-481-3295-9_11

Liang, S., Gliniewicz, K., Gerritsen, A.T., McDonald, A.G., 2016. Analysis of microbial community variation during the mixed culture fermentation of agricultural peel wastes to produce lactic acid. Bioresource Technology 208, 7–12. https://doi.org/10.1016/j.biortech.2016.02.054

Mathias, R. dos S., Pedro, P.M. de M., Eliana, F.C.S., 2014. Solid wastes in brewing process: A review. Journal of Brewing and Distilling 5, 1–9. https://doi.org/10.5897/JBD2014.0043

Mathias, T.R. dos S., Alexandre, V.M.F., Cammarota, M.C., de Mello, P.P.M., Sérvulo, E.F.C., 2015. Characterization and determination of brewer's solid wastes composition: Brewer's waste characterization. Journal of the Institute of Brewing 121, 400–404. https://doi.org/10.1002/jib.229

Mussatto, S.I., 2009. Biotechnological Potential of Brewing Industry By-Products, in: Singh nee' Nigam, P., Pandey, A. (Eds.), Biotechnology for Agro-Industrial Residues Utilisation. Springer Netherlands, Dordrecht, pp. 313–326. https://doi.org/10.1007/978-1-4020-9942-7_16

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59, 695–700.

Nakamura, L.K., 1981. Lactobacillus amylovorus, a New Starch-Hydrolyzing Species from Cattle Waste-Corn Fermentations. International Journal of Systematic and Evolutionary Microbiology 31, 56–63. https://doi.org/10.1099/00207713-31-1-56

Neilson, J.W., Jordan, F.L., Maier, R.M., 2013. Analysis of Artifacts Suggests DGGE Should Not Be Used for Quantitative Diversity Analysis. J Microbiol Methods 92, 256–263. https://doi.org/10.1016/j.mimet.2012.12.021

Perimenis, A., Nicolay, T., Leclercq, M., Gerin, P.A., 2017. Comparison of the acidogenic and methanogenic potential of agroindustrial residues. Waste Management. https://doi.org/10.1016/j.wasman.2017.11.033

Probst, M., Walde, J., Pümpel, T., Wagner, A.O., Insam, H., 2015. A closed loop for municipal organic solid waste by lactic acid fermentation. Bioresource Technology 175, 142–151. https://doi.org/10.1016/j.biortech.2014.10.034

RedCorn, R., Engelberth, A.S., 2016. Identifying conditions to optimize lactic acid production from food waste co-digested with primary sludge. Biochemical Engineering Journal 105, 205–213. https://doi.org/10.1016/j.bej.2015.09.014

Roos, S., Engstrand, L., Jonsson, H., 2005. Lactobacillus gastricus sp. nov., Lactobacillus antri sp. nov., Lactobacillus kalixensis sp. nov. and Lactobacillus ultunensis sp. nov., isolated from human stomach mucosa. International Journal of Systematic and Evolutionary Microbiology 55, 77–82. https://doi.org/10.1099/ijs.0.63083-0

Sakamoto, K., Konings, W.N., 2003. Beer spoilage bacteria and hop resistance. International Journal of Food Microbiology 89, 105–124.

https://doi.org/10.1016/S0168-1605(03)00153-3

Sokollek, S.J., Hertel, C., Hammes, W.P., 1998. Description of Acetobacter oboediens sp. nov. and Acetobacter pomorum sp. nov., two new species isolated from industrial vinegar fermentations. International Journal of Systematic and Evolutionary Microbiology 48, 935–940. https://doi.org/10.1099/00207713-48-3-935

Suzuki, K., 2011. 125th Anniversary Review: Microbiological Instability of Beer Caused by Spoilage Bacteria. Journal of the Institute of Brewing 117, 131–155. https://doi.org/10.1002/j.2050-0416.2011.tb00454.x

Timmery, S., Hu, X., Mahillon, J., 2011. Characterization of Bacilli Isolated from the Confined Environments of the Antarctic Concordia Station and the International Space Station. Astrobiology 11, 323–334. https://doi.org/10.1089/ast.2010.0573

Vaishampayan, P., Miyashita, M., Ohnishi, A., Satomi, M., Rooney, A., La Duc, M.T., Venkateswaran, K., 2009. Description of Rummeliibacillus stabekisii gen. nov., sp. nov. and reclassification of Bacillus pycnus Nakamura *et al*. 2002 as Rummeliibacillus pycnus comb. nov. International Journal of Systematic and Evolutionary Microbiology 59, 1094–1099. https://doi.org/10.1099/ijs.0.006098-0

Walter, J., Hertel, C., Tannock, G.W., Lis, C.M., Munro, K., Hammes, W.P., 2001. Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 67, 2578–2585. https://doi.org/10.1128/AEM.67.6.2578-2585.2001

Wang, Y., Qian, P.-Y., 2009. Conservative Fragments in Bacterial 16S rRNA Genes and Primer Design for 16S Ribosomal DNA Amplicons in Metagenomic Studies. PLoS ONE 4, e7401. https://doi.org/10.1371/journal.pone.0007401

Wu, Y., Ma, H., Zheng, M., Wang, K., 2015. Lactic acid production from acidogenic fermentation of fruit and vegetable wastes. Bioresource Technology 191, 53–58. https://doi.org/10.1016/j.biortech.2015.04.100

Xiros, C., Christakopoulos, P., 2012. Biotechnological Potential of Brewers Spent Grain and its Recent Applications. Waste Biomass Valor 3, 213–232. https://doi.org/10.1007/s12649-012-9108-8

Yansanjav, A., Siegumfeldt, H., Jespersen, L., Vancanneyt, M., Swings, J., Hollerová, I., Leisner, J.J., 2004. Detection of resistance of lactic acid bacteria to a mixture of the hop analogue compounds tetrahydroiso-alpha-acids by noninvasive measurement of intracellular pH. J. Appl. Microbiol. 96, 1324–1332. https://doi.org/10.1111/j.1365-2672.2004.02261.x

Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnology and Bioengineering 89, 670–679. https://doi.org/10.1002/bit.20347

6.7 Supplementary material

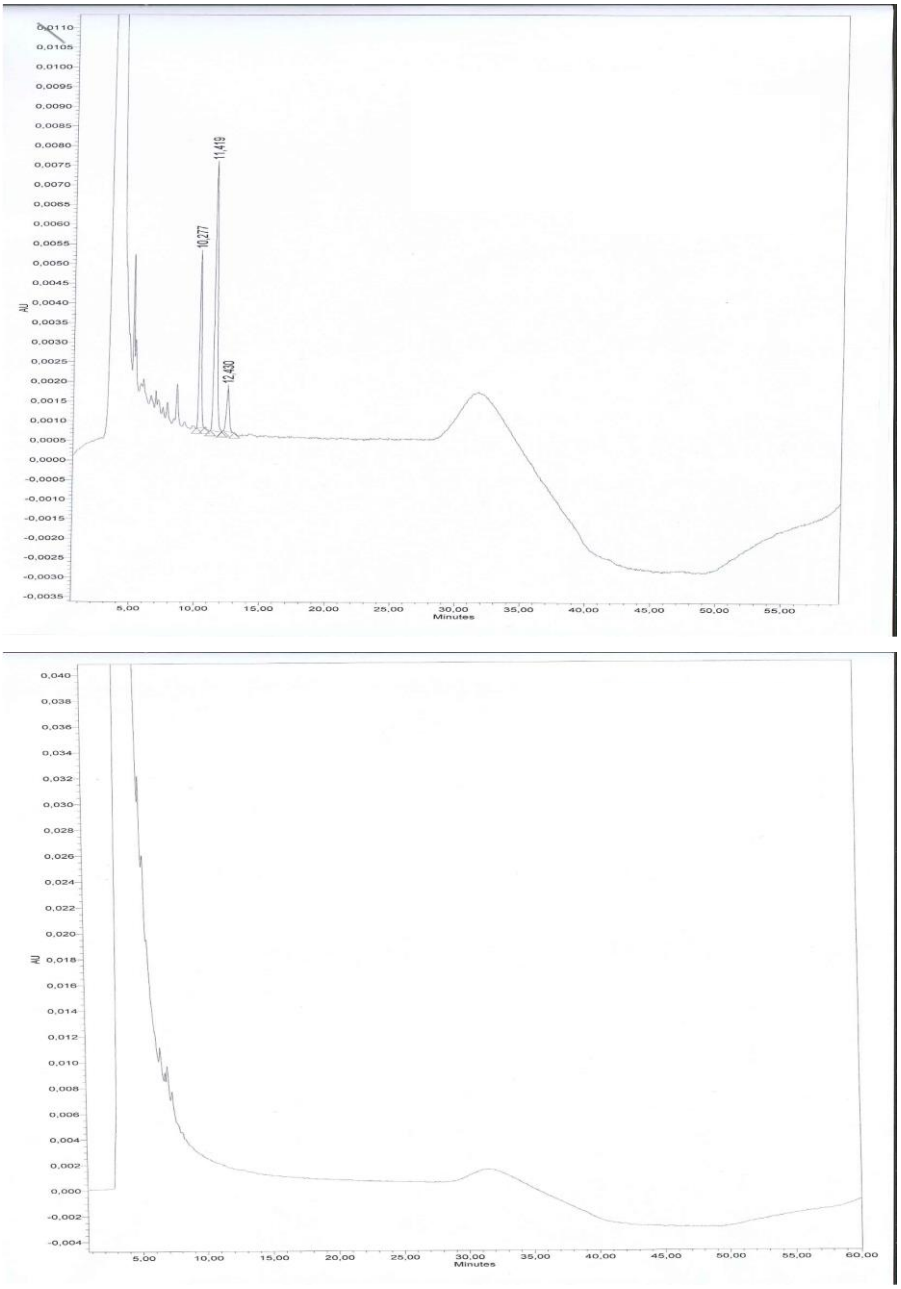


Figure 6-5: Chromatogram of R1 from d0 (up) and d62 (down).

Correspondence of retention times 10,277: co-iso-humulone; 11,419: ad-iso-humulone; 12,430: n-iso-humulone)

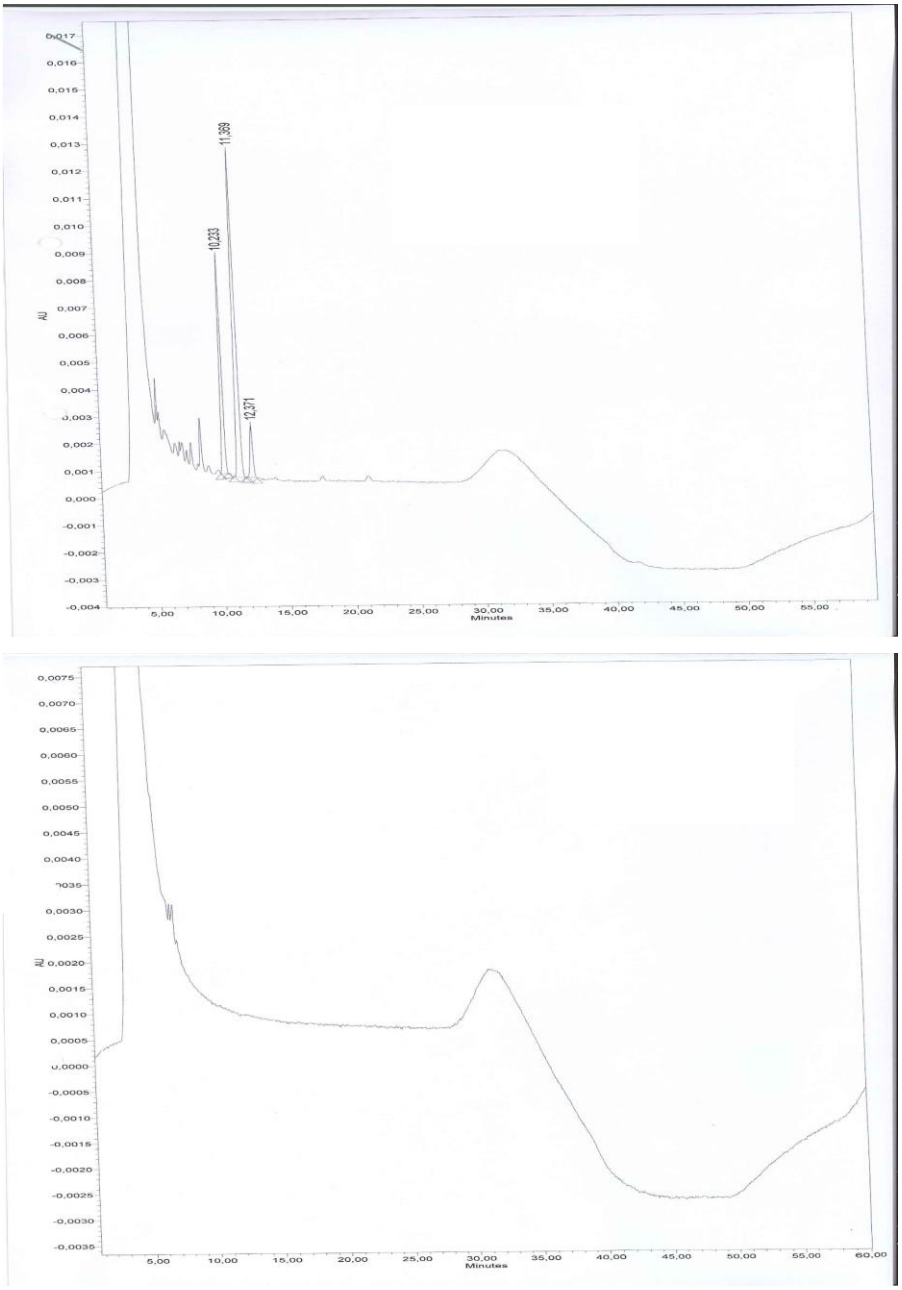


Figure 6-6: Chromatogram of R3 from d0 (up) and d27 (down).

Correspondence of retention times (10,233: co-iso-humulone; 11,369: as-iso-humulone; 12,371: n-iso-humulone)

7 Discussion & perspectives

7.1 Context of the scientific approach

The general context of the thesis is the EU's expressed commitment on sustainable use of natural resources, reduction of waste and promotion of viable circular economy and bioeconomy concepts. The use of agroindustrial residual fractions as source of bio-based products can contribute to all of these goals, thereby providing added value to an otherwise unexploited resource. Agroindustrial residues were the focus of this thesis, due to their organic potential, their large production volumes and their low cost. Their diverse nature and complex structure, asks for deployment of integrated processes that can harness the maximum out of a given feedstock and/or out of different feedstocks at the same time, i.e. the biorefinery scheme (Venkata Mohan *et al*., 2016). There is most probably no "one-process concept" for the sustainable exploitation of such complex residues; a process integration strategy is more likely to succeed by combining processes that exploit the maximum of a given feedstock, by reusing by-products and waste of one process as input (energetic or material) for the other and by combining infrastructures (Ferreira, 2017). Among other existing processes that could be part of such integrated schemes, this thesis focused on mixed acidogenic fermentation for the production of carboxylates (i.e. volatile fatty acids, lactic acid) as intermediate and/or end-products with a variety of potential applications (Singhania *et al*., 2013). The use of mixed microbial communities increases the capacity for substrate conversion, but also adds to the general complexity of the process (Kleerebezem and van Loosdrecht, 2007).

As mentioned in section 1.1.4, a series of open research challenges prevent at the time being the scale-up of mixed acidogenic fermentation, namely (i) the control of the complexity of the fermentation system (i.e. the triptych "substrate-inoculum-conditions") and the effect on the evolution of the microbiota and (ii) the downstream processing needs for the recovery and purification of the carboxylates from the fermentation broth. This thesis focused on the first challenge. The rationale behind this decision is that mastering the complexity "inside" the fermentation system would allow a better control of the outcome, hence a better determination of the characteristics an efficient DSP should have. As Figure 1-3 illustrates, the microbiome of the reactor is the central element that determines the metabolic performance. If its evolution and its link to the operational parameters and the metabolic products are not well understood, directing fermentation towards a desired product will not be possible.

The following paragraphs develop answers to the research questions defined as objectives of the thesis in section 1.2.

7.2 Appropriateness of mixed acidogenic fermentation in biorefining processes

7.2.1 Can mixed acidogenic fermentation efficiently treat complex agroindustrial waste?

Eleven residues have been tested in this thesis under batch conditions, namely apple pulp, date pulp, pear pulp, chicory roots (Annex 3), hot trub, spent yeast, spent grain, wheat bran, dried sugar beet pulp, miscanthus and distiller's dried grain with solubles, showing the adaptive nature of the process. With the exception of spent yeast and chicory roots, all other substrates had a total solids (TS) content ranging from 0.24 to 0.91 g_{TS}/g_{fresh_matter}. The TS content of the reactors in our experiments was within the range of 3.5-5.2 % (w/w), above which Gameiro *et al*. (2016) suggested that substrate conversion yields are decreasing. The **highest concentrations and conversion yields** obtained are summarised in Table 7-1.

Table 7-1: Highest product concentrations and conversion yields obtained from batch acidogenic fermentations

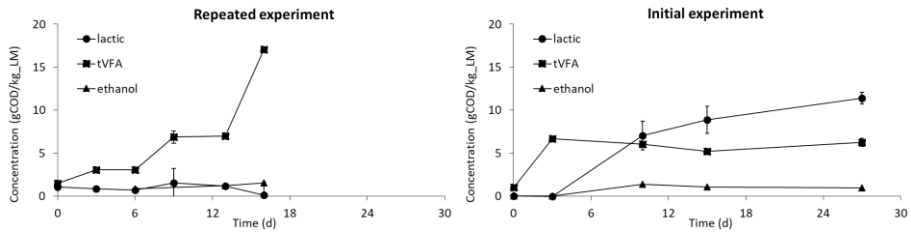
Substrate (S)	Inoculum (I)	pH	Initial total concentration (g_{CODt}/kg_{ML})	Product concentration (g_{COD_tVFA}/kg_{ML})	Conversion yield ($g_{COD\_tVFA}/g_{COD\_substrate}$)
Hot trub	Pre-treated activated sludge	5.5	60.7	29.7	0.56
Chicory roots	Acidogenic slurry	7	80.0	41.1	0.51*
Dried sugar beet pulp	Anaerobic sludge	6.5	19.9	9.1	0.64

* *unit:* g_{COD_tVFA}/g_{CODt}

The aforementioned values are situated at the highest range of values reported in literature for mixed fermentation of complex solid residues under batch conditions (Arslan *et al.*, 2016; Lee *et al.*, 2014). Concentrations reaching approximately 25 g_{COD_tVFA}/kg_{ML} were also achieved during the initial phase of semi-continuous operation, but it was not possible to maintain these levels further at steady state (see Annex 4). Acetic and butyric acids were the **predominant VFA** in almost all fermentation series, thereby agreeing with the majority of acidogenic fermentation studies (Arslan *et al.*, 2016; Lee *et al.*, 2014). In some cases (Chapters 2 and 4), further acids like caproic acid and propionic acid were produced at important concentrations. Propionic acid showed resistance to degradation contrary to the other VFA products (Chapter 4); this resistance has been reported before (Batstone and Jensen, 2011) and could constitute an advantage when trying to inhibit methanogenesis in fermentation systems that are specifically targeting this acid. Caproic acid is gaining increasing research attention because it has a higher molecular weight and it is easier to separate from water than the other VFA (Agler *et al*. (2014);

see also section 2.3.2).

Overall the **metabolic reproducibility** (herewith defined as the variation in metabolite concentration between reactors of the same fermentation series) of the fermentations was good given that the coefficient of variation was maximum 18.5% and in the majority of series was at the level of 10%, which is in agreement with values reported in other complex biological systems (Bosch *et al*., 2017). While reproducibility within the same series could be confirmed, we were not able to confirm **repeatability**, herewith defined as conducting the same experiment at a later time. More specifically, in the context of Chapter 2, an additional series was tested (besides the ones presented), containing trub inoculated with pre-treated activated sludge without pH adjustment (other than initially at 5.5). This series led to the unexpected results of lactic acid production despite the inhibitory effect of hop acids in trub and was the driver for the work conducted in Chapter 6. When we repeated the experiment, we obtained a different metabolic profile (Figure 7-1). Contrary to the original experiment where lactic acid was the main metabolic product followed by tVFA, the repetition showed tVFA as the main metabolic product with negligible lactic acid production. We have attributed in Chapter 6 the presence of lactic acid, to the collective action of the mixed consortia that could degrade hop acids in the trub and allow lactic acid bacteria to develop. We could assume that this collective action was not efficient enough in the second experiment and/or that the selection of sporulating acidogenic microorganisms after pretreatment of the inoculum was quite pronounced, not allowing the development of lactic acid bacteria. Furthermore, trub has proven to be a substrate whose conversion can easily follow different pathways depending on the conditions imposed (see also 6.3.1) and we can assume that even small differences between the two experiments could have led to a different fermentation profile. This result highlights once again that the complexity of mixed fermentations is not yet sufficiently mastered and that selective carboxylate production is not yet possible at industrial scale.



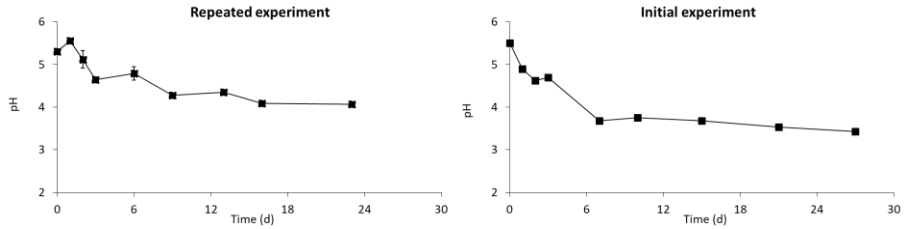


Figure 7-1: Comparison of metabolic profile from two trub fermentation experiments conducted under the same conditions at different times.
Evolution of metabolite concentration in the upper row; evolution of pH in the lower row.

Ultimately, it is the final product use that will define the acceptable levels of reproducibility at industrial scale, especially for applications that rely on a consistent product output (e.g. monomers for green chemistry). On the other hand, one could envisage a flexible scheme where the diversified microbiome is capable of treating a variety of feedstocks in the same reactor to generate VFAs that can be used as group product (e.g. industrial solvents, biofuels, soluble COD for microbial fuel cells and nutrient removal, etc; see Section 1.1.5). In that case, reproducibility of individual VFA concentration may not be of critical importance since VFA- rich streams will be consistently produced, even if this is at different proportions of individual acids. The challenge of such a concept would lie in the flexibility of market uptake of a constantly altered VFA stream.

Whether as a stand-alone process (Chapters 2, 4, 5) or as a process integrated into a bio-cascading scheme (following fractionation, Chapter 3), acidogenic fermentation was implemented for the treatment of **recalcitrant waste fractions**. Excluding substrates like spent yeast, trub and chicory roots, the lignocellulosic content of the residues tested (including lignin, hemicellulose and cellulose) ranged from 0.47 to 0.86 $g_{lignocellulose}/g_{TS}$. In particular, Chapter 3 showed that when fractionation processes (in this case steam explosion) are employed to recover digestible compounds of the lignocellulosic matrix (in this case hemicellulose) then the acidogenic performance of the solid remainder declines considerably compared to the non-treated substrate. For such highly recalcitrant substrates, conversion yields into VFA could hardly exceed 10% of the initial COD in the reactors, thus rendering acidogenic fermentation probably not efficient enough compared to standard anaerobic digestion (see also section 7.2.2). Indeed, acidogenic fermentation is a process that partly depends on the ability of a rapid release of VFA to contribute in maintaining the pH at acidic levels and avoid methanogenesis (Batstone and Jensen, 2011). The absence of easily digestible components in the substrate renders acidogenic fermentation less attractive.

7.2.2 Is the biomethane potential of agroindustrial waste a representative assessment of their acidogenic potential?

Chapter 2 has shown that **acidogenic fermentation exhibits systematically lower conversion yields** compared to standard anaerobic digestion (i.e. 36-69% lower, especially for substrates with high lignocellulosic content). We can, therefore conclude that the biomethane potential is not accurately representing the acidogenic potential as well Nevertheless, and based on a simplified calculation, acidogenic fermentation remains an attractive stand-alone option for waste valorisation when considering only **product value,** mainly because of the finer applications that are envisaged for VFA compared to methane.

The reason for the lower conversion yield of acidogenic fermentation are most probably substrate overloading and product inhibition from VFA accumulation (Arslan et al., 2016). This is the cost of trying to increase product concentrations when VFA are considered as end-products. In analogy to standard methanogenesis, where VFA are consumed to yield methane thus allowing the overall process to continue without inhibition, it is crucial to combine a high acidogenic performance with efficient downstream processes (DSP) for the recovery of VFA from the fermentation broth and their subsequent concentration, separation and purification. The challenge of efficient DSP is high due to the high complexity of the broth's matrix and to the solubility of VFA in this broth (Zacharof and Lovitt, 2014; see also Section 7.4.1).

At the same time, a high performing acidogenesis has as prerequisite the inhibition of methanogenic activity, without using chemical inhibitors. In the experiments of this thesis, **methanogenesis was successfully inhibited** for the majority of tested series but not for all of them. For example, Chapter 4 shows a net decrease of VFA (and soluble COD in general) during fermentation and although biogas was not measured it is reasonable to assume that methanogenesis started taking place. This could be attributed to elevated pH values (around 6.5) where methanogenic archaea are favourably developing (Batstone and Jensen, 2011). Whenever inhibition of methanogenesis was indeed achieved, this was due to the **combined effect** of (i) the pre-treatment of the inoculum that aims at selecting sporulating acidogenic bacteria; (ii) the maintenance of an acidic pH around 5.0-5.5 and (iii) the increased substrate concentration leading to a rapid release of VFA from the readily digestible fraction, thus allowing the maintenance of an acidic environment. None of the aforementioned conditions sufficed to reach the same result on its own. For example, all the inocula controls of Chapter 3 resulted in methane production, despite the fact that the inoculum had been pre-treated. Also, the apple pulp inoculated with rumen fluid in Chapter 5

resulted in the production of methane, despite the adjustment of the pH at 5.0-5.5. Finally, the series 7/80 (i.e. pH 7, total initial concentration 80 g_{COD}/kg_{ML}) of chicory roots in Annex 3 resulted in high VFA concentrations without detection of methane, due to high reactor loading and despite neutral pH that is favourable to methanogens.

7.2.3 Is there a residue that is particularly promising for mixed acidogenic fermentation?

From all substrates tested in this thesis, hot trub has shown particularly interesting results. This, of course, does not exclude the fact that other substrates not tested in this thesis might exhibit equally interesting results.

Hot trub is a common residue of the brewing process, but its valorisation potential through bioconversion is highly understudied (Mathias *et al*., 2014; Mussatto, 2009). It was shown in Chapters 2 and 6 that, despite the existence of bacteriostatic substances like hop-acids, trub exhibits the unique **capacity of being converted to a multitude of metabolites** (i.e., VFA, lactic acid, ethanol) **by altering the imposed conditions** (e.g. pH adjustment, substrate concentration, inoculum addition and pre-treatment). VFA concentrations of approximately 30 g_{COD_tVFA}/kg_{ML} and lactic acid concentrations of approximately 20 g_{COD}/kg_{ML} were achieved with trub as substrate and are promising for potential up-scaling. Owing to its high sugar content (see Table 2-1), conversion was often resulting to simultaneous production of different metabolites of interest (see Table 6-2), indicating that no clear consortium dominance has been established.

Most particularly, it appears to be a **promising substrate for the production of caproic acid** that has gained increasing attention the last years due to its potential applications and easier recoverability from the broth compared to lighter VFA. Indeed, caproic acid is produced through chain elongation, a reverse β-oxidation process where a reduced compound (i.e. ethanol or lactate) is converted to acetyl CoA, which is then added through a cyclic process to the carboxylic acid chain, thus elongating it (Cavalcante *et al*., 2017; Spirito *et al*., 2014) (Figure A.2-5). Literature sources are mainly achieving chain elongation through external addition of reduced compounds like ethanol or lactic acid (Agler *et al*., 2014; Angenent *et al*., 2016; Grootscholten *et al*., 2013; Vasudevan *et al*., 2014), while we have shown in Chapter 2 that, for hot trub, this is possible **without external addition of the reduced molecule**, probably as a result of the aforementioned simultaneous production those molecules.

Of note, the initial production of caproic acid during semi-continuous operation (see Annex 4) at low HRT, even if not possible to maintain further on, confirms batch experiments and suggests that trub could be a substrate of

interest for this particular VFA also in continuous mode. On the other hand, soluble COD concentration was approximately 10 times higher than the concentrations of the metabolites of interest (VFA, lactic acid, ethanol) at the end of the experiments indicating that part of the substrate was not converted. This could be the consequence of a potential detrimental effect that hop substances had to bacterial growth that led to microbial wash-out not observed in batch cultures.

7.3 System complexity and microbial diversity

7.3.1 Is the addition of a mixed external inoculum useful for the improvement of fermentation performance?

Four different inocula have been tested throughout this thesis, namely pretreated activated sludge, anaerobic sludge, granular sludge and acidogenic slurry. In all series where both inoculated and non-inoculated (i.e. control) substrates were tested (i.e. Chapters 2, 3, 5), **the addition of an external inoculum always improved tVFA production**, sometimes dramatically (e.g. from 0.7 to 30 g_{COD_tVFA}/kg_{ML} in the case of trub inoculated with pre-treated activated sludge), others marginally (e.g. from 8.9 to 9.6 g_{COD_tVFA}/kg_{ML} in the case of apple pulp inoculated with ruminal fluid). This gain is due to the reinforcement of the microbial diversity in the fermentation system and the associated improvement of the hydrolytic and metabolic capacity of the microbial consortia (Temudo *et al.*, 2008). Often, a thermal and acidic pretreatment is used to select spore-forming acidogenic species, thereby increasing even more the chances that acidogenic metabolic pathways are established (Arslan *et al.*, 2016). This pre-treatment proved to be more efficient than the mere addition of an inoculum that is supposedly adapted to acidogenic conditions (ruminal fluid) but where the microorganisms find themselves outside of their natural environment (Shin *et al*., 2011). As shown in Chapter 5, however, the inherent microbial consortia of the substrate are not to neglect as they, too, contribute in the conversion of the substrate. The wide range in potential gain in digestibility highlights that the addition, if at all necessary, of external microbial consortia is of importance and the source should be carefully selected, especially in view of a process scale-up (Golub *et al*., 2013).

This thesis has also added another argument in the discussion of the advantages of mixed fermentations (Kleerebezem and van Loosdrecht, 2007). As shown from the unexpected production of lactic acid from hot trub in Chapter 6, a **mixed consortium may not only be regarded as a producer of metabolites but as a degrader of unwanted substances**, too. More specifically a fraction of the microorganisms may deal with the degradation of inhibitory compounds (in this case hop acids) while another fraction is

liberated to produce the desired metabolite (in this case lactic acid). This is an interesting finding whose applicability needs to be tested in different fermentation systems and may open up new ways of "pre-fermentation" strategies for a selective production of a target molecule.

7.3.2 How unique is each fermentation system and what this uniqueness could be attributed to?

Besides the inoculum, another essential pillar of the triptych "*substrate-inoculum-conditions*", as mentioned in section 1.1.4, is the substrate. In fact, it was shown in Chapter 4, that every substrate/inoculum combination is *statistically* unique in terms of metabolic performance and that both elements have an effect on the fermentation profile. The microbiome in the reactor originates from these two elements and is further developing on the basis of imposed fermentation conditions. It is therefore reasonable to **attribute this uniqueness to the evolution and dynamics of the underlying microbiome** that is, in turn, affecting the acidogenic metabolic products (see also Figure 1-3). This uniqueness is also linked to the reproducibility of each fermentation. Indeed, the more reproducible the results of a fermentation, the lower the deviation among the replicates in the concentration of a given product. This leads to a decreased probability to find series whose concentrations overlap, hence the metabolic uniqueness (see chapter 4).

But metabolic reproducibility is not necessarily linked to microbial reproducibility. Indeed, the same microorganism may adapt its metabolism as a result of a change, thus leading to non-reproducible metabolic results but a stable microbial structure; or different microorganisms can contribute to the production of the same metabolite, thus masking possibly diverse microbiota behind a reproducible metabolic profile (Ye *et al.*, 2007). We have confirmed this in Chapter 5, where two reactors of apple pulp inoculated with pretreated activated sludge had an average (throughout fermentation) coefficient of variation of 8% in the tVFA concentration, while the similarity coefficient of their DGGE profiles was not higher than 55%. This divergence shows that monitoring of the microbial communities is crucial to understand how they relate to the metabolic products, especially when a constant production line is envisaged.

7.3.3 Can a robust and direct link between the metabolic products and microbial species be established?

Table A.1-1 shows that the microbial diversity of acidogenic species reported in studies with a microbial analysis component is very large (without considering unclassified, uncultured or unknown bacteria which was a considerable fraction, often more than 50% of the reported OTU, in the studies included in the table). It is striking that, at genus level, only 8 out of 152 entries

have been reported by more than one author and only 1 out of 152 by more than two. But at higher taxonomic level, there is a certain number of families that are ubiquitous (e.g. *Clostridiaceae*) or very frequently reported (e.g. *Lactobacillaceae, Bacteroidaceae*). Furthermore, the majority of the references in Table 1-3 examined the microbial structure and only few have tried to correlate microbial species with metabolic products (Cheng *et al.*, 2014; Hollister *et al.*, 2010, 2011; Shen *et al.*, 2014; Sträuber *et al.*, 2012; Yun and Cho, 2016; Zhou *et al.*, 2016).

Chapters 5 and 6 have contributed to existing knowledge by identifying dominant microorganisms in the respective fermentation systems. To a certain extent **we could identify plausible links but not robust correlations between microorganism and metabolite**, for example the detection of *Selenomonas ruminantium* could be linked to the observed production of propionic acid and the absence of hydrogen (Chapter 5), or the dominance of *Clostridia* members coincides with marked tVFA increase (Chapter 5), or the absence of hop-resistant lactic acid bacteria in the fermentation broth of hot trub (Chapter 6). However, in the last experiment of this thesis (Annex 3, manuscript under preparation) we employed a metagenomic approach with next generation sequencing (Illumina, MiSeq) for a more holistic view of the dominant OTU in the fermentation system and a better correspondence between microbial species and metabolic products. Such an approach allows not only to follow the evolution of the microbial consortia better but also to increase confidence in establishing direct links between microorganisms and products. As mentioned in Annex 3, it was possible to link pronounced lactic acid production to four particularly abundant lactic acid bacteria species, to juxtapose metabolic performance and microbial abundance at the class, order and family taxonomic level and to monitor the similarity of the microbial structure in duplicate reactors. These findings are bringing us a step closer into correlating in a robust way the effect that a modification of an operational parameter (in our case pH and initial concentration) has to the microbial community dynamics (at different taxonomic levels) and thereby to the metabolic performance (Vanwonterghem *et al.*, 2014)

7.4 Perspectives

The discussion in chapters 7.1-7.3 suggests that mixed acidogenic fermentation has a true potential for sustainable valorisation of a variety of agroindustrial waste. Owing to the complexity of the system, a number of aspects require further research to bring this process closer to industrial environments. Figure 7-2 presents an illustrated overview of the scope of this thesis and of the areas where further research should focus on. These areas are discussed below.

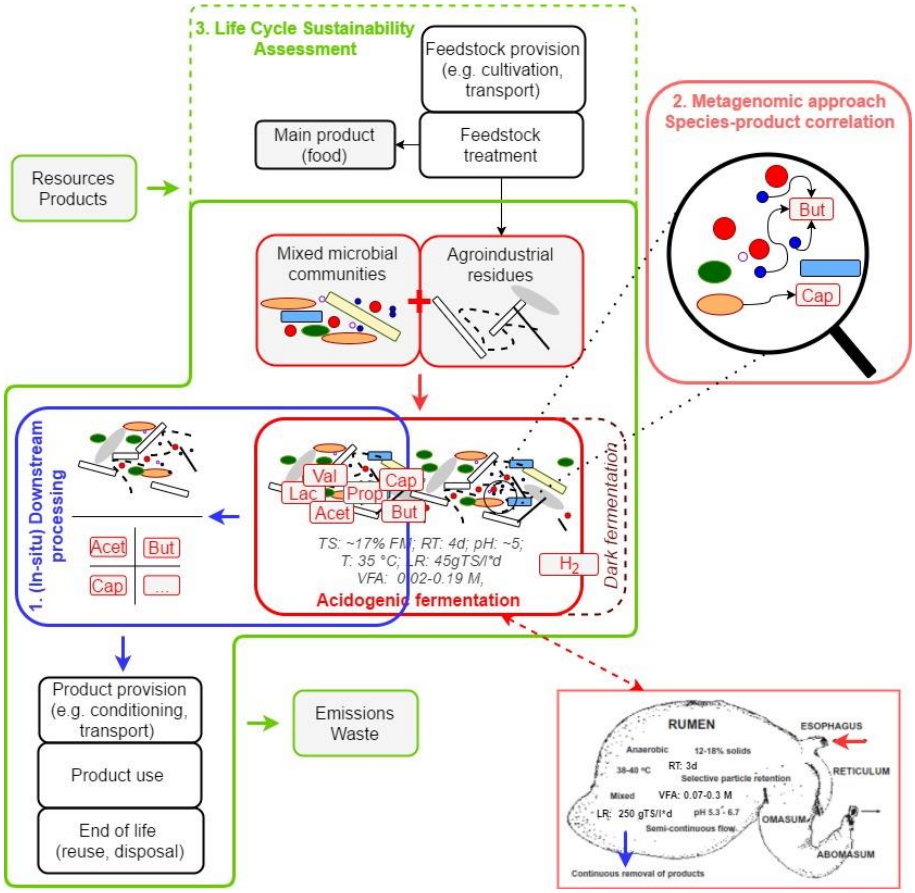


Figure 7-2: Scheme of future focus in the acidogenic fermentation value chain

TS: Total solids; FM: Fresh matter; VFA: Volatile fatty acids; RT: Residence time; LR: Loading rate; T: Temperature. Coloured frames represent different aspects of the value chain, where further research is necessary. Processes are represented by white background, while inputs and outputs are represented by grey background. Red frame is used for elements that have been examined in this thesis. The operational values in the acidogenic fermentation red frame are based on Annex 4, i.e. semi-continuous fermentation of trub. Black frame is used for upstream and downstream stages that are out of the scope of this thesis, but relevant for the life cycle perspective. Blue frame is used for the 1st perspective of section 7.4, i.e. the coupling of fermentation and downstream processing. Light red frame is used for the 2nd perspective of section 7.4, i.e. the metagenomic approaches to correlate functionally dominant species to metabolic products; light red is used because this aspect is only partially considered in this thesis (Annex 3, fermentation of chicory roots). Green frame is used for the 3rd perspective of section 7.4, i.e. the Life Cycle Sustainability Assessment of the process. The figure of the rumen, a natural acidogenic bioreactor, serves only as an analogy to a laboratory bioreactor and not direct comparison; it is taken from Weimer et al. (2009) and adapted based on information in the article; it is framed in light red because the aspect of ruminal inoculation is only partially treated in this thesis. Dark fermentation is framed with a dashed line, because it is considered outside the scope of this thesis, although its context is very similar (production of H_2 and VFA). Indeed, dark fermentation is typically aiming at optimising hydrogen as main product and VFA

are typically measured as by- or co-products of the process, while this thesis considers VFA as the main products and gas production and composition as a factor influencing their production (see Figure 1-3). It is, nevertheless, an important process to consider when trying to decipher mixed fermentation and this is discussed in section 7.4.2).

7.4.1 Continuous operation coupled with downstream processing

The discussion above provided elements to showcase the appropriateness of mixed acidogenic fermentation for the treatment of agroindustrial waste. Further work should focus on **continuous or semi-continuous operation** for the most promising substrates (e.g. trub) to determine the parameters that will allow acidogenic fermentation to be maintained at steady state and the potential inhibitory role of hop acids. Preliminary results from our (semi)-continuous experiments at HRT of 4 and 7 days have shown that acidogenic fermentation starts off at the beginning but cannot be further maintained at steady state and this is something that merits further investigation. Especially when passing to continuous operation, it will be important to couple fermentation with an efficient DSP process to avoid product inhibition, as mentioned in section 7.2.2.

Various authors have been focusing on this aspect, currently in systems that allow fermentation and VFA extraction within the same reactor, i.e. **in-situ product recovery**. Indicatively, Arslan *et al*. (2017) integrated a bipolar membrane electrodialysis system (EDBM) for in-situ VFA recovery and simultaneous control of the fermentation pH during continuous mixed fermentation of potato waste. They reported an increase of the substrate's conversion yield (74% increase to reach approximately 0.44 g_{COD_tVFA}/g_{COD_fed}), but also a drop in VFA concentration (58% drop to reach approximately 5_{gCOD_tVFA}/l) when coupling the EDBM system to the continuous reactor. Interestingly, they also observed a selective recovery of acetate and propionate during this coupling, which could be an important step into steering mixed fermentations towards a target molecule. In another study, Andersen *et al*. (2015) integrated membrane electrolysis to a fermenter for extractive electro-fermentation of thin stillage. Applying a current of 100 mA at HRT of 3 days led to an increase of VFA production rate by 5.5 (approximately 10.4 g_{COD_tVFA}/l*d), VFA concentration by 1.7 (approximately 18.9 g_{COD_tVFA}/l) and CODs to VFA conversion yield by 2 (approximately 0.60 g_{COD_tVFA}/g_{CODs}) compared to no current application. Additionally, they observed a tendency towards elongated VFA (butyrate, caproate, heptanoate), which is also an important sign towards selective VFA production. In both studies, natural pH control through coupled electrolysis/fermentation (without external acid/base addition) was possible. Such integrated extractive fermentation concepts are gaining attention thanks also to lower equipment needs and more sustainable pH control, compared to more conventional concepts of sequential fermentation and DSP (López-Garzón and Straathof,

2014).

It is, therefore, clear that an **equilibrium between a performant fermentation** leading to high VFA concentrations **and an efficient downstream VFA recovery process is key** to increasing the attractiveness of acidogenic fermentation as a stand-alone process with scale-up potential.

7.4.2 Deciphering microbial diversity

Deciphering the large microbial diversity in an acidogenic reactor is a major step towards better understanding the aforementioned uniqueness of the fermentation and better controlling the outcome. While it is already possible to establish direct links between microorganisms and metabolic products, this is not yet feasible in a systematic way for every taxonomic level at any given fermentation system. Given the complexity of the system, every single contribution in this area is of importance to reach critical mass of knowledge. Further work should first focus on **reviewing microbial-metabolic correlations** that have been already reported in acidogenic fermentation systems. For that, Annex 1 should be extended to systems of similar context (e.g. dark fermentation for hydrogen production as in Huang *et al.* (2010); first stage of two-stage anaerobic digestion as in Maspolim *et al.* (2015); first stage of PHA production as in Shen *et al.* (2014), mixed fermentation of simple substrates as in Tamis *et al.* (2015)). A recent meta-analysis by Bier *et al.* (2015) on the links between microbial communities and processes presents well the challenging nature of this task.

Moreover, it is important to investigate whether it is possible to establish direct and robust correlations between microbial species and metabolic products; and whether there is a core group of microbial species that is ubiquitous under different fermentation settings (i.e. triptych "*substrate-inoculum-conditions*") and is mostly contributing to the metabolic profile, particularly at steady state. Combination of meta-omics approaches (metagenomics, metatranscriptomics, etc.) can contribute to achieving these objectives as they offer now the possibility for deep community profile analysis for both highly and less abundant populations, thus making a clearer distinction between abundance and function (Vanwonterghem *et al.*, 2014). As de los Reyes III *et al.* (2015) claim, function can be defined differently for each given system. In our case, it would not only be the production of a given VFA but also the degradation of unwanted substances (i.e. hop acids). A possible strategy to achieve this could be through the combination of (i) mixed fermentations and (ii) gnotobiotic fermentations by species isolated from mixed fermentations and under the same conditions as mixed fermentations. Indeed, Vanwonterghem *et al.* (2014) claim that genomic information generated through -omics approaches could provide the basis for the design of

isolation strategies for tailored communities dedicated to a specific function. This information on the precise role and function of the different microorganisms involved could bring us a step closer to the development of a **mixed culture biotechnology,** where several microbial species can be managed in one single reactor to perform selective conversion of complex substrates, while preventing the establishment of undesired strains (Agler *et al.*, 2011).

7.4.3 Life Cycle Sustainability Assessment

A complete assessment of the economic and environmental (and potentially also societal) factors of mixed acidogenic fermentation is necessary to evaluate whether it could indeed represent a sustainable option at larger scale. This would require the conceptualisation of a complete process chain that would cover different technical configurations of fermentation and DSP and include upstream (feedstock/waste provision) and downstream (product provision) stages. A Life Cycle Assessment (LCA) is the most adequate tool for the assessment of environmental impacts as it considers all inputs and outputs during the entire life cycle of a product[6]. In this case, a stricter (compared to the one applied in this thesis) definition of the agroindustrial substrate as true "waste" (i.e. need to be disposed of, no foreseeable use) or "by-product" (i.e. possible further use and market uptake) will be an essential element for the selection of the appropriate LCA methodology (i.e. attributional, consequential, system boundaries, allocation, etc.). Extended to economic and societal impacts, it is referred to as **Life Cycle Sustainability Assessment** (LCSA), that is increasingly gaining attention as a more holistic and beneficial approach (Hellweg and Canals, 2014).

Chapter 2 only provided a simplified economic calculation based on the product value, without taking into consideration neither upstream stages (waste collection & transport) nor the downstream investment required to bring the final product to its usable form, as this is strongly dependent on its final use. For instance, application of a tVFA-rich stream as soluble COD feed for microorganisms performing nutrient removal in wastewaters would require less downstream processing efforts than the concentration, separation and purification of a specific VFA for use as a monomer for the green chemistry. On the other hand, potential economic advantages like the profit margin for biobased production were also not considered. As more

[6] See EU Commission Communication COM(2003)302 on Integrated Product Policy (p.10), available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2003:0302:FIN:en:PDF

information on mixed fermentation become available, such an analysis will become of high priority (Weimer et al., 2016)

7.5 Conclusions

This thesis is attempting to bridge two "worlds" of biotechnological applications: on the one side, the world of environmental biotechnology with the use of natural mixed consortia and non-axenic conditions for treating and valorising composite waste fractions; on the other side, the world of industrial biotechnology, where this complexity should be mastered and the process should be put at work at large scale for the sustainable production of marketable products for real-life applications.

This thesis advocates that the complexity of the fermentation system and the underlying microbial diversity ask for continuous research and every contribution in this respect brings us closer to deciphering mixed acidogenic fermentation of composite substrates.

Considering the multitude of potential applications of the generated product, acidogenic fermentation can earn its place as a stand-alone process for the conversion of complex substrates. VFA concentrations up to 41 g_{COD_tVFA}/kg_{ML} and substrate conversion yields of the magnitude of 0.65 $g_{COD\_tVFA}/g_{COD\_substrate}$ have been achieved after testing a variety of substrates. Substrates with limited digestible compounds (i.e. highly lignocellulosic substrates), did not perform very well indicating that when acidogenic fermentation follows upstream processes that exploited the digestible compounds of the substrates, its effectiveness in conversion is reduced.

Owing to its high sugar content, hot trub, a common brewery residue, proved to be particularly interesting at batch conditions. Apart from high product concentrations during fermentation it was also a very flexible substrate resulting in different metabolic products (VFA, lactic acid, ethanol), often simultaneously, depending on the fermentation conditions. Particularly interesting was the production of caproic acid at considerable concentrations (approximately 13 $gCOD/kg_{ML}$) and that without the external addition of ethanol or lactic acid for the chain elongation process.

Compared to standard anaerobic digestion, acidogenic fermentation exhibited systematically lower conversion yields, probably as a result of overloading and product inhibition. Even so, it is more attractive in terms of product value as carboxylic acids can be used in finer applications compared to methane. Acidogenic conversion yields can be improved if (in analogy to VFA consumption by methanogens in anaerobic digestion or VFA extraction through the rumen wall during rumination), efficient (in-situ) downstream processes are developed to recover, concentrate and separate VFA.

The important role of mixed consortia is evidenced by (i) the improvement (significant or marginal) of acidogenic performance whenever a mixed external inoculum was added and (ii) the apparent degradation of inhibitory hop substances in trub through a collective degradative activity, which allowed other microorganisms to convert the substrate into lactic acid.

The fermentation system, comprised of the substrate with its inherent flora, the external inoculum and the operational conditions is highly complex and this complexity leads to unique metabolic profiles. The central element behind this uniqueness is the microbiota of the reactor and how they evolve during fermentation. Ultimately the microbiome of the reactor is the responsible for the metabolic output. Deciphering the huge diversity and being able to correlate microbial species to metabolic products will be instrumental into achieving selective carboxylate production. Currently, such links are possible but not systematic enough and not for all taxonomic levels.

7.6 References

Agler, M.T., Spirito, C.M., Usack, J.G., Werner, J.J., Angenent, L.T., 2014. Development of a highly specific and productive process for n-caproic acid production: applying lessons from methanogenic microbiomes. Water Sci. Technol. 69, 62–68. https://doi.org/10.2166/wst.2013.549

Agler, M.T., Wrenn, B.A., Zinder, S.H., Angenent, L.T., 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends Biotechnol. 29, 70–78. https://doi.org/10.1016/j.tibtech.2010.11.006

Andersen, S.J., Candry, P., Basadre, T., Khor, W.C., Roume, H., Hernandez-Sanabria, E., Coma, M., Rabaey, K., 2015. Electrolytic extraction drives volatile fatty acid chain elongation through lactic acid and replaces chemical pH control in thin stillage fermentation. Biotechnology for Biofuels 8. https://doi.org/10.1186/s13068-015-0396-7

Angenent, L.T., Richter, H., Buckel, W., Spirito, C.M., Steinbusch, K.J.J., Plugge, C.M., Strik, D.P.B.T.B., Grootscholten, T.I.M., Buisman, C.J.N., Hamelers, H.V.M., 2016. Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology to Produce Biochemicals. Environ. Sci. Technol. 50, 2796–2810. https://doi.org/10.1021/acs.est.5b04847

Arslan, D., Steinbusch, K.J.J., Diels, L., Hamelers, H.V.M., Strik, D.P.B.T.B., Buisman, C.J.N., De Wever, H., 2016. Selective short-chain carboxylates production: A review of control mechanisms to direct mixed culture fermentations. Critical Reviews in Environmental Science and Technology 46, 592–634. https://doi.org/10.1080/10643389.2016.1145959

Arslan, D., Zhang, Y., Steinbusch, K.J.J., Diels, L., Hamelers, H.V.M., Buisman, C.J.N., De Wever, H., 2017. In-situ carboxylate recovery and simultaneous pH control with tailor-configured bipolar membrane electrodialysis during continuous mixed culture fermentation. Separation and Purification Technology 175, 27–35. https://doi.org/10.1016/j.seppur.2016.11.032

Batstone, D.J., Jensen, P.D., 2011. Anaerobic Processes, in: Wilderer, P. (Ed.), Treatise on Water Science. Elsevier, Oxford, pp. 615–639. https://doi.org/10.1016/B978-0-444-53199-5.00097-X

Bier, R.L., Bernhardt, E.S., Boot, C.M., Graham, E.B., Hall, E.K., Lennon, J.T., Nemergut, D.R., Osborne, B.B., Ruiz-González, C., Schimel, J.P., Waldrop, M.P., Wallenstein, M.D., 2015. Linking microbial community structure and microbial processes: an empirical and conceptual review. FEMS Microbiology Ecology, 91, 10. https://doi.org/10.1093/femsec/fiv113

Bosch, G., Heesen, L., de Melo Santos, K., Pellikaan, W.F., Cone, J.W., Hendriks, W.H., 2017. Evaluation of an in vitro fibre fermentation method using feline faecal inocula: repeatability and reproducibility. J Nutr Sci 6. https://doi.org/10.1017/jns.2017.22

Cheng, W., Chen, H., Yan, S., Su, J., 2014. Illumina sequencing-based analyses of bacterial communities during short-chain fatty-acid production from food waste and sewage sludge fermentation at different pH values. World Journal of Microbiology and Biotechnology 30, 2387–2395. https://doi.org/10.1007/s11274-014-1664-6

de los Reyes III, F., Weaver J.E., Wang, L., 2015. A methodological framework for linking bioreactor function to microbial communities and environmental conditions. Current Opinion in Biotechnology 2015, 33, 112-118. http://dx.doi.org/10.1016/j.copbio.2015.02.002

Ferreira, A.F., 2017. Biorefinery Concept, in: Rabaçal, M., Ferreira, A.F., Silva, C.A.M., Costa, M. (Eds.), Biorefineries, Lecture Notes in Energy. Springer International Publishing, Cham, pp. 1–20. https://doi.org/10.1007/978-3-319-48288-0_1

Gameiro, T., Lopes, M., Marinho, R., Vergine, P., Nadais, H., Capela, I., 2016. Hydrolytic-Acidogenic Fermentation of Organic Solid Waste for Volatile Fatty Acids Production at Different Solids Concentrations and Alkalinity Addition. Water Air Soil Pollut 227, 391. https://doi.org/10.1007/s11270-016-3086-6

Grootscholten, T.I.M., Kinsky dal Borgo, F., Hamelers, H.V.M., Buisman, C.J.N., 2013. Promoting chain elongation in mixed culture acidification reactors by addition of ethanol. Biomass and Bioenergy 48, 10–16. https://doi.org/10.1016/j.biombioe.2012.11.019

Golub, K.W., Forrest, A.K., Wales, M.E., Hammett, A.J.M., Cope, J.L., Wilkinson, H.H., Holtzapple, M.T., 2013. Comparison of three screening methods to select mixed-microbial inoculum for mixed-acid fermentations. Bioresource Technology 130, 739–749. https://doi.org/10.1016/j.biortech.2012.10.010

Hellweg, S., Canals, L.M. i, 2014. Emerging approaches, challenges and opportunities in life cycle assessment. Science 344, 1109–1113. https://doi.org/10.1126/science.1248361

Hollister, E. b., Hammett, A. m., Holtzapple, M. t., Gentry, T. j., Wilkinson, H. h., 2011. Microbial community composition and dynamics in a semi-industrial-scale facility operating under the MixAlco™ bioconversion platform. Journal of Applied Microbiology 110, 587–596. https://doi.org/10.1111/j.1365-2672.2010.04919.x

Hollister, E.B., Forrest, A.K., Wilkinson, H.H., Ebbole, D.J., Malfatti, S.A., Tringe, S.G., Holtzapple, M.T., Gentry, T.J., 2010. Structure and dynamics of the microbial communities underlying the carboxylate platform for biofuel production. Applied Microbiology and Biotechnology 88, 389–399. https://doi.org/10.1007/s00253-010-2789-7

Huang, Y., Zong, W., Yan, X., Wang, R., Hemme, C.L., Zhou, J., Zhou, Z., 2010. Succession of the bacterial community and dynamics of hydrogen producers in a hydrogen-producing bioreactor. Appl. Environ. Microbiol. 76, 3387–3390. https://doi.org/10.1128/AEM.02444-09

Kleerebezem, R., van Loosdrecht, M.C., 2007. Mixed culture biotechnology for bioenergy production. Current Opinion in Biotechnology 18, 207–212. https://doi.org/10.1016/j.copbio.2007.05.001

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

López-Garzón, C.S., Straathof, A.J.J., 2014. Recovery of carboxylic acids produced by fermentation. Biotechnology Advances 32, 873–904. https://doi.org/10.1016/j.biotechadv.2014.04.002

Maspolim, Y., Zhou, Y., Guo, C., Xiao, K., Ng, W.J., 2015. Comparison of single-stage and two-phase anaerobic sludge digestion systems - Performance and microbial community dynamics. Chemosphere 140, 54–62. https://doi.org/10.1016/j.chemosphere.2014.07.028

Mathias, T.R. dos S., Alexandre, V.M.F., Cammarota, M.C., de Mello, P.P.M., Sérvulo, E.F.C., 2015. Characterization and determination of brewer's solid wastes composition: Brewer's waste characterization. Journal of the Institute of Brewing 121, 400–404. https://doi.org/10.1002/jib.229

Mussatto, S.I., 2009. Biotechnological Potential of Brewing Industry By-Products, in: Singh nee' Nigam, P., Pandey, A. (Eds.), Biotechnology for Agro-Industrial Residues Utilisation. Springer Netherlands, Dordrecht, pp. 313–326. https://doi.org/10.1007/978-1-4020-9942-7_16

Shen, L., Hu, H., Ji, H., Cai, J., He, N., Li, Q., Wang, Y., 2014. Production of poly(hydroxybutyrate–hydroxyvalerate) from waste organics by the two-stage process: Focus on the intermediate volatile fatty acids. Bioresource Technology 166, 194–200. https://doi.org/10.1016/j.biortech.2014.05.038

Shin, S.G., Yoo, S., Hwang, K., Song, M., Kim, W., Han, G., Hwang, S., 2011. Dynamics of transitional acidogenic community along with methanogenic population during anaerobic digestion of swine wastewater. Process Biochemistry 46, 1607–1613. https://doi.org/10.1016/j.procbio.2011.05.001

Singhania, R.R., Patel, A.K., Christophe, G., Fontanille, P., Larroche, C., 2013. Biological upgrading of volatile fatty acids, key intermediates for the valorization of biowaste through dark anaerobic fermentation. Bioresour. Technol. 145, 166–174. https://doi.org/10.1016/j.biortech.2012.12.137

Sträuber, H., Schröder, M., Kleinsteuber, S., 2012. Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a

leach-bed process. Energ Sustain Soc 2, 13. https://doi.org/10.1186/2192-0567-2-13

Tamis, J., Joosse, B. m., Loosdrecht, M.C.M. va., Kleerebezem, R., 2015. High-rate volatile fatty acid (VFA) production by a granular sludge process at low pH. Biotechnol. Bioeng. 112, 2248–2255. https://doi.org/10.1002/bit.25640

Temudo, M.F., Muyzer, G., Kleerebezem, R., van Loosdrecht, M.C.M., 2008. Diversity of microbial communities in open mixed culture fermentations: impact of the pH and carbon source. Appl. Microbiol. Biotechnol. 80, 1121–1130. https://doi.org/10.1007/s00253-008-1669-x

Vanwonterghem, I., Jensen, P.D., Ho, D.P., Batstone, D.J., Tyson, G.W., 2014. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Current Opinion in Biotechnology, 27, 55–64. https://doi.org/10.1016/j.copbio.2013.11.004

Vasudevan, D., Richter, H., Angenent, L.T., 2014. Upgrading dilute ethanol from syngas fermentation to n-caproate with reactor microbiomes. Bioresour. Technol. 151, 378–382. https://doi.org/10.1016/j.biortech.2013.09.105

Venkata Mohan, S., Nikhil, G.N., Chiranjeevi, P., Nagendranatha Reddy, C., Rohit, M.V., Kumar, A.N., Sarkar, O., 2016. Waste biorefinery models towards sustainable circular bioeconomy: Critical review and future perspectives. Bioresource Technology 215, 2–12. https://doi.org/10.1016/j.biortech.2016.03.130

Weimer, P.J., Russell, J.B., Muck, R.E., 2009. Lessons from the cow: What the ruminant can teach us about consolidated bioprocessing of cellulosic biomass. Bioresource Technology 100, 5323-5331. https://doi.org/10.1016/j.bioretech.2009.04.075

Weimer, P.J., Kohn, R.A., 2016. Impacts of ruminal microorganisms on the production of fuels: how can we intercede from the outside? Applied Microbiology and Biotechnology 100, 3, 3389-3398. https://doi.org/10.1007/s00253-016-7358-2

Ye, N.-F., Lü, F., Shao, L.-M., Godon, J.-J., He, P.-J., 2007. Bacterial community dynamics and product distribution during pH-adjusted fermentation of vegetable wastes. J. Appl. Microbiol. 103, 1055–1065. https://doi.org/10.1111/j.1365-2672.2007.03321.x

Yun, J., Cho, K.-S., 2016. Effects of organic loading rate on hydrogen and volatile fatty acid production and microbial community during acidogenic hydrogenesis in a continuous stirred tank reactor using molasses wastewater. Journal of Applied Microbiology 121, 1627–1636. https://doi.org/10.1111/jam.13316

Zacharof, M.-P., Lovitt, R.W., 2014. Recovery of volatile fatty acids (VFA) from complex waste effluents using membranes. Water Sci. Technol. 69, 495–503. https://doi.org/10.2166/wst.2013.717

Zhou, A., Zhang, J., Wen, K., Liu, Z., Wang, G., Liu, W., Wang, A., Yue, X., 2016. What could the entire cornstover contribute to the enhancement of waste activated sludge acidification? Performance assessment and microbial community analysis. Biotechnology for Biofuels 9. https://doi.org/10.1186/s13068-016-0659-y

ANNEXES

Annex 1

Acidogenic bacteria reported in mixed acidogenic fermentation studies

Table A.1-1: Microorganisms reported in acidogenic fermentation studies

Reference numbers correspond to the studies of Table 1-3

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*		*Clostridium*	*1, 2, 12, 13, 17, 18, 20, 22, 23, 27, 28, 29, 30, 31, 32*	*C. acetobutylicum*	*3*
							C. cellulosi	*4, 15*
							C. thermopalmarium	*4*
							C. ljungdahlii	*5*
							C. asidisoli	*5*
							C. mesophilum	*15*
							C. novyi	*6*
							C. magnum	*3*
							C. propionicum	*3*
							C. sticklandii	*3*
							C. tertium	*3*
							C. disporicum	*7*
							C. quinii	*7*
							C. tyrobutyricum	*34*
							C. lavalense	*15*
							C. jejuense	*15*
							C. xylanovorans	*15*
							C. cellulolyticum	*15*
							C. stercorarium	*15*

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
							C. aminovalericum	*15*
							C. perfringens	*31*
							C. viride	*16*
					Ethanoligenens	*2, 14, 23, 27*	*E. harbinense*	*15, 34*
					Lutispora		*L. thermophila*	*8*
					Geosporobacter	*11*	*G. subterraneus*	*16*
					Alkaliphilus	*21, 26*	*A. peptidofermentans*	*31*
					Proteiniclasticum	*11, 19*		
					Brassicibacter	*21*		
					Clostiriisalibacter	*31*		
			Gracilibacteraceae		*Gracilibacter*	*21*	*G. thermotolerans*	*8*
			Ruminococcaceae	*4, 2, 11*	*Ruminococcus*	*11, 22, 27, 30*	*R. albus*	*12*
							R. flavefaciens	*16*
					Anaerofilum	*17, 30, 34*	*A. agile*	*3*
							A. pentosovorans	*34*
					Saccharofermentans	*11, 19*	*S. acetigenes*	*12*
					Acetanaerobacterium	*11*		
					Acetivibrio	*11*		
					Faecalibacterium	*11*		
					Sporobacter		*S. termitidis*	*16*
					Anaerotruncus	*17*		
					Oscillospira	*22, 30, 34*		
					Fastidiosipila	*23, 26*		
			Lachnospiraceae	*4, 11, 16, 31,*	*Roseburia*	*11*		
					Butyrivibrio	*12, 29*		
					Lachnoclostridium		*L. phutofermentans*	*15*

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
				34	*Lachnospira*		*L. pectinoschiza*	*16*
					Coprococcus		*C. eutactus*	*16*
					Syntrophococcus		*S. sucromutans*	*16, 34*
			Syntrophomonadaceae		*Thermanaerovibrio*		*T. acidaminovorans*	*4*
					Dethiobacter		*D. aklaliphilus*	*31*
			Peptostreptococcaceae	*2, 26*	*Proteocatella*	*11*	*P. sphenisci*	*3*
					Acetoanaerobium	*11, 19*		
					Terrisporobacter		*T. mayombei*	*28**
					Peptostreptococcus	*22, 29*		
			Peptococcaceae	*21*	*Desulfotomaculum*	*29*	*D. reducens*	*3c*
							D. thermosapovorans	*16*
							D. hydrothermale	*31*
							D. kuznetsovii	*16*
					Desulfonispora	*31*	*D. thiosulfatigenes*	*31*
					Sporotomaculum		*S. syntrophicum*	*16*
					Pelotomaculum	*30*		
			Eubacteriaceae		*Eubacterium*	*28, 29*	*E. pyruvativorans*	*6*
							E. eligens	*34*
							E. cellulosolvens	*12*
					Alkalibacter		*A. saccharofermentans*	*7*
					Alkalibaculum	*32*		
					Garciella	*21, 31*		
			Symbiobacteriaceae		*Symbiobacterium*	*14*	*S. toebii*	*15*
			Oscillospiraceae		*Oscillibacter*	*17, 24, 32*		
			Christensenellaceae	*21*				
			Proteinivoraceae		*Anaerobranca*	*20, 32*	*A. gottschalkii*	*31*

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
							A. californiensis	*31*
							A. horikoshii	*31*
							A. zavarzinii	*31*
			unclassified		*Anaerovirgula*	*11*		
					Proteiniborus	*23, 32*		
			incertae sedis		*Guggenheimella*	*11, 26, 32*	*G. bovis*	*31*
					Anaerovorax	*30*		
					Fusibacter	*11*		
		Thermoanaerobacterales	*Thermoanaerobacterac*	*8*				
			Family III Incertae Sedis		*Thermoanaerobacterium*	*14*	*T. aotearoense*	*15*
							T. saccharolyticum	*15*
					Caldicellulosiruptor	*15*	*C. hydrothermalis*	*15*
			Thermodesulfobiaceae		*Coprothermobacter*	*14, 20*		
		Halanaerobiales	*Halanaerobiaceae*	*4*				
	Bacilli	*Lactobacillales*	*Lactobacillaceae*		*Lactobacillus*	*1, 9, 6, 13, 21, 22, 24, 27, 3*	*L. acidophilus*	*5, 7*
							L. delbrueckii	*5*
							L. panis	*5*
							L. mucosae	*5*
							L. acetotolearns	*9, 34*
							L. casei	*34*
							L. paracasei	*34*
							L. zeae	*34*
							L. camelliae	*34*
							L. similis	*34*
							L. buchneri	*34*
							L. parafarraginis	*34*

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
							L. frumenti	*34*
							L. brevis	*34*
							L. fructivorans	*9*
							L. homohiochii	*9, 34*
			Streptococcaceae		*Lactococcus*	*9, 22, 30*		
					Streptococcus	*22, 29*	*S. bovis*	*3b*
			Leuconostocaceae		*Weissella*	*13*	*W. cibaria*	*5*
					Leuconostoc	*9, 21, 22, 30*	*L. mesenteroides*	*9*
			Enterococcacea	*4*	*Enterococcus*	*11, 21, 22, 30*	*E. faecium*	*34*
			Carnobacteriaceae		*Trichococcus*		*T. flocculiformis*	*3*
							T. collinsii	*33*
					Carnobacterium	*22*		
					Granulicatella	*29*		
			Aerococcaceae		*Abiotrophia*	*29*	*A. para-adiacens*	*16*
		Bacillales	*Bacillaceae*	*4, 5*	*Bacillus*	*15, 22, 23, 29*	*B. coagulans*	*4*
							B. clausii	*8*
							B. halodurans	
							B. circulans	*15*
							B. thuringiensis	*33*
							B. subtilis	*33*
							B. thermozeamaize	*15*
					Anaerobacillus	*21*		
					Amphibacillus	*21*		
					Lysinibacillus	*30*		
					Geobacillus	*14, 15*	*G. stearothermophilus*	*31*
			Planococcaceae		*Ureibacillus*	*1, 14*		

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
					Bhargavaea	*14*		
					Rummeliibacillus	*17, 22*		
			Thermoactinomycetaceae		*Thermoflavimicrobium*		*T. dichotomicum*	*8*
					Planifilum	*14*		
			Sporolactobacillaceae		*Sporolactobacillus*	*13*		
			Paenibacillaceae		*Paenibacillus*	*29*	*P. brasilensis*	*15*
					Brevibacillus	*29*		
			Listeriaceae		*Brochothrix*	*21*		
			Staphylococcaceae		*Staphylococcus*	*22, 29*		
			incertae sedis		*Exiguobacterium*	*17, 29*		
	Erysipelotrichi	*Erysipelotrichales*	*Erysipelotrichidae*		*Erysipelothrix*	*2, 18, 23, 26*	*E. rhusiopathiae*	*31*
	Negativicutes	*Veillonellales*	*Veillonellaceae*	*9*	*Megasphaera*	*9, 24*	*M.cerevisiae*	*9*
							M. paucivorans	*7*
							M. sueciensis	*7*
					Pectinatus	*9, 24*		
					Veillonella	*9, 29*		
					Dialister	*24*	*D. invisus*	*7*
							D. succinatiphilus	*16*
					Anaeroglobus		*A. geminatus*	*7*
		Selenomonadales	*Selenomonadaceae*		*Selenomonas*	*9*	*S. lacticifex*	*9*
					Mitsuokella	*1*		
			Sporomusaceae		*Sporomusa*		*S. ovata*	*16*
					Propionispora		*P. hippei*	*31*
		Acidaminococcales	*Acidaminococcaceae*		*Acidaminococcus*	*11, 30*		
					Phascolarctobacterium	*30, 32*	*P. faecium*	*16*
	Tissierellia	*Tissierellales*	*Tissierellaceae*		*Tissierella*	*2, 11, 21, 23,*	*T. creatinophila*	*31*

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
						26, 32	T. praeacuta	31
					Soehngenia	22, 30		
		Unclassified			Sporanaerobacter	17, 22, 23	S. acetigenes	6, 16
					Sedimentibacter	8, 11, 19, 22,		
					Tepidimicrobium	14, 20, 22		
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	4	Parabacteroides	9, 12, 23, 27,	P. goldsteinii	
					Petrimonas	11, 19, 23, 32		
			Bacteroidaceae	4	Bacteroides	4, 9, 15, 17, 19, 22, 23, 30, 32	B. graminisolvens	4, 12
							B. uniformis	10, 12
							B. coprophilus	7
							B. caccae	15
			Dysgonamonadaceae		Dysgonomonas	11, 30		
					Proteiniphilum	32	P. acetatigenes	28
			Prevotellaceae		Prevotella	1, 9, 15, 24, 30, 32	P. ruminicolla	12
							P. brevis	12
							P. albensis	15
					Hallella	24, 32		
			Paludibacteraceae		Paludibacter	2, 11, 19, 22,	P. propionicigenes	31
			Tannerellaceae		Parabacteroides	17, 19, 32		
			Odoribacteraceae		Butyricimonas	28		
		Marinilabiliales	Marinilabiliaceae		Mangroviflexus	19		
					Alkaliflexus	26, 31		
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae		Cloacibacterium	9		
			Crocinitomicaceae		Fluviicola	19		
	Chitinophagia	Chitinophagales	Chitinophagaceae		Terrimonas	11		
	Sphingobacteriia	Sphingbacteriles	Sphingobacteriaceae		Sphingbacterium	30		

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*		*Defluviicoccus*	*2*		
					Dongia	*11*		
			Acetobacteraceae	*13*	*Acidocella*	*23*		
					Gluconobacter		*G. albidus*	*31*
					Roseomonas		*R. stagni*	*31*
							R. genomospecies	*31*
					Acetobacter	*27, 29*	*A. lovaniensis*	*34*
			Erythrobacteraceae	*4*				
		Sphingomonadales	*Sphingomonadaceae*		*Novosphingobium*	*11, 26*		
					Sphingopyxis	*11*		
					Sphingobium	*30*		
					Zymomonas	*29*		
					Sphingomonas	*11*		
		Caulobacterales	*Caulobacteraceae*		*Brevundimonas*	*9, 22, 30*	*B. aveniformis*	*9*
		Rickettsiales	*Rickettsiaceae*		*Wolbachia*	*9*		
		Rhizobiales	*Methylocystaceae*		*Pleomorphomonas*	*9*		
			Hyphomicrobiaceae		*Devosia*	*11, 26*		
					Hyphoomicrobium	*11,21*		
					Filomicrobium	*11*		
			Phyllobacteriaceae		*Mesorhizobium*	*11, 26, 33*	*M. amorphae*	*31*
			Rhizobiaceae		*Sinorhizobium*	*31*		
					Agrobacterium		*A. tumefaciens*	*33*
			Bruxellaceae		*Ochrobactrum*		*O.*	*33*
			Bradyrhizobiaceae		*Bradyrhizobium*	*11, 31*	*B. japonicum*	*31*
					Bosea	*30*		
		Rhodobacterales	*Rhodobacteraceae*		*Paracoccus*	*11, 30*		

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
					Rhodobacter	*11*		
					Thioclava	*17*		
	Betaproteobacteria	*Burkholderiales*	*Burkholderiaceae*	*4*	*Limnobacter*	*27*		
					Pandoraea	*22*		
			Alcaligenaceae		*Alcaligenes*	*2, 18, 26, 33*		
					Achromobacter	*9, 17*	*A. anxifer*	*9*
							A. dolens	*9*
							A. denitrificans	*9*
					Derxia	*11*		
					Pussilimonas	*31*		
					Advenella	*11, 17*		
			Oxalobacteraceae		*Massilia*	*9*		
					Janthinobacterium	*13*		
			Comamonadaceae		*Acidovorax*	*11, 31*	*A. avenae*	*31*
					Curvibacter	*11*		
					Comamonas	*11, 17, 22, 30*	*C. denitrificans*	*31*
					Diaphorobacter	*11*		
					Ottowia	*18, 26*		
					Hylemonella	*17*		
					Variovorax	*25, 33*		
			unclassified		*Piscinibacter*	*11*		
					Ideonella	*11*		
					Methylibium	*31*		
					Aquabacterium	*11*		
		Rhodocyclales	*Rhodocyclaceae*		*Thauera*	*2, 11, 19, 23, 31*	*T. phenylacetica*	*31*
							T. aminoaromatica	*31*

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
					Dechloromonas	*2, 23, 26*		
					Azovibrio		*A. restrictus*	*3a*
					Azospira	*11*		
			Zoogloeaceae		*Zoogloea*	*2, 11, 23, 31*		
		Nitrosomonadales	*Sterolibacteriaceae*		*Sulfuritalea*	*11*		
					Georgfuchsia	*11*		
			Nitrosomonadaceae		*Nitrosospira*	*11, 19*		
	Gammaproteobacteria	*Pseudomonadales*	*Pseudomonadaceae*		*Pseudomonas*	*1, 9, 8, 13, 17, 18, 26, 29, 30, 33*	*P. aeruginosa*	*25*
							P. fluorescens	*10, 6, 3*
							P. antarctica	*28*
							P. luteola	*33*
							P. knackmussii	*33*
							P. psychrophilla	*28*
							P. indica	*15*
					Azotobacter		*A. chroococcum*	*15*
							A. tropicalis	*15*
			Moraxellaceae	*4*	*Enhydrobacter*	*33*		
					Psychrobacter		*P. maritimus*	*6*
					Acinetobacter	*17, 22, 27, 30*	*A. radioresistens*	*31*
		Enterobacterales	*Enterobacteriaceae*	*9*	*Sodalis*	*9*		
					Enterobacter	*9*		
					Citrobacter	*9*		
					Klebsiella	*15*	*K. oxytoca*	*3*
					Escherichia	*17, 29*	*E. coli*	*15*
			Hafniaceae	*21*				
			Morganellaceae		*Proteus*	*29, 30*		

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
					Providenzia	*30*		
		Aeromonodales	*Aeromonodaceae*		*Aeromonas*	*29*	*A. veronii*	*9*
							A. hydrophila	*3*
		Xanthomonadales	*Xanthomonadaceae*		*Thermomonas*		*T. fusca*	*8*
							T. haemolytica	*8*
							T. Koreensis	*8*
					Pseudoxanthomonas	*11*		
					Stenotrophomonas	*27, 30*		
			Rhodanobacteraceae		*Aquimonas*	*11*		
					Dokdonella	*11, 31*		
		Methlycoccales	*Methyloccocae*		*Methylobacter*	*31*		
	Deltaproteobacteria	*Desulfobacterales*	*Desulfobacteraceeah*		*Desulfobotulus*		*D. sapovorans*	*3*
		Desulfovibrionales	*Desulfovibrionaceae*		*Desulfovibrio*	*17, 24, 29, 30*	*D. vulgaris*	*3*
		Desulfuromonadales	*Geobacteraceae*		*Geobacter*	*30*		
		Desulfarculales	*Desulfarculaceae*		*Desulfarculus*	*30*		
		Myxococcales	*Polyangiaceae*		*Byssovorax*	*11*		
		Syntrophobacterales	*Syntrophobacteraceae*		*Syntrophobacter*	*22, 30*		
	Epsilonproteobacteria	*Campylobacterales*	*Campylobacteraceae*		*Arcobacter*	*9, 17*		
					Sulfurospirillum		*S. halorespirans*	*3c*
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*		*Actinomyces*	*2, 23*		
					Actinotignum		*A. schaalii*	*15*
		Actionobacteriales	*Actinomycineae*		*Actinobaculum*	*15, 16*		
		Bifidobacteriales	*Bifidobacteriaceae*		*Bifidobacterium*	*9, 24*	*B. thermacidophilum*	*5,7*
							B. boum	*7*
			Intrasporangiaceae		*Tetrasphaera*	*2, 11*		
					Ornithinimicrobium	*11*		

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
					Ornithinibacter		*O. aureus*	*25*
		Micrococcales	*Micrococcineae*	*13*				
			Micrococcaceae		*Micrococcus*	*29*		
			Microbacteriaceae		*Leucobacter*	*26*		
					Microbacterium		*M. hominis*	*33*
		Propionibacteriales	*Nocardioidaceae*		*Propionicicella*	*11*		
			Propionibacteriaceae		*Propionibacterium*	*24, 29*		
		Corynebacteriales	*Corynebacteriacea*		*Corynebacterium*	*26, 30*	*C. kroppenstedtii*	*7*
							C. aurimucosum	*28*
							C. hemireducens	*28*
			Mycobacteriaceae		*Mycobacterium*	*20, 29*		
			Nocardiaceae		*Rhodococcus*	*27*		
	Coriobacteriia	*Coriobacteriales*	*Coriobacteriaceae*		*Olsenella*		*O. profusa*	*9*
					Atopobium	*9*	*A. parvulum*	*9*
		Eggerthellales	*Eggerthellaceae*		*Eggerthella*	*30*		
	Acidimicrobiia	*Acidimicrobiales*	*Iamiaceae*		*Iamia*	*2, 11*		
			Acidimicrobiaceae		*Ilumatobacter*	*11*		
			Microthrixaceae		*Microthrix*	*26*		
Thermotogae	*Thermotogae*	*Thermotogales*	*Thermotogaceae*	*4*				
		Kosmotogales	*Kosmotogaceae*		*Kosmotoga*	*22, 30*		
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*4, 21*	*Cloacibacillus*	*11, 19*		
					Aminobacterium	*22*		
					Aminiphilum	*30*		
					Acetomicrobium	*20*		
Spirochaetes	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*		*Treponema*	*1, 11, 23, 30*		
					Spirochaeta	*11*		

Phylum	Class	Order	Family	Ref.	Genus	Ref.	Species	Ref
					Sphaerochaeta	*28*		
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*		*Prosthecobacter*	*2*		
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*21*	*Anaerolinea*	*2, 23, 26*		
					Bellilinea	*11*		
					Leptolinea	*2, 23*		
					Longilinea	*19, 21, 30*		
					Levilinea	*19, 32*		
					Ornatilinea	*21*		
	Caldilineae	*Caldilineales*	*Caldilineaceae*		*Caldilinea*	*11*		
					Litorilinea	*19*		
	Chloroflexia	*Chloroflexales*	*Roseiflexaceae*		*Roseiflexus*	*11*		
	Ardenticatenia	*Ardenticatenales*	*Ardenticatenaceae*		*Ardenticatena*			
Nitrospirae	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*		*Nitrospira*	*2, 11, 30*		
Tenericutes	*Mollicutes*	*Acholeplasmatales*	*Acholeplasmataceae*		*Acholeplasma*	*26*	*morum*	*31*
Acidobacteria	*Blastocatellia*	*Blastocatellales*	*Blastocatellaceae*	*26*	*Blastocatella*			
Planctomycetes	*Planctomycetia*	*Planctomycetales*	*Planctomycetaceae*		*Planctomyces*	*27, 31*		
Caldiserica	*Caldisericia*	*Caldisericales*	*Caldisericaceae*		*Caldisericum*	*32*		

** reclassification from the original species in the reference*

Annex 2

Metabolic pathways of short chain carboxylic acids

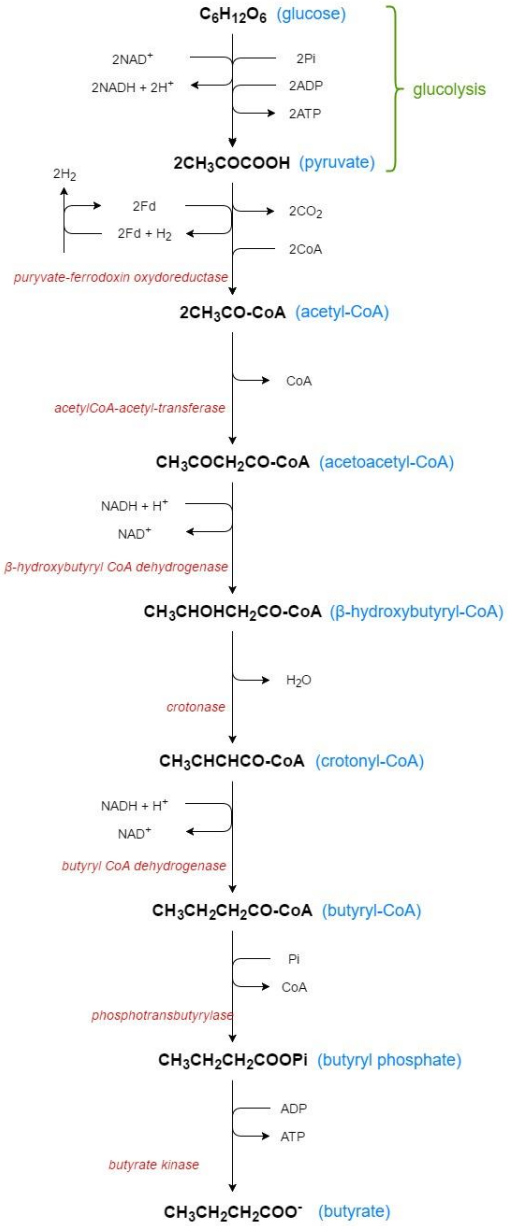


Figure A.2-1: Metabolic pathway of butyric acid from glucose
(based on Zidwick et al., 2013)

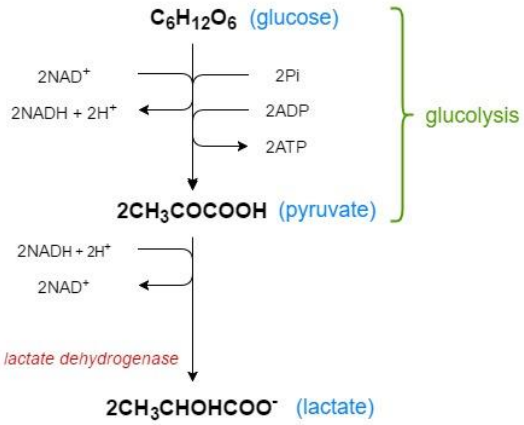


Figure A.2-2: Metabolic pathway of lactic acid from glucose
(based on Nelson and Cox, 2005)

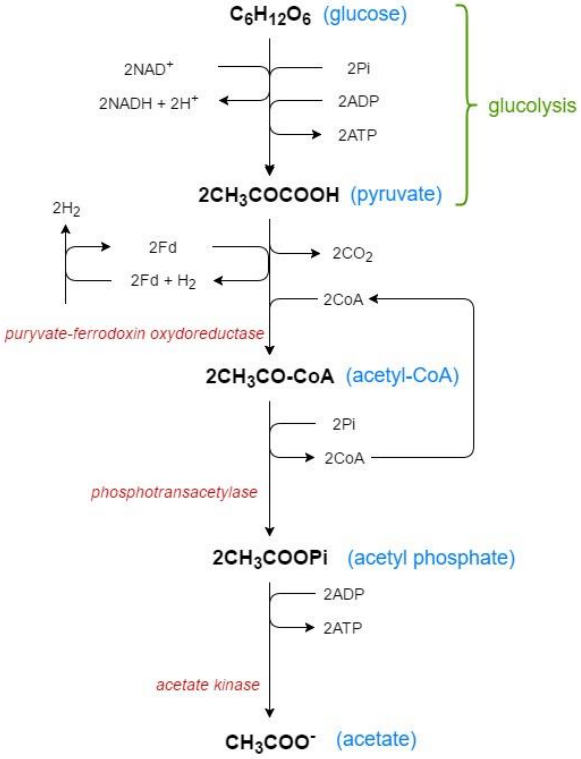


Figure A.2-3: Metabolic pathway of acetic acid from glucose
(based on Zidwick et al., 2013)

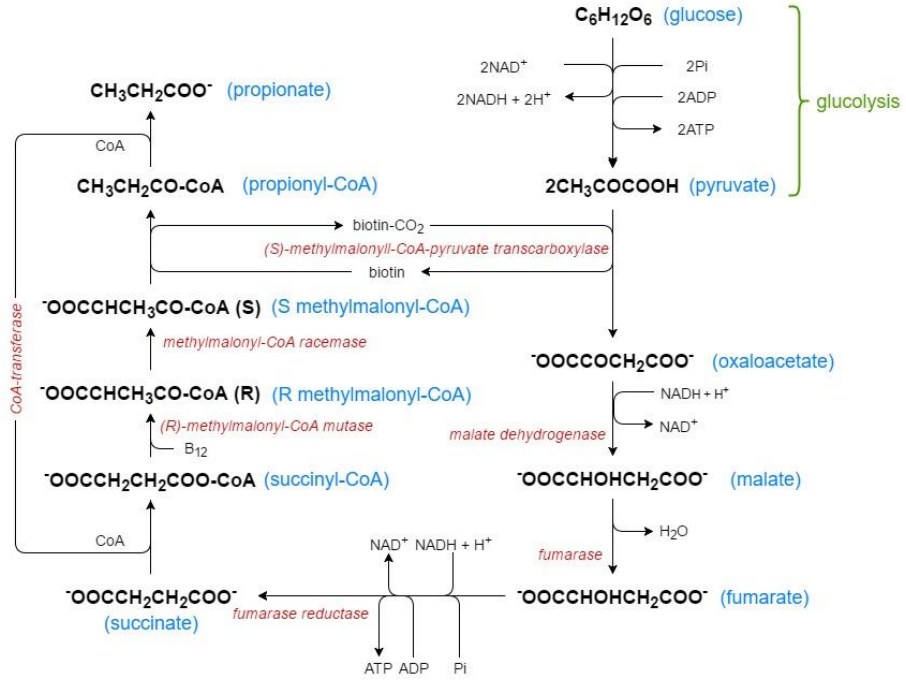


Figure A.2-4: Metabolic pathway of propionic acid from glucose
(based on Denman et al., 2015)

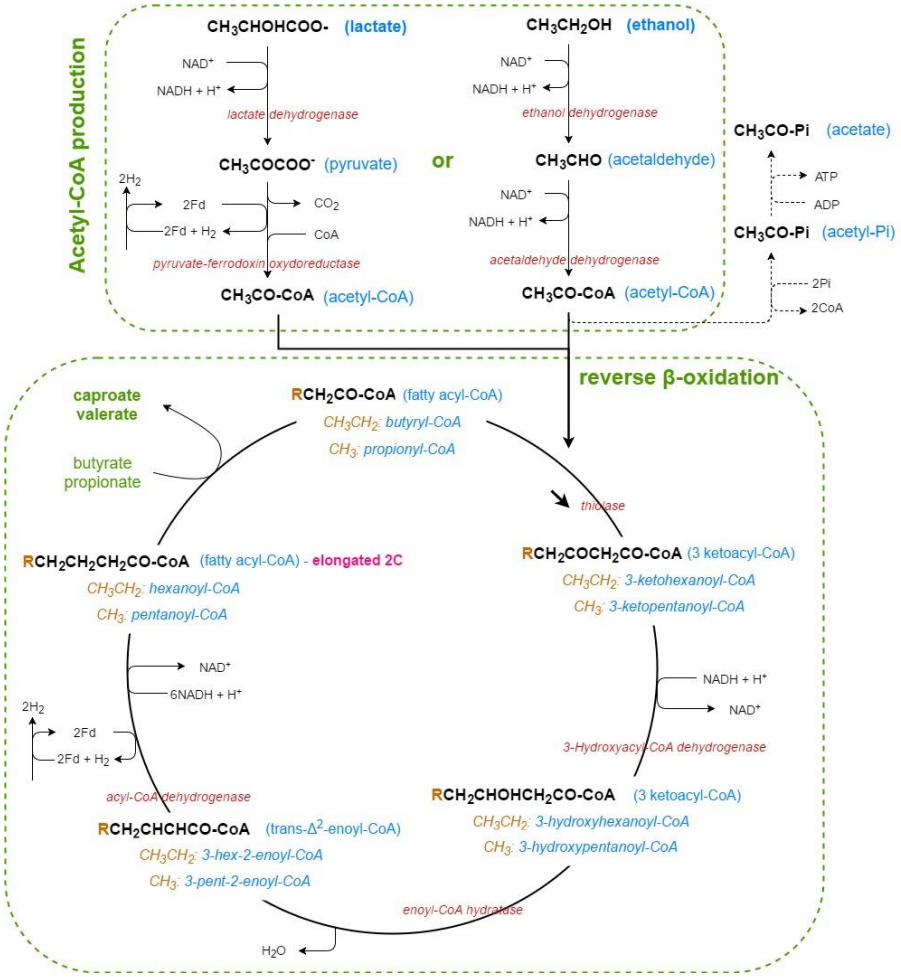


Figure A.2-5: Metabolic pathway of valeric and caproic acids based on reverse β-oxidation (chain elongation)
(based on Cavalcante et al., 2017; Horton et al., 1992; Spirito et al., 2014)

References

Cavalcante, W. de A., Leitão, R.C., Gehring, T.A., Angenent, L.T., Santaella, S.T., 2017. Anaerobic fermentation for n-caproic acid production: A review. Process Biochemistry 54, 106–119. https://doi.org/10.1016/j.procbio.2016.12.024

Denman, S.E., Martinez Fernandez, G., Shinkai, T., Mitsumori, M., McSweeney, C.S., 2015. Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.01087

Nelson, D., Cox, M., 2005. Lehninger Principles of Biochemistry, Fourth. ed. W. H. Freeman and Company, New York, p.538.

Horton, R., Moran, L., Ochs, R., Rawn, D., Scrimgeour, G., 1992. Principles of Biochemistry, Second. ed. Prentice Hall, p. 17:8.

Spirito, C.M., Richter, H., Rabaey, K., Stams, A.J., Angenent, L.T., 2014. Chain elongation in anaerobic reactor microbiomes to recover resources from waste. Current Opinion in Biotechnology 27, 115–122. https://doi.org/10.1016/j.copbio.2014.01.003

Zidwick, M.J., Chen, J.-S., Rogers, P., 2013. Organic Acid and Solvent Production, in: Dr, M.D.P., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes - Applied Bacteriology and Biotechnology. Springer New York, pp. 511–755.

Annex 3

Preliminary results of metagenomic approach

Introduction

The purpose of the experiment was twofold: (i) examine the maximum VFA concentration that can be obtained from acidogenic fermentation of ground forced chicory roots for a better control of the methanogenic stage of an existing two-stage anaerobic digestion plant in the Walloon Region fed with this substrate (Greenwatt, Belgium) (ii) obtain a holistic view of the evolution of microorganisms in the fermentation system and their response to the change of two basic operational parameters: the pH and the initial total concentration.

Materials and Methods

Three different pH values (i.e., 4.5, 5.5 and 7; adjusted daily) and three different initial total COD concentrations (i.e., 26.7, 53.3 and 80 g_{COD}/kg_{ML}; substrate/inoculum COD ratio: 3/1) have been tested for the fermentation of forced chicory roots (COD: 0.151 gCOD/GFM; TS: 12.8 %FM; VS: 92.7 %TS). The inoculum used was acidogenic slurry (COD: 0.057 gCOD/gFM; TS: 3.2 %FM; VS: 73.2 %TS) from the first stage of a pilot two-stage anaerobic digestion plant mixed with methanogenic effluent (COD: 0.003 gCOD/gFM; TS: 0.6 %FM; VS: 36.5 %TS) from the second stage of the same pilot plant (Greenwatt, Belgium). The mixing ratio was 3:1 on a fresh weight basis and the purpose of the mixing was to simulate actual recirculation of the methanogenic effluent in the acidogenic reactor in the existing installation.

Sampling of the mixed liquor took place at days 0, 1, 2, 3, 7, 10, 14. pH adjustment Biogas production and composition as well as soluble COD and VFA in the liquid phase of the centrifuged samples were measured the same way as described in previous chapters of the thesis. The solid phase after sample centrifugation was used for microbial analysis (i.e. DNA extraction, PCR amplification and Illumina MiSeq).

Microbial analysis was conducted for samples from the last day of every condition. Additionally, for condition 5.5/26.7 (i.e. highest conversion yield to tVFA) and for condition 7/80 (i.e. highest tVFA concentrations), microbial analysis of samples from d0, d3, d10 was also conducted. In all those analyses the extracted DNA from duplicate reactors was pooled together, therefore results represent average values. Exception to this was the last day of the two aforementioned conditions, where the two reactors were analysed separately to examine microbial reproducibility.

Results

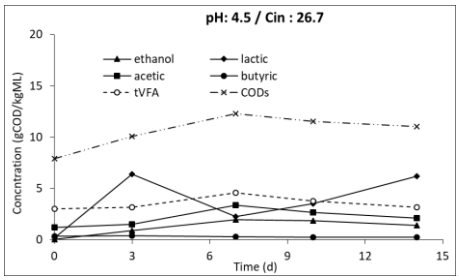
Highest tVFA concentrations and conversion yields are shown in Table A.3-1:

Table A.3-1: Highest tVFA concentrations and corresponding conversion yields during acidogenesis of forced chicory roots

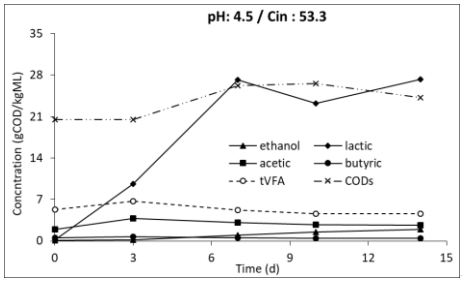
pH	C_{in} : 26.7 (g_{COD}/kg_{ML})		C_{in} : 53.3 (g_{COD}/kg_{ML})		C_{in} : 80 (g_{COD}/kg_{ML})	
	tVFA (g_{COD}/kg_{ML})	Yield (g_{COD}/g_{COD_t})	tVFA (g_{COD}/kg_{ML})	Yield (g_{COD}/g_{COD_t})	tVFA (g_{COD}/kg_{ML})	Yield (g_{COD}/g_{COD_t})
4.5	4.5	0.12	6.7	0.09	10.8	0.09
5.5	15.9	0.59	19.3	0.36	20.2	0.25
7	15.3	0.58	22.3	0.42	41.1	0.51

Coefficient of variance between duplicates for tVFA concentration ranged between 2 and 13%

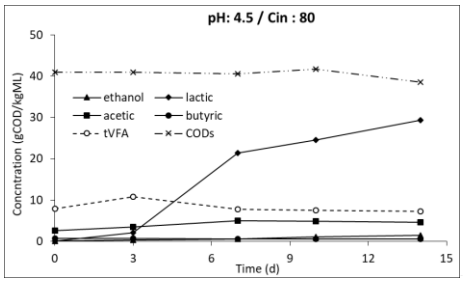
Figure A.3-1 to Figure A.3-5 present the results of the metabolic and microbial analysis of the 9 series examined (code "pH/Cin"). Only the 15 most abundant OTUs are presented in Figure A.3-1 to Figure A.3-5.



Phylum	Genus	Species	Abundance
Firmicutes	*Lactobacillus*	*Lactobacillus delbrueckii*	39.3%
Firmicutes	*Lactobacillus*	*Lactobacillus plantarum*	21.2%
Firmicutes	*Weissella*	*Weissella soli*	5.4%
Firmicutes	*Lactobacillus*	*Lactobacillus similis*	4.8%
Proteobacteria	*Acetobacter*	*Acetobacter syzygii*	4.7%
Firmicutes	*Lactobacillus*	*Lactobacillus bifermentans*	3.8%
Firmicutes	*Lactobacillus*	*Lactobacillus mucosae*	3.1%
Firmicutes	*Lactobacillus*	*Lactobacillus fermentum*	2.4%
Firmicutes	*Lactobacillus*	*Lactobacillus coryniformis*	2.1%
Firmicutes	*Lactobacillus*	*Lactobacillus casei*	1.4%
Firmicutes	*Lactobacillus*	*Lactobacillus buchneri*	1.1%
Firmicutes	*Leuconostoc*	*Leuconostoc sp*	1.0%
Cyanobacteria	*Oscillatoria*	*Oscillatoria sp*	1.0%
Firmicutes	*Pediococcus*	*Pediococcus acidilactici*	0.9%
Firmicutes	*Lactobacillus*	*Lactobacillus sp*	0.9%



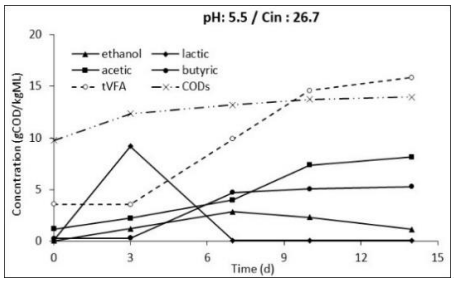
Phylum	Genus	Species	Abundance
Firmicutes	*Lactobacillus*	*Lactobacillus delbrueckii*	54.2%
Firmicutes	*Lactobacillus*	*Lactobacillus pontis*	7.1%
Firmicutes	*Pediococcus*	*Pediococcus acidilactici*	6.4%
Firmicutes	*Lactobacillus*	*Lactobacillus similis*	6.3%
Firmicutes	*Lactobacillus*	*Lactobacillus amylovorus*	5.2%
Firmicutes	*Lactobacillus*	*Lactobacillus plantarum*	3.5%
Proteobacteria	*Acetobacter*	*Acetobacter syzygii*	3.4%
Firmicutes	*Lactobacillus*	*Lactobacillus fermentum*	3.2%
Firmicutes	*Lactobacillus*	*Lactobacillus songhuajiangensis*	2.3%
Firmicutes	*Lactobacillus*	*Lactobacillus mucosae*	1.4%
Firmicutes	*Lactobacillus*	*Lactobacillus ghanensis*	1.1%
Firmicutes	*Lactobacillus*	*Lactobacillus buchneri*	0.8%
Firmicutes	*Lactobacillus*	*Lactobacillus reuteri*	0.7%
Firmicutes	*Lactobacillus*	*Lactobacillus harbinensis*	0.6%
Cyanobacteria	*Oscillatoria*	*Oscillatoria sp*	0.5%



Phylum	Genus	Species	Abundance
Firmicutes	*Lactobacillus*	*Lactobacillus amylovorus*	42.5%
Firmicutes	*Lactobacillus*	*Lactobacillus delbrueckii*	30.3%
Firmicutes	*Lactobacillus*	*Lactobacillus pontis*	7.8%
Firmicutes	*Lactobacillus*	*Lactobacillus similis*	7.6%
Cyanobacteria	*Oscillatoria*	*Oscillatoria sp*	3.4%
Firmicutes	*Pediococcus*	*Pediococcus acidilactici*	2.6%
Unclassified	*Unclassified*	*Unclassified*	1.3%
Firmicutes	*Lactobacillus*	*Lactobacillus crispatus*	0.7%
Firmicutes	*Bacillus*	*Bacillus sp*	0.6%
Firmicutes	*Lactobacillus*	*Lactobacillus mucosae*	0.5%
Firmicutes	*Lactobacillus*	*Lactobacillus buchneri*	0.4%
Firmicutes	*Lactobacillus*	*Lactobacillus kitasatonis*	0.3%
Cyanobacteria	*Oscillatoria*	*Oscillatoria sp*	0.1%
Proteobacteria	*Serratia*	*Serratia sp*	0.1%
Bacteroidetes	*Unclassified*	*Unclassified*	0.1%

Figure A.3-1: Metabolic profile of acidogenesis of chicory roots at pH 4.5 and varying initial concentrations (left side) and the fifteen most abundant corresponding OTUs (right side).

Microbial analysis only at d14. The 15 most abundant OTU correspond to 93.3%, 96.7% and 98.3% relative abundance in pH/Cin: 4.5/26.7, 4.5/53.3 and 4.5/80, respectively.



d0

Phylum	Genus	Species	Abundance
Cyanobacteria	*unclassified*	*Unclassified*	18.4%
Unclassified	*Unclassified*	*Unclassified*	11.0%
Proteobacteria	*Pseudomonas*	*Pseudomonas sp*	7.5%
Proteobacteria	*Pseudomonas*	*Pseudomonas sp*	6.7%
Proteobacteria	*Serratia*	*Serratia sp*	5.6%
Proteobacteria	*Enterobacter*	*Enterobacter sp*	3.9%
Firmicutes	*Weissella*	*Weissella soli*	2.8%
Proteobacteria	*Pseudomonas*	*Pseudomonas sp*	2.4%
Proteobacteria	*Pantoea*	*Pantoea sp*	1.8%
Proteobacteria	*Arcobacter*	*Arcobacter skirrowii*	1.6%
Unclassified	*Unclassified*	*Unclassified*	1.5%
Synergistetes	*Synergistes*	*Synergistes sp*	1.4%
Proteobacteria	*Acinetobacter*	*Acinetobacter sp*	1.3%
Proteobacteria	*Duganella*	*Duganella sp*	1.2%
Proteobacteria	*Duganella*	*Duganella sp*	1.1%

d3

Phylum	Genus	Species	Abundance
Firmicutes	*Lactobacillus*	*Lactobacillus plantarum*	20.5%
Firmicutes	*Lactobacillus*	*Lactobacillus coryniformis*	15.7%
Firmicutes	*Lactobacillus*	*Lactobacillus herbarum*	12.9%
Firmicutes	*Lactobacillus*	*Lactobacillus mucosae*	10.7%
Proteobacteria	*Acetobacter*	*Acetobacter fabarum*	10.4%
Firmicutes	*Lactobacillus*	*Lactobacillus songhuajiangensis*	7.7%
Firmicutes	*Weissella*	*Weissella soli*	4.5%
Firmicutes	*Lactobacillus*	*Lactobacillus vaccinostercus*	3.2%
Firmicutes	*Lactobacillus*	*Lactobacillus fermentum*	2.9%
Firmicutes	*Lactobacillus*	*Lactobacillus camelliae*	1.8%
Firmicutes	*Lactobacillus*	*Lactobacillus pontis*	1.0%
Proteobacteria	*Acetobacter*	*Acetobacter syzygii*	0.9%
Firmicutes	*Lactobacillus*	*Lactobacillus bifermentans*	0.7%
Firmicutes	*Lactobacillus*	*Lactobacillus buchneri*	0.6%
Cyanobacteria	*Oscillatoria*	*Oscillatoria sp*	0.6%

d10

Phylum	Genus	Species	Abundance
Firmicutes	*Unclassified*	*Unclassified*	26.1%
Firmicutes	*Eubacterium*	*Eubacterium eligens*	17.0%
Firmicutes	*Eubacterium*	*Eubacterium eligens*	16.8%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	9.5%
Firmicutes	*Clostridium*	*Clostridium sp*	7.7%
Unclassified	*Unclassified*	*Unclassified*	2.1%
Firmicutes	*Clostridium*	*Clostridium luticellarii*	2.1%
Unclassified	*Unclassified*	*Unclassified*	1.9%
Proteobacteria	*Acetobacter*	*Acetobacter fabarum*	1.7%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	1.1%
Proteobacteria	*Acetobacter*	*Acetobacter syzygii*	1.0%
Firmicutes	*Unclassified*	*Unclassified*	1.0%
Firmicutes	*Clostridium*	*Clostridium tyrobutyricum*	0.9%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	0.6%
Firmicutes	*Unclassified*	*Unclassified*	0.5%

d14_R1

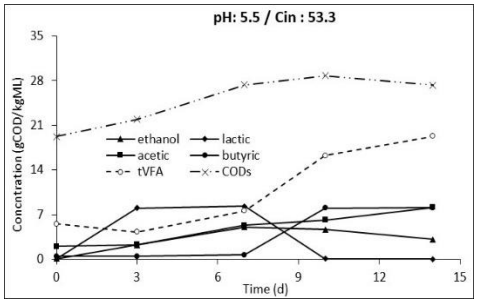
Phylum	Genus	Species	Abundance
Firmicutes	*Unclassified*	*Unclassified*	20.3%
Firmicutes	*Eubacterium*	*Eubacterium eligens*	18.8%
Firmicutes	*Eubacterium*	*Eubacterium eligens*	13.4%
Proteobacteria	*Acetobacter*	*Acetobacter fabarum*	7.1%
Firmicutes	*Clostridium*	*Clostridium sp*	7.0%
Firmicutes	*Clostridium*	*Clostridium sp*	5.7%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	4.7%
Unclassified	*Unclassified*	*Unclassified*	4.4%
Firmicutes	*Rummeliibacillus*	*Rummeliibacillus suwonensis*	3.0%
Firmicutes	*Unclassified*	*Unclassified*	1.8%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	1.3%
Firmicutes	*Clostridium*	*Clostridium luticellarii*	1.3%
Proteobacteria	*Acetobacter*	*Acetobacter syzygii*	1.3%
Unclassified	*Unclassified*	*Unclassified*	1.1%
Firmicutes	*Lactobacillus*	*Lactobacillus bifermentans*	1.0%

d14_R2

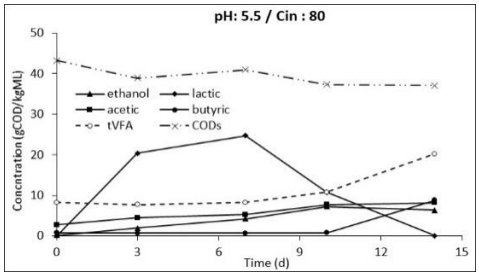
Phylum	Genus	Species	Abundance
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	16.3%
Firmicutes	*Eubacterium*	*Eubacterium eligens*	15.0%
Firmicutes	*Unclassified*	*Unclassified*	12.5%
Firmicutes	*Clostridium*	*Clostridium sp*	7.8%
Firmicutes	*Clostridium*	*Clostridium sp*	5.3%
Unclassified	*Unclassified*	*Unclassified*	5.2%
Unclassified	*Unclassified*	*Unclassified*	3.7%
Proteobacteria	*Acetobacter*	*Acetobacter syzygii*	3.3%
Firmicutes	*Unclassified*	*Unclassified*	3.1%
Firmicutes	*Lactobacillus*	*Lactobacillus bifermentans*	2.7%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	2.6%
Firmicutes	*Eubacterium*	*Eubacterium eligens*	2.5%
Unclassified	*Unclassified*	*Unclassified*	1.6%
Firmicutes	*Clostridium*	*Clostridium tyrobutyricum*	1.6%
Firmicutes	*Clostridium*	*Clostridium sp*	1.4%

Figure A.3-2: Metabolic profile of acidogenesis of chicory roots at condition 5.5/53.3 (upper left corner) and the fifteen most abundant OTUs for days 0, 3, 10 and 14.

Duplicate reactors at d14 were analysed separately. The 15 most abundant OTU correspond to 68,4%, 94.0%, 89.9%, 92.6% and 84.4% relative abundance at d0, d3, d10, d14_R1 and d14_R2, respectively.



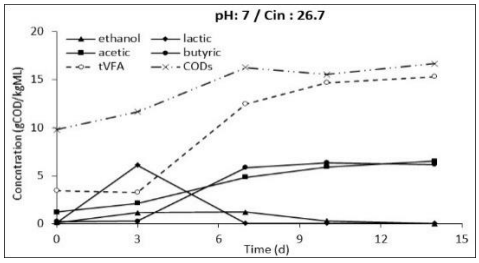
Phylum	Genus	Species	Abundance
Firmicutes	*Unclassified*	*Unclassified*	23.1%
Firmicutes	*Eubacterium*	*Eubacterium eligens*	20.3%
Unclassified	*Unclassified*	*Unclassified*	10.6%
Firmicutes	*Unclassified*	*Unclassified*	10.1%
Firmicutes	*Eubacterium*	*Eubacterium eligens*	5.9%
Firmicutes	*Clostridium*	*Clostridium sp*	3.2%
Firmicutes	*Clostridium*	*Clostridium sp*	3.0%
Firmicutes	*Clostridium*	*Clostridium luticellarii*	2.7%
Firmicutes	*Clostridium*	*Clostridium tyrobutyricum*	2.6%
Firmicutes	*Lactobacillus*	*Lactobacillus mucosae*	1.8%
Unclassified	*Unclassified*	*Unclassified*	1.6%
Firmicutes	*Lactobacillus*	*Lactobacillus bifermentans*	1.6%
Firmicutes	*Lactobacillus*	*Lactobacillus delbrueckii*	1.5%
Proteobacteria	*Acetobacter*	*Acetobacter fabarum*	1.2%
Actinobacteria	*Neoscardovia*	*Neoscardovia arbecensis*	1.0%



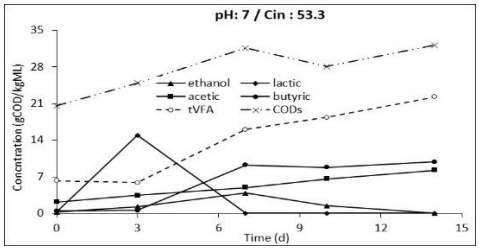
Phylum	Genus	Species	Abundance
Unclassified	*Unclassified*	*Unclassified*	25.2%
Firmicutes	*Clostridium*	*Clostridium sp*	22.0%
Firmicutes	*Eubacterium*	*Eubacterium eligens*	11.2%
Firmicutes	*Rummeliibacillus*	*Rummeliibacillus suwonensis*	8.2%
Firmicutes	*Clostridium*	*Clostridium sp*	3.4%
Firmicutes	*Clostridium*	*Clostridium sp*	3.2%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	2.8%
Firmicutes	*Clostridium*	*Clostridium tyrobutyricum*	2.8%
Firmicutes	*Clostridium*	*Clostridium luticellarii*	2.8%
Firmicutes	*Lactobacillus*	*Lactobacillus bifermentans*	1.5%
Firmicutes	*Lactobacillus*	*Lactobacillus coryniformis*	1.3%
Firmicutes	*Lactobacillus*	*Lactobacillus similis*	1.3%
Firmicutes	*Lactobacillus*	*Lactobacillus mucosae*	1.2%
Firmicutes	*Lactobacillus*	*Lactobacillus delbrueckii*	1.1%
Firmicutes	*Lactobacillus*	*Lactobacillus mucosae*	1.1%

Figure A.3-3: Metabolic profile of acidogenesis of chicory roots for conditions 5.5/53.3 (left, top) and 5.5/80 (left, bottom) and the fifteen most abundant corresponding OTUs (right side).

Microbial analysis has been conducted only at d14 for these conditions. The 15 most abundant OTU correspond to 90.0%, and 89.2% relative abundance in the conditions 5.5/53.3 and 5.5/80, respectively.



Phylum	Genus	Species	Abundance
Unclassified	*Unclassified*	*Unclassified*	48.8%
Unclassified	*Unclassified*	*Unclassified*	14.2%
Firmicutes	*Oscillibacter*	*Oscillibacter ruminantium*	4.1%
Firmicutes	*Caloramator*	*Caloramator proteoclasticus*	1.8%
Firmicutes	*Unclassified*	*Unclassified*	1.2%
Firmicutes	*Erysipelatoclostridium*	*Erysipelatoclostridium ramosum*	1.1%
Firmicutes	*Ruminiclostridium*	*Ruminiclostridium thermocellum*	0.9%
Firmicutes	*Unclassified*	*Unclassified*	0.9%
Firmicutes	*Clostridium*	*Clostridium sp*	0.9%
Firmicutes	*Sedimentibacter*	*Sedimentibacter sp*	0.9%
Firmicutes	*Unclassified*	*Unclassified*	0.8%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	0.8%
Unclassified	*Unclassified*	*Unclassified*	0.7%
Firmicutes	*Unclassified*	*Unclassified*	0.6%
Firmicutes	*Unclassified*	*Unclassified*	0.6%

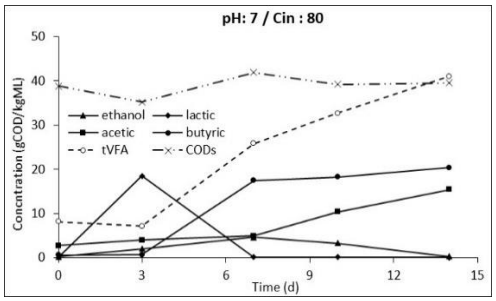


Phylum	Genus	Species	Abundance
Firmicutes	*Unclassified*	*Unclassified*	25.3%
Firmicutes	*Unclassified*	*Unclassified*	20.2%
Firmicutes	*Clostridium*	*Clostridium sp*	9.5%
Firmicutes	*Unclassified*	*Unclassified*	4.0%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	3.7%
Firmicutes	*Oscillibacter*	*Oscillibacter ruminantium*	3.3%
Firmicutes	*Clostridium*	*Clostridium sp*	2.5%
Firmicutes	*Clostridium*	*Clostridium sp*	2.2%
Firmicutes	*Lachnoclostridium*	*Lachnoclostridium phytofermentans*	1.8%
Firmicutes	*Clostridium*	*Clostridium sp*	1.7%
Firmicutes	*Clostridium*	*Clostridium kluyveri*	1.7%
Firmicutes	*Unclassified*	*Unclassified*	1.4%
Unclassified	*Unclassified*	*Unclassified*	1.3%
Firmicutes	*Lachnoclostridium*	*Clostridium celerecrescens*	1.2%
Firmicutes	*Lachnoclostridium*	*Clostridium aminovalericum*	1.1%

Figure A.3-4: Metabolic profile of acidogenesis of chicory roots for conditions 7/26.7 (left, top) and 7/53.3 (left, bottom) and the fifteen most abundant corresponding OTUs (right side).

Microbial analysis has been conducted only at d14 for these conditions. The 15 most abundant OTU correspond to 78.3%, and 81.1% relative abundance in the conditions 7/26.7 and 7/53.3,

respectively



d0

Phylum	Genus	Species	Abudance
Firmicutes	*Weissella*	*Weissella soli*	24.5%
Proteobacteria	*Serratia*	*Serratia sp*	15.3%
Proteobacteria	*Enterobacter*	*Enterobacter sp*	13.4%
Cyanobacteria	*Oscillatoria*	*Oscillatoria sp*	9.2%
Proteobacteria	*Pseudomonas*	*Pseudomonas sp*	6.8%
Unclassified	*Unclassified*	*Unclassified*	5.2%
Proteobacteria	*Pantoea*	*Pantoea sp*	5.0%
Proteobacteria	*Pseudomonas*	*Pseudomonas sp*	3.6%
Proteobacteria	*Pseudomonas*	*Pseudomonas sp*	2.3%
Proteobacteria	*Acinetobacter*	*Acinetobacter sp*	1.4%
Proteobacteria	*Stenotrophomonas*	*Stenotrophomonas sp*	1.0%
Proteobacteria	*Acinetobacter*	*Acinetobacter sp*	0.9%
Proteobacteria	*Pectobacterium*	*Pectobacterium carotovorum*	0.8%
Proteobacteria	*Unclassified*	*Unclassified*	0.7%
Proteobacteria	*Pseudomonas*	*Pseudomonas sp*	0.6%

d3

Phylum	Genus	Species	Abundance
Firmicutes	*Lactobacillus*	*Lactobacillus plantarum*	47.9%
Firmicutes	*Lactobacillus*	*Lactobacillus songhuajiangensis*	24.3%
Firmicutes	*Lactobacillus*	*Lactobacillus fermentum*	11.0%
Firmicutes	*Lactobacillus*	*Lactobacillus delbrueckii*	5.1%
Firmicutes	*Weissella*	*Weissella soli*	3.7%
Firmicutes	*Lactobacillus*	*Lactobacillus coryniformis*	2.1%
Firmicutes	*Lactobacillus*	*Lactobacillus sp*	1.3%
Firmicutes	*Lactobacillus*	*Lactobacillus mucosae*	0.9%
Firmicutes	*Lactobacillus*	*Lactobacillus similis*	0.7%
Firmicutes	*Pediococcus*	*Pediococcus acidilactici*	0.3%
Firmicutes	*Leuconostoc*	*Leuconostoc sp*	0.3%
Firmicutes	*Leuconostoc*	*Leuconostoc pseudomesenteroides*	0.3%
Firmicutes	*Lactobacillus*	*Lactobacillus paracasei*	0.3%
Firmicutes	*Lactobacillus*	*Lactobacillus vaccinostercus*	0.2%
Firmicutes	*Lactobacillus*	*Lactobacillus herbarum*	0.2%

d10

Phylum	Genus	Species	Abundance
Firmicutes	*Lachnoclostridium*	*Clostridium aminovalericum*	11.7%
Firmicutes	*Clostridium*	*Clostridium sp*	8.9%
Firmicutes	*Clostridium*	*Clostridium sp*	8.2%
Firmicutes	*Clostridium*	*Clostridium sp*	5.9%
Firmicutes	*Clostridium*	*Clostridium sp*	5.2%
Firmicutes	*Lachnoclostridium*	*Clostridium celerecrescens*	4.2%
Firmicutes	*Clostridium*	*Clostridium sp*	4.2%
Firmicutes	*Robinsoniella*	*Robinsoniella peoriensis*	3.4%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	3.0%
Firmicutes	*Unclassified*	*Unclassified*	2.9%
Firmicutes	*Clostridium*	*Clostridium sp*	2.6%
Firmicutes	*Clostridium*	*Clostridium sp*	2.5%
Unclassified	*Unclassified*	*Unclassified*	2.4%
Firmicutes	*Bacillus*	*Bacillus sp*	2.2%
Firmicutes	*Bacillus*	*Bacillus sp*	2.2%

d14_R1

Phylum	Genus	Species	Abundance
Firmicutes	*Unclassified*	*Unclassified*	58.4%
Firmicutes	*Clostridium*	*Clostridium sp*	5.3%
Unclassified	*Unclassified*	*Unclassified*	3.6%
Firmicutes	*Ruminococcus*	*Ruminococcus champanellensis*	2.7%
Firmicutes	*Alkalibacterium*	*Alkalibacterium sp*	2.4%
Firmicutes	*Clostridium*	*Clostridium sp*	2.1%
Firmicutes	*Anaerosalibacter*	*Anaerosalibacter bizertensis*	2.0%
Firmicutes	*Clostridium*	*Clostridium sp*	1.8%
Firmicutes	*Clostridium*	*Clostridium sp*	1.7%
Firmicutes	*Lachnoclostridium*	*Clostridium asparagiforme*	1.7%
Firmicutes	*Unclassified*	*Unclassified*	1.6%
Firmicutes	*Clostridium*	*Clostridium sp*	0.9%
Firmicutes	*Lachnoclostridium*	*Clostridium aminovalericum*	0.9%
Firmicutes	*Unclassified*	*Unclassified*	0.8%
Firmicutes	*Unclassified*	*Unclassified*	0.7%

d14_R2

Phylum	Genus	Species	Abundance
Firmicutes	*Unclassified*	*Unclassified*	46.2%
Unclassified	*Unclassified*	*Unclassified*	10.4%
Firmicutes	*Clostridium*	*Clostridium sp*	8.2%
Firmicutes	*Ruminococcus*	*Ruminococcus champanellensis*	4.6%
Firmicutes	*Clostridium*	*Clostridium sp*	3.0%
Firmicutes	*Alkalibacterium*	*Alkalibacterium sp*	2.8%
Firmicutes	*Proteiniborus*	*Proteiniborus sp*	2.2%
Firmicutes	*Lachnoclostridium*	*Clostridium aminovalericum*	2.0%
Firmicutes	*Clostridium*	*Clostridium sp*	1.9%
Firmicutes	*Unclassified*	*Unclassified*	1.2%
Firmicutes	*Clostridium*	*Clostridium sp*	1.2%
Firmicutes	*Clostridium*	*Clostridium kluyveri*	1.1%
Firmicutes	*Clostridium*	*Clostridium sp*	1.0%
Firmicutes	*Anaerosporobacter*	*Anaerosporobacter mobilis*	0.9%
Firmicutes	*Terrisporobacter*	*Terrisporobacter glycolicus*	0.8%

Figure A.3-5: Metabolic profile of acidogenesis of chicory roots at condition 7/80 (upper left corner) and the fifteen most abundant OTUs for days 0, 3, 10 and 14. *Duplicate reactors at d14 were analysed separately. The 15 most abundant OTU correspond to 90.1%, 98.4%, 69.4%, 86.5% and 87.6% relative abundance at d0, d3, d10, d14_R1 and d14_R2, respectively.*

Discussion

Based on the results above, the following paragraphs present indicative examples, where links between microbial species and metabolic performance can be derived, at different taxonomic levels:

Condition 4.5/80**:** At d14, the following four *Lactobacillus* species represented about 88.2% of all OTU reads of the sample (i.e. *L. amylovorus* with 42.5%, *L. delbrueckii* with 30.3%, *L. pontis* with 7.8% and *L. similis* with

7.6%). We can therefore confidently link the marked lactic acid production to those species.

Condition 7/80**:** The evolution of the microbial dynamics during fermentation is shown in Figure A.3-6 at order and family level, in conjunction with the metabolic profile. While at the beginning of fermentation there was a higher diversity of orders present in the medium, this status gives place to an almost complete dominance of the *Lactobacillales* order (and *Lactobacillaceae* family), coinciding with a marked production of lactic acid. After day 7, this dominance is switched over to the *Clostridiales* order with a simultaneous production of acetic and butyric acids as the predominant soluble metabolites (see also CODs line in Figure A.3-5; upper left corner) lactic acid. The majority of the *Unclassified* and *Unknown* members at family level at days 10 and 14 belong to the *Clostridiales* order.

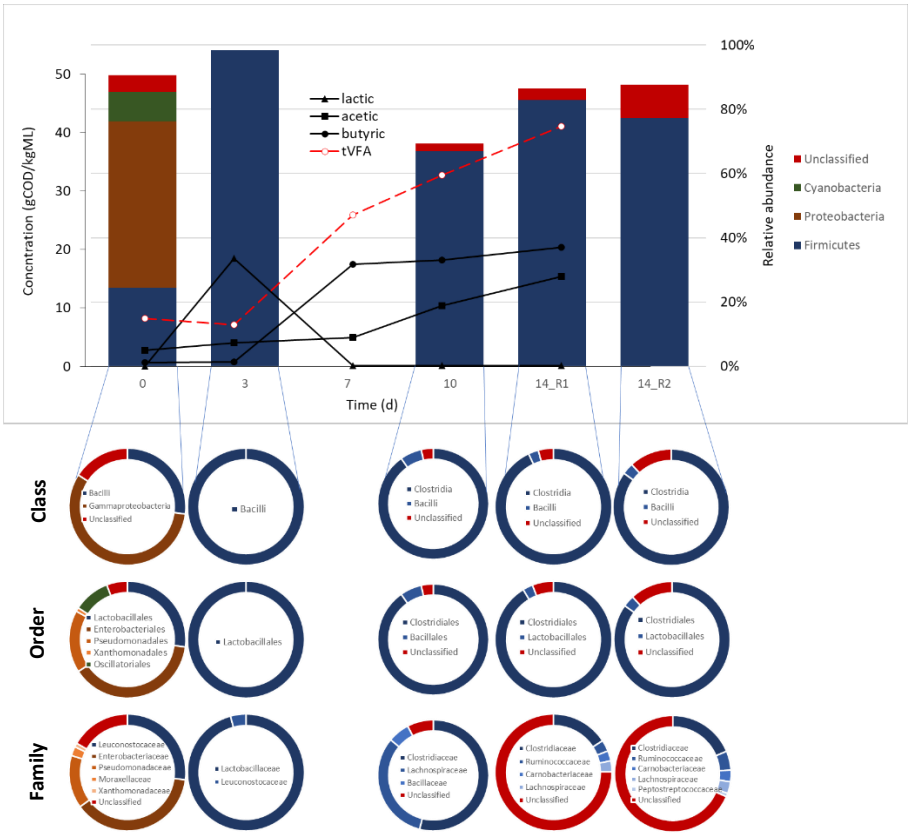


Figure A.3-6: Evolution of relative abundance of microbial communities at the phylum (secondary axis at the right) and other taxonomic levels (bottom) in conjunction with the metabolic profile of acidogenic fermentation of chicory roots

pH: 7, initial concentration: 80 gCOD/kgML; The plots of lactic, acetic, butyric and tVFA correspond to the left y axis; The columns of cumulated OTU abundance correspond to the right y axis; Only the 15 most abundant OTU are shown; They correspond to 90.1%, 98.4%, 69.4%, 86.5% and 87.6% of the total reads at d0, d3, d10, d14_R1 and d14_R2, respectively;

Condition 7/80: A microbial analysis of each reactor at day 14 reveals that out of the first 15 OTU, 9 of them are common, corresponding to a cumulated 78% and 80% of the total relative abundance of the two reactors, respectively (Figure A.3-7). Interestingly, an unclassified member of the *Clostridiales* order is the most dominant microorganism and most probably responsible for the high tVFA concentration observed at d14.

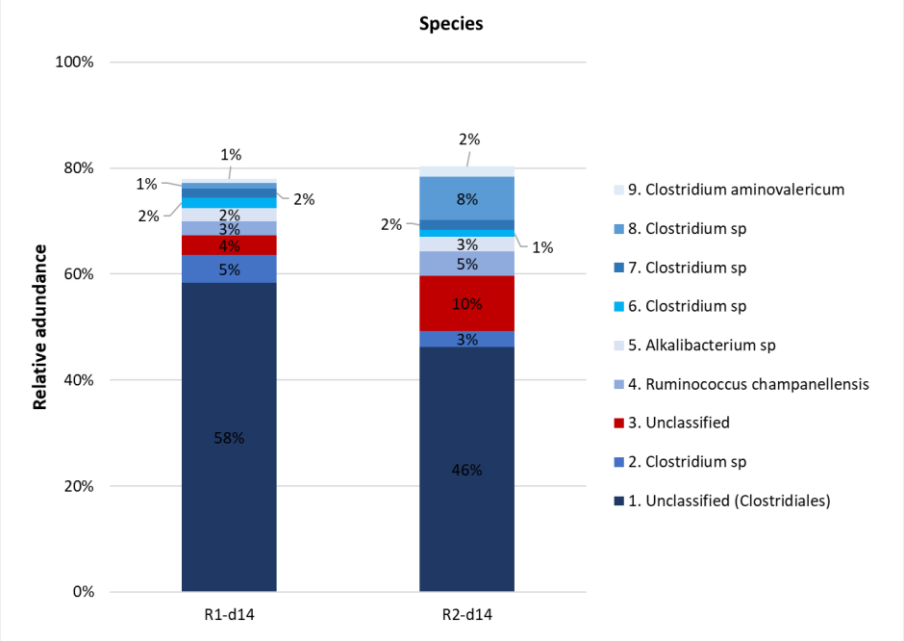


Figure A.3-7: Common microbial species between duplicates at d14 of the acidogenic fermentation of chicory roots

It is noteworthy that in many of the conditions were tVFA were the predominant metabolic products (indicatively conditions 5.5/53.5 at d14, 7/26.7 at d14, 7/53.3 at d14, etc.), the most abundant OTU corresponded to unclassified species, thus confirming the discussion of section 1.1.7 on the major role of unknown/unclassified/uncultured species in understanding the microbial complexity of the fermentation system.

Annex 4

Preliminary results of semi-continuous experiments

Introduction

This thesis focused almost exclusively on experimentation at batch conditions, given that the complexity of the fermentation system is not yet fully mastered. Nevertheless, the purpose of every bioconversion process should be its scaling-up from laboratory to industrial scale for real-life applications. To do so, continuous operation should be investigated to evaluate the challenges of maintaining high-performing acidogenic fermentation at steady state in a reproducible way.

At the final stage of this thesis, we dedicated limited resources to evaluate acidogenic fermentation at semi-continuous operation. We chose trub as substrate, given the interesting results it showed at batch conditions (Chapters 2 and 6). The purpose of the experiment is to evaluate the appropriate Hydraulic Retention (HRT) to maximise tVFA concentration.

Materials and Methods

Semi-continuous operation was achieved by withdrawing a fixed amount of mixed liquor from the reactor and feeding back the reactor with the same amount of fresh trub. Experiments took place in 1 l glass reactors in duplicates, in a thermostatic room at 35 °C. The experimental conditions imposed are shown in Table A.4-1. Soluble and gaseous metabolites were analysed the same way as previous experiments presented in this thesis.

Table A.4-1: Identity of semi-continuous mixed acidogenic fermentation experiments of trub

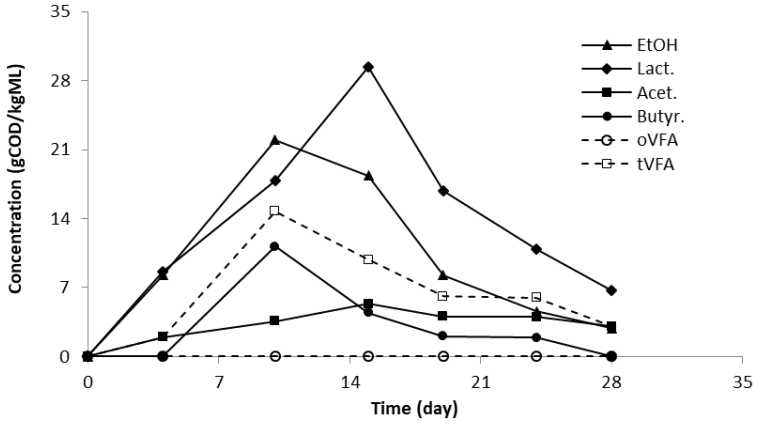
	Unit	**Experiment HRT4**	**Experiment HRT7**
Start-up			
Substrate: trub	g_{COD}/g_{FM}	0.213	0.221
Inoculum: pretreated activated sludge (AS)	g_{COD}/g_{FM}	0.019	0.021
Working volume	ml	500	500
Substrate/Inoculum	g_{COD_trub}/g_{COD_AS}	4	4
Acclimatisation	days	4	6
Duration	days	32	28
Follow-up			
Hydraulic retention time (HRT)	days	4	7
Organic loading rate (OLR)	$g_{COD}/(l*d)$	53.3	31.6

Feeding frequency	times/day	1	1
pH		5.0-5.5	5.0-5.5

pH was adjusted at the desired value whenever necessary with the addition of HCl and NaOH 2M. Gaseous and soluble metabolites were analysed with the same methods as described in previous chapters of the thesis.

Results

Figure A.4-1 and Figure A.4-2 present the evolution of metabolites as function of time for the two replicate reactors at HRT 4 and 7 days, respectively. The results show that it was not possible to maintain a well performing acidogenic fermentation). Although during the first approximately 10 days tVFA concentration was increasing, we observe a steep decrease during the remaining period. Final tVFA concentrations (average of two reactors) were 7.5 and 2.7 g_{COD_tVFA}/l for experiments HRT4 and HRT7, respectively. This corresponds to substrate conversion yields of 0.03 and 0.01 $g_{COD\_tVFA}/g_{COD\_trub}$, respectively. Maximum tVFA concentrations observed were 24.9 and 13.9 g_{COD_tVFA}/l, respectively. Furthermore, at the end of experiment the soluble COD (CODs) values were between 145 and 172 g_{CODs}/l for all four reactors of both experiments. This value includes sugars (i.e. fructose, maltose, glucose, saccharose, maltotriose).



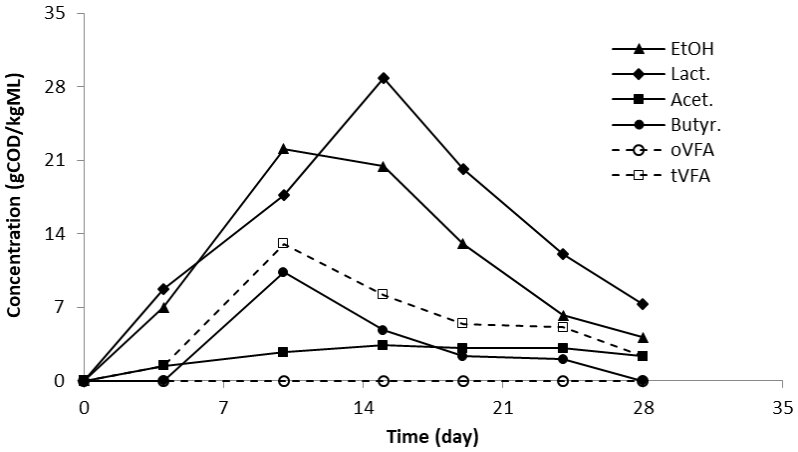
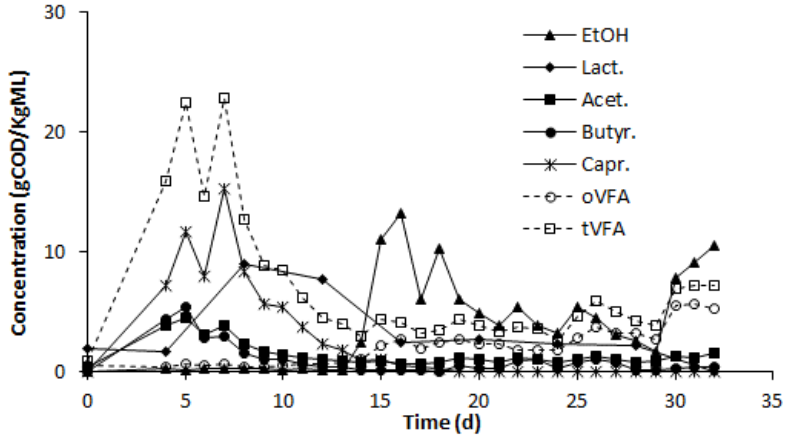


Figure A.4-1: Metabolic profile of semi-continuous mixed acidogenic fermentation of trub (HRT: 4d)

Top: reactor 1; bottom: reactor 2; oVFA: other VFA (propionic, iso-butyric, iso-valeric, valeric and caproic acids)



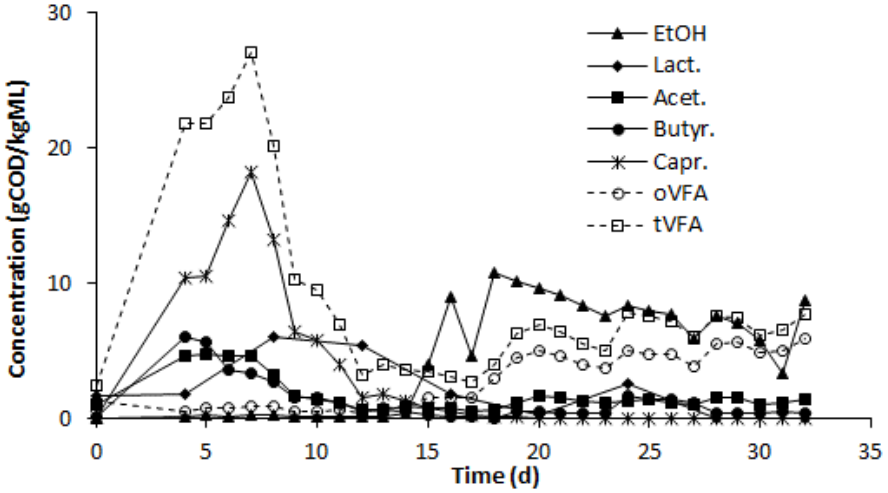


Figure A.4-2: Metabolic profile of semi-continuous mixed acidogenic fermentation of trub (HRT: 7d)
Top: reactor 1; bottom: reactor 2; oVFA: other VFA (propionic, iso-butyric, iso-valeric and valeric acids)

Discussion

The large difference between CODs and measured metabolites, is an indication that the substrate is not being properly converted through either of the metabolic pathways of relevance for this thesis (i.e. acidogenic, ethanolic, lactic). Further experimentation is necessary to determine the reasons for this low performance (e.g. lower HRT, better pH management, reactor configuration and mixing, etc.). Also, analysis of microbial communities would be very valuable to examine the evolution and dynamics of the reactor microbiota, especially during marked changes in the metabolic profiles (e.g. sudden decrease of tVFA concentration).