Faculteit Bio-ingenieurswetenschappen

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pH Hysteresis & electrolysis for zero-chemical input control of fermentation processes

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Promotor: Prof. dr. ir. Korneel Rabaey

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Masterproef voorgedragen tot het behalen van de graad van
Master in de bio-ingenieurswetenschappen: Milieutechnologie

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Abstract

The biorefinery concept attempts to alleviate fossil fuel dependency by sustainable bio-production of fuels and chemicals. The bio-ethanol process is well established but a large portion of incoming organics remains unconverted. Part of this rest fraction can be fermented to volatile fatty acids, valuable as industrial precursor chemicals. However, large amounts of chemicals are needed for fermentation management and the obtained volatile fatty acids are difficult to extract and recover. This study demonstrates the conversion of biorefinery thin stillage – the bottoms or waste fraction of a bio-ethanol distillation column – into short chain carboxylates using zero chemical input techniques. A stream characterisation of thin stillage was performed to gather knowledge on this stream. Membrane Electrolysis (ME) – an electrochemical membrane-based extraction technique – was applied to investigate the possibility of extraction of the target products. ME produces H_2 and OH^- in the fermentation broth. The first of these was shown to influence the obtained volatile fatty acids, resulting in a higher-value product output, while the second could be used for pH-management with zero-chemical input. Methanogenic activity, converting volatile fatty acids to methane, is unwanted in this application considering the goal of chemicals production. The influence of pH changes on methanogenesis was examined using a zero-chemical input system by decreasing pH with acidogenesis and increasing pH with electrolytic extraction. Further experiments studied the long-term influence of pH changes on the fermenting community. This study demonstrates the potential of biorefinery thin stillage as a substrate for production of volatile fatty acids as well as the potential of ME for product recovery and pH-control in a fermenter.

Nederlandse samenvatting

De bio-raffinaderij poogt door duurzame, biologische productie van brandstoffen en chemicaliën de afhankelijkheid van fossiele bronnen te verlichten. Het bio-ethanol proces is al vaak toegepast, maar zet een groot deel van het organisch materiaal niet om naar het eindproduct ethanol. Een deel van de resterende organische fractie kan gefermenteerd worden tot vluchtige vetzuren, waardevol als bouwstenen voor de chemische industrie. Deze fermentatie vereist echter grote hoeveelheden chemicaliën voor controle van het proces en extractie en herwinning van vluchtige vetzuren is duur. Deze studie demonstreert de omzetting van 'thin stillage' – de restfractie uit een bio-ethanol distillatiekolom – naar vluchtige vetzuren met technieken die geen chemicaliëngebruik vereisen. Om kennis te vergaren over thin stillage werd de stroom gekarakteriseerd. Een elektrochemische membraangebaseerde extractietechnologie, membraanelektrolyse (ME), werd onderzocht op de mogelijkheid voor extractie van de bekomen producten. ME produceert H_2 en OH^- in het fermentorcompartiment. Er werd aangetoond dat H_2 een invloed heeft op de bekomen vluchtige vetzuren, wat een meer waardevolle mix van vluchtige vetzuren opleverde. De geproduceerde OH^- kon gebruikt worden om pH te controleren zonder chemicaliëninput. De conversie van vluchtige vetzuren naar methaan door methanogene activiteit is ongewenst als chemische bouwstenen beoogd worden. De invloed van pH-veranderingen op deze methanogene activiteit werd bestudeerd gebruikmakend van een systeem zonder input van chemicaliën door de pH te laten zakken m.b.v. acidogenese terwijl stijgingen bekomen werden door elektrolytische extractie. De langetermijninvloed van dergelijke pH-veranderingen op fermenterende gemeenschap werd ook bestudeerd. Deze studie demonstreert het potentieel van thin stillage uit een bioraffinaderij als substraat voor de productie van vluchtige vetzuren evenals het potentieel van ME voor herwinning van de doelproducten en pH-controle in de fermentor.

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List of acronyms

Ac	Acetic Acid/Acetate
AD	Anaerobic Digestion
AEM	Anion Exchange Membrane
But	Butyric Acid/Butyrate
Cap	Caproic Acid/Caproate
CE	Coulombic Efficiency
COD	Chemical Oxygen Demand
CSTR	Continuously Stirred Tank Reactor
DDG	Distillers' Dried Grains
DDGS	Distillers' Dried Grains with Solubles
dsDNA	Double-stranded DNA
EC	Electrical Conductivity
EF	Electro-Fermentation
EtOH	Ethanol
Hep	Heptanoic Acid/Heptanoate
HRT	Hydraulic Retention Time
iBut	Isobutyric Acid/Isobutyrate
iCap	Isocaproic Acid/Isocaproate
iVal	Isovaleric Acid/Isovalerate
LCA	Life Cycle Analysis
LCFA	Long Chain Fatty Acids
MBR	Membrane Bioreactor
ME	Membrane Electrolysis
NREL	National Renewable Energy Laboratory
Oct	Octanoic Acid/Octanoate
OLR	Organic Loading Rate
PPP	Pentose Phosphate Pathway
PKP	Phosphoketolase Pathway
Pr	Propionic Acid/Propionate
PV	Photovoltaic
sCOD	Soluble COD
SRT	Sludge Retention Time
tCOD	Total COD
TKN	Total Kjeldahl Nitrogen
TS	Total Solids
TSS	Total Suspended Solids
Val	Valeric Acid
VFA	Volatile Fatty Acids
VS	Volatile Solids
VSS	Volatile Suspended Solids

1. Introduction

1.1. Biorefineries

The biorefinery concept tries to implement a shift from fossil sources towards sustainable sources (i.e. biomass) for production of chemicals to substitute conventional petrochemical refineries (Ghatak, 2011). Not only chemicals, but also biofuels and bioenergy can be produced in a biorefinery. As the American National Renewable Energy Laboratory (NREL) defines the concept: "A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass. The biorefinery concept is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum" (NREL, 2009).

There are multiple reasons for implementing this concept. A first motivation is the reduction of the environmental impact of human activity by moving toward a carbon neutral society (Ghatak, 2011). At the same time, renewable sources of fuels and materials will aid in reducing the dependency on finite fossil fuel reserves (Fernando et al., 2006; Kamm & Kamm, 2004; Sanders et al., 2007; Ghatak, 2011).

The type of feedstock used in a biorefinery is a key factor in how the refinery will be designed and operated. In this way, two generations of feedstocks can be defined. The first generation consists of dedicated crops, such as corn, sorghum, soy, etc. From these crops, a wide array of products can be obtained. Vegetable oils (e.g. rapeseed oil, soybean oil, etc.) can be converted to biodiesel through transesterification (Naik et al., 2010). Carbohydrate sources (e.g. sugar cane, corn) can be converted to ethanol (EtOH) through fermentation, conventionally using yeast cultures such as *Saccharomyces cerevisiae* (Wilkie et al., 2000). Through Anaerobic Digestion (AD), first generation feedstocks can also be converted to biogas or biomethane (Naik et al., 2010). Both the EtOH-production process and AD will be discussed in greater detail, in Section 1.2 and 1.3 respectively. Waste streams from these processes can also be used to produce biomaterials. For instance, EtOH production from corn allows recovery of corn fiber oil, corn fiber gum and zein, all of which have commercial value and specific applications. A key advantage of using first generation feedstocks is its ease of use for fermentation, as pretreatment steps are limited. The downside of these convenient feedstocks is the competition with food production. Production of biofuels requires either food being directly used as a feedstock or valuable land area, water and energy being used to produce non-food crops for the same purpose, implying an indirect loss of food (Ghatak, 2011).

Second generation - or lignocellulosic - feedstocks consist mostly of agricultural residues and woody biomass. The simplest path of valorising this type of raw material is heat energy generation through combustion of wood or residues, however this releases carbon back into the atmosphere. Large amounts of carbon, in the form of lignin and polysaccharides (i.e. hemicellulose and cellulose), are stored in these feedstocks. The carbon complexes embedded in these feedstocks could be better utilized when converted into complex organic molecules, as opposed to CO_2 (via oxidation). Production of materials and biofuels from these sources could then be very promising. Woody biomass has already been used for centuries to produce paper, but other materials and fuels could be produced from the lignin, hemicellulose and cellulose in the wood. Lignin is the crucial

component in unlocking the potential of lignocellulosic raw materials. Since lignin blocks the access to the cellulose for chemicals and micro-organisms, there are several technical barriers that need to be overcome before agricultural residues and wood can be used to produce fuels and materials. For instance, the production of EtOH from lignocellulosic biomass requires a physical pretreatment (e.g. steam or ammonia treatment) to remove the lignin from the cellulose. Next, enzymatic hydrolysis of the (hemi)cellulose is necessary to obtain the sugar building blocks of these polysaccharides, i.e. the sugar monomers. These monosaccharides can then be fermented to EtOH by organisms such as *S. cerevisiae* (Naik et al., 2010).

'Platforms' can also be used to make a classification of biorefineries, distinguishing between platforms on the basis of their target products. Two well-established platforms are the sugar and the syngas platform. In the sugar platform, biomass is broken down into five- and six-carbon sugars which are then converted into fuels and chemicals, such as EtOH, by fermentation. The syngas platform consists of a thermochemical process in which biomass is converted to syngas; this is a mix of CO, CO_2 and H_2. This syngas can then be converted into energy or fuels by means of catalysers. A third platform, the carboxylate platform, has been emerging in recent years. This platform uses anaerobic fermentation to produce carboxylic acids, which can be used as building blocks for other chemicals and organics. This will be discussed at length in Section 1.4 (Agler et al., 2011).

Some examples of biorefineries can already be found worldwide. ALCO BioFuel in Ghent, Belgium produces EtOH and other byproducts from corn, a process which will be discussed in detail in Section 1.2. The MixAlco-process, developed by Terrabon in Texas, USA, produces alcohols from biomass such as wood, municipal solid waste or sorghum (Holtzapple & Granda, 2009; Granda et al., 2009). The Inbicon demonstration plant in Kalundborg, Denmark shows the potential of producing EtOH from lignocellulosic feedstocks with the Integrated Biomass Utilization System-process. In this case wheat straw is the main feedstock, but sugar bagasse, corn stover, empty fruit bunches, etc. have also been used as feedstocks (Larsen et al., 2012; Larsen et al., 2008; Inbicon, 2014).

1.2. Bio-ethanol production from corn

As mentioned in the previous section, corn grains can be used as substrate for the production of bio-EtOH. While the key step in this process is the fermentation of sugars to EtOH, multiple pre-treatment steps are required to convert starch in the corn grains into sugars. After fermentation, EtOH is distilled from the broth which results in a high EtOH concentration distillate and a low EtOH-concentration bottoms stream or 'whole stillage'. A schematic overview of the different steps in this process and any co-products can be seen in Figure 1.

The first step in the process is to mill the corn grains to reduce the particle size, obtaining a meal. This meal is then mashed, cooked and liquefied. The mashing entails mixing of the meal with water, containing α-amylase enzymes, to obtain the thick mash. This mash is then cooked and the high temperatures liquefy the starch in the mash. The starch is broken down into dextran, a branched polysaccharide, by the α-amylase enzymes. After cooking, the mash is cooled down and gluco-

amylase is added, which can break down the dextran into fermentable sugars (Alco BioFuel, 2014; Wilkie et al., 2000).

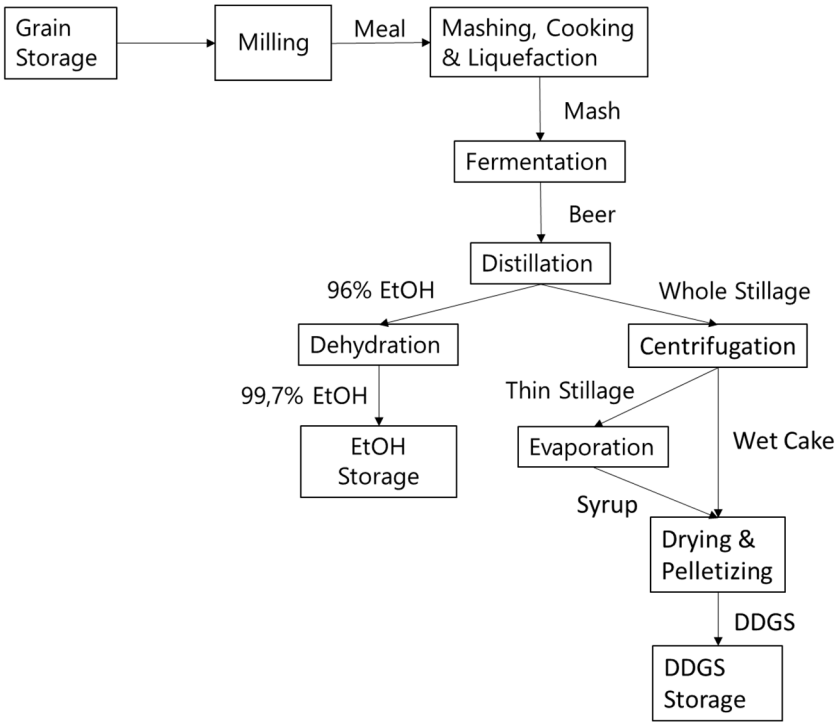

Figure 1. Schematic overview of Alco BioFuel production process (adapted from Alco BioFuel, 2014)

After the degradation of the starch to sugars, fermentation can proceed. In the Alco BioFuel process this is done in a batch process at 30-32 °C for 20 to 30 hours. This results in a 'beer' with an EtOH-content of approximately 12% (Alco BioFuel, 2014). Literature describes a very similar process, allowing batch fermentation to take place for generally less than 2 days, resulting in a beer containing 2 to 12% EtOH. For batch processes a yeast culture inoculum needs to be added to the mash in order to induce fermentation. The volume of this inoculum is often close to 10% of the total batch reactor volume. Fermentation can also be performed in continuous reactors where yeast is either immobilized or recycled. This increases fermenter productivity and EtOH yield (Wilkie et al., 2000).

After fermentation the beer is distilled. This results in a distillate containing most of the EtOH, at 96% purity due to azeotropic effects. For automotive applications the EtOH should have a water content lower than 1%, which cannot be achieved through distillation. For this purpose the EtOH is dehydrated after distillation. Several technologies are available for dehydration, with molecular sieves being the most frequently applied in fuel production. This molecular sieve consists of synthetic zeolites that preferentially absorb water, dehydrating the EtOH passing through the sieve (Wilkie et al., 2000).

The distillation process also produces a 'bottoms' or 'whole stillage' stripped of most of the EtOH. This whole stillage can still contain 0,1-0,2% EtOH when distillation runs efficiently, and can increase to 1% when distillation is suboptimal. This stillage still contains high amounts of organics, with literature reporting organics concentrations of 55 g Chemical Oxygen Demand (COD) per L

of stillage. This COD is the amount of O_2 needed to oxidize all organics to CO_2, quantifying the organics in a sample. This stream is also valorised by centrifuging the stillage to obtain separation of the solid fraction from the liquid fraction. The solid fraction is called the 'wet cake' while the liquid fraction is 'thin stillage'. The wet cake can be dried and pelletized resulting in Dried Distiller's Grains (DDG). Thin stillage can be concentrated to a syrup by evaporation. Addition of this thin stillage to DDG results in Dried Distiller's Grains and Solubles (DDGS) (Wilkie et al., 2000).

1.3. Anaerobic Digestion & Fermentation

1.3.1. Biochemical processes

AD is one of the most basic and mature biorefinery technologies. In this process, biomass is broken down to methane (CH_4). This CH_4 can then be valorised either by burning it to generate heat and/or electricity or it can be used as natural gas after purification. The breakdown of biomass occurs over several steps, as shown in Figure 2.

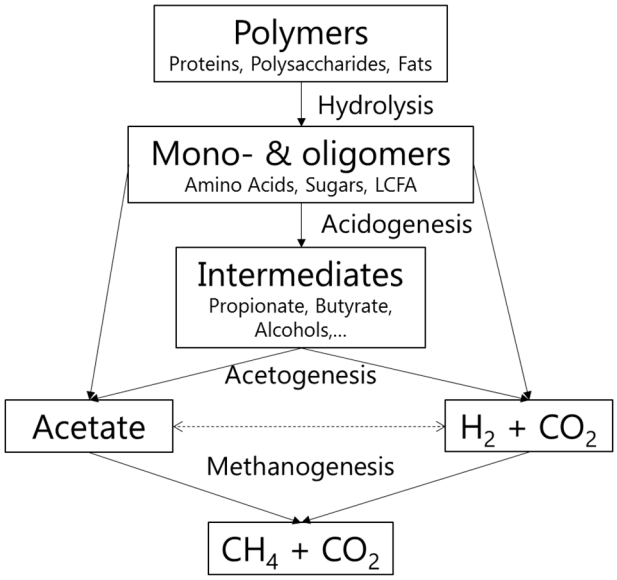

Figure 2. Overview of biomass degradation for production of CH_4 (adapted from Angenent et al., 2004)

In a first step, the biomass is hydrolysed. This means the complex organic matter is broken down to the organic building blocks, i.e. sugars (from carbohydrates), amino acids (from proteins) and Long Chain Fatty Acids (LCFA) from fats. This breakdown occurs extracellularly by a combination of processes, such as non-enzymatic and physical breakdown, physical separation and lysis (Batstone & Keller, 2002). With second generation lignocellulosic feedstocks this step is a major barrier. Due to the complex structure of this feedstock, the polysaccharides in the lignocellulose are not available for the extracellular enzymes. For this reason pretreatment of lignocellulosic feedstocks is necessary to increase the efficiency of the process. However, it is this pretreatment that is one of the major technological barriers for the use of lignocellulosic feedstocks in AD and biorefineries in general. A wide range of techniques has been studied, but these techniques are often found to be either very expensive or very slow, limiting the commercialisation of second generation feedstocks in AD (Taherzadeh & Karimi, 2008).

The second step of the AD-process is acidogenesis. In this step sugars and amino acids are converted into a mix of Volatile Fatty Acids (VFA), Carbon Dioxide (CO_2) and hydrogen. These VFA include propionate, valerate and butyrate. The VFA from the acidogenesis are then broken down into acetate, hydrogen and CO_2 by the acetogenesis-step. The acetogens can be divided into three groups. One group breaks down LCFA, a second group is responsible for the degradation of butyrate and valerate and the last group breaks down propionate.

Table 1. Overview of main pathways for methanogenesis and the associated standard changes in energy (Source: Liu & Whitman, 2008)

Pathway	Reaction	ΔG^0 (kJ.mol CH_4^{-1})
Hydrogenotrophic	$CO_2+4\ H_2 \rightarrow CH_4 + 2H_2O$	-135
Acetoclastic	$CH_3COOH \rightarrow CH_4 + CO_2$	-33
Methylotrophic	$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	-105

From this mix of acetate, CO_2 and hydrogen, methanogens can produce CH_4. This occurs through three main pathways: hydrogenotrophic, acetoclastic and methylotrophic methanogenesis. All of these pathways are exclusively executed by organisms belonging to the *Archaea*-domain. The hydrogenotrophic pathway uses hydrogen and CO_2 as substrates for the CH_4-production, as can be seen in Table 1. This pathway can be performed by most methanogens. Some hydrogenotrophic methanogens can also use formate or some alcohols (e.g. 4-isopropanol) as substrates. The acetoclastic pathway produces CH_4 from the reduction of acetate, as can be seen in Table 1. Even though this pathway is the energetically least attractive of the three and can only be performed by two genera (*Methanosaeta* and *Methanosarcina*), it provides up to two thirds of all the biologically produced CH_4. The last pathway is the methylotrophic pathway, which can only be performed by species belonging to the order of Methanosarcinales, with the exception of *Methanosphaera*. In this pathway a wide range of methylated compounds, such as methanol (see Table 1) and methylated amines and sulphides, can be used to produce CH_4 (Liu & Whitman, 2008).

1.3.2. Important parameters

In this section, several important parameters for anaerobic digestion will be discussed in detail, focussing on why these parameters are relevant to the AD process.

1.3.2.1. Temperature

AD processes can be divided into two categories based on temperature. Mesophilic digestion occurs at temperatures of approximately 35°C while thermophilic digestion occurs at 55°C. The higher temperature can increase COD removal, biogas yield and net energy output and allows for a higher Organic Loading Rate (OLR) (Ward et al., 2008). There are several effects at play with increasing temperature in AD. The increase of biological and chemical reaction rates has a positive effect on AD, as does the increased solubility of organic compounds. On the other hand the increasing temperature increases the fraction of free ammonia and raises the pKa of VFA. Both of these effects increase the risk of methanogen inhibition and subsequent process failure (Appels et al., 2008).

1.3.2.2. pH

The different groups of micro-organisms involved in AD each have their own optimal operating pH. The fermentative organisms (i.e. those performing the first three steps of AD) are tolerant to a wider range of pH than the methanogens. While the fermenters can function between pH 4 and 8.5, methanogens in digesters are limited to a pH-range between 6.5 and 7.2. pH not only directly influences the methanogens, but can also influence the toxicity of some substances (e.g. VFA, ammonia) as will be explained in detail in Sections 1.4.2.2 and 1.5. pH can also influence the VFA produced by the acidogenic fermenters; lower pH results in acetate and butyrate while higher pH results in acetate and propionate. These VFA decrease the pH, which is usually countered by methanogenesis, since the CO_2, HCO_3^- and NH_4^+ produced by methanogenesis can act as alkalinity buffer (Appels et al., 2008).

1.3.2.3. Organic Loading Rate

The OLR is the rate of organics loading per volume of reactor, usually expressed in organics per L reactor per day, organics being expressed as gCOD, g Volatile Solids (VS), g Volatile Suspended Solids (VSS),… This parameter determines the rate of organics conversion to CH_4 and is preferentially set as high as possible to increase potential CH_4 production. Increase of the OLR has limitations as well. Sánchez et al. (2005) showed that at increasing OLR, organics removal efficiencies decrease sharply at a certain threshold OLR. This decrease was due to the simultaneous accumulation of VFA and decrease in alkalinity, resulting in a pH decrease. These effects are detrimental to methanogens, as will be discussed at length in Sections 1.4.2.2 and 1.5.

1.3.2.4. Retention Times

The average time solids spend in the digester is called the Sludge Retention Time (SRT) or sludge age. This parameter affects the required growth rate of methanogens in the community. Lowering of the SRT implies a larger fraction of the sludge being removed per unit of time, which in turn means cell growth should be faster to compensate for the lost sludge. Therefore it is a key parameter for AD. For instance, when a Continuously Stirred Tank Reactor (CSTR) is operated with a SRT lower than five days the methanogenic community will be lost due to wash-out, which results in increasing VFA concentrations. It is only when the SRT becomes larger than eight days that digestion can be run stably (Appels et al., 2008). The average retention time of water in a reactor is called the Hydraulic Retention Time (HRT) and determines to what extent the community can convert substrate in the influent to CH_4. The main importance of this parameter is sizing of the reactor, as a smaller HRT requires smaller reactor volume for the same flow (Larsson et al., 2015).

1.4. Carboxylate Platform

1.4.1. Goal

AD as a biorefinery technology has been well established and even though it produces heat and energy, it may not be the most profitable way of valorising a waste stream. Using waste-streams to make valuable products, i.e. chemicals, could create economic opportunities. This is what the

carboxylate platform attempts. This can then be seen as a separate biorefinery platform, next to the sugar, syngas and other platforms (Agler et al., 2011).

In the carboxylate platform anaerobic fermentation is used to produce VFA, otherwise known as carboxylates when present as dissociated acids. This anaerobic fermentation is generally performed with an undefined mixed culture to allow the treatment of variable streams such as waste or biomass and reduce operation costs by removing the need for axenic conditions (Agler et al., 2011).

Since the anaerobic fermentation to VFA, also called acidogenic fermentation (Lee et al., 2014), is similar to AD, the last step of AD, methanogenesis, has to be inhibited to avoid conversion of VFA to CH_4. Various methods of inhibiting methanogenesis will be discussed in the next section.

1.4.2. Inhibition of methanogenesis

1.4.2.1. Chemical inhibition

Several chemicals can be applied to inhibit methanogens. These chemicals can be divided into two groups: specific and nonspecific inhibitors. Specific inhibitors act on enzymes only found in methanogens, thus leaving other micro-organisms unaffected. Examples of specific inhibitors are 2-bromoethanesulfonate, and mevastatin. Nonspecific inhibitors affect methanogens as well as other fermenting micro-organisms so care should be taken to preserve the acidogenic community. Examples include ethane, acetylene, chloroform (Lee et al., 2014).

Other compounds can also disrupt methanogenesis and AD as a whole. For instance, NH_3 is very toxic for micro-organisms due to its free migration through membranes after which it can disrupt proton balances. NH_3 originates from biological degradation of nitrogen-containing matter. Sulphides (HS^- and H_2S) can also be toxic, although there is no clear mechanism of this toxicity. These sulphides originate from the reduction of sulphates (SO_4^{2-}) by sulphate reducing bacteria (Chen et al., 2008).

1.4.2.2. pH

Due to the small pH-range in which methanogens can survive - between 6.5 and 7.2 (Appels et al., 2008) or even 6.8 to 7.2 (Chen et al., 2008) - as opposed to the wide pH range for acidogens - pH 4 to 8.5 (Appels et al., 2008) - it is clear that a suitable choice of pH can inhibit methanogens. This results in pH optima for VFA producers of pH 8 to 11 in case of sludge fermentation and pH 5.25 to 6 when wastewater is used (Lee et al., 2014). There are several effects at play with changing pH.

At high pH, i.e. larger than 8, NH_3 becomes a serious problem for toxicity. This is because the fraction NH_3 of the total ammonia nitrogen, i.e. the sum of NH_3 and NH_4^+, increases with increasing pH. In this way, an increasing pH increases the concentration of NH_3 and causes toxicity for micro-organisms (Chen et al., 2008).

H_2S can also affect methanogenic activity, although there is still no consensus on the mechanism behind H_2S-toxicity. The protonated form, representing a higher fraction of total sulfide at low pH, can diffuse into the cell and potentially denature proteins. This mechanism is still unproven as studies have found no clear link between H_2S-toxicity and pH (Chen et al., 2008).

At low pH, toxicity due to VFA comes into play. A decrease of pH causes a larger fraction of VFA to become protonated. This protonated, electrically neutral form can migrate through the cell membrane. After migration, the acids can dissociate, lowering the pH inside the cell. This can cause homeostatic pH controlling mechanisms to fail, resulting in the death of the micro-organism (Harris et al., 2001). This effect will be discussed in detail later in Section 1.5.

1.4.2.3. Retention Times

As discussed earlier (see Section 1.3.2.4), retention times of both sludge and water influence AD. Due to slow growth, methanogenesis is especially sensitive to low SRT. As mentioned before, at SRT lower than 8 days, acidogenesis prevails due to washout of methanogens. Only at SRT higher than 10 days stable methanogenesis could be obtained. For the case of acidogenic wastewater fermentation, no clear link between SRT and VFA production has been found, due to the limited amount of study (Lee et al., 2014).

1.4.2.4. Organic Loading Rate

As discussed before (see Section 1.3.2.3), OLR is an important parameter for AD. For VFA production OLR, can be chosen with the goal of inhibiting methanogens. The mechanism behind this inhibition is as follows: due to the high OLR, VFA production rate increases. When the VFA production rate is higher than the consumption by methanogens, VFA start to accumulate, causing acidification of the reactor. This combination of high VFA concentrations and low pH inhibits the methanogens (Yan et al., 1993). Despite this, OLR should not be excessively high because acidogens are affected by this parameter as well. According to Lee et al. (2014) VFA production has an optimum OLR. At lower OLR, VFA production increases linearly with OLR while at higher OLR, production deteriorates. While mechanisms behind this process failure are not well understood, mass transfer limitations could be at play.

1.4.3. Examples and practical challenges

Practical applications of the carboxylate platform are still rare. One example in development is MixAlco, currently operating a demonstration plant. The MixAlco uses the carboxylate platform to produce alcohols, ketones and other chemicals. Carboxylates are produced by fermenting lignocellulosic biomass (e.g. crops, municipal solid waste,…) to VFA in piles. Methanogenesis is inhibited by using chemical inhibitors, in this case iodoform, even though high NH_3 concentrations should be sufficient to inhibit methanogens. Residues after fermentation can be gasified to produce H_2. The fermentation broth is dewatered by vapour compression and subsequently esterified with high molecular-weight alcohols. The esters formed in this esterification are subsequently hydrogenolysed with H_2 from fermentor gas, gasification of residues or new biomass, through natural gas reforming or through electrolysis. This hydrogenolysis results in high molecular-weight alcohols, reusable for the carboxylate esterification, and saleable low-molecular-weight alcohols(Granda et al., 2009).

The carboxylate platform faces some challenges which Agler et al. (2011) reduced to three major challenges: the separation barrier, the methanogen barrier and the ecology barrier. The methanogen barrier was discussed earlier with Methods for inhibition of methanogenesis can help overcome

the methanogen barrier, as discussed in Section 1.4.2). Methanogenesis is undesirable for chemicals production because of the loss of multi-carbon compounds to CH_4. The separation barrier alludes to the fact that even though VFA may be produced, they still have to be separated from the reaction broth. Several methods have been attempted for separation, one of them being the extraction through ion-exchange membranes. VFA produced in the cathode compartment can then be transferred to the anode compartment. This technique will be discussed in greater detail in Section 1.6. Another possibility for separation is production of longer VFA (also called medium-chain carboxylates) such as caproate and caprylate, which are easier to separate than shorter carboxylates (Agler et al., 2011). The economic importance of this barrier should be noted. Downstream processing, including the crucial separation step, can often account for more than 60% of total production costs in fermentation-based processes (Bechthold & Bretz, 2008). Lastly, the ecology barrier is the challenge to direct the microbial process in order to generate the desired products at sufficient rates and high concentrations. The thermodynamic feasibility of the processes generating these products is crucial and is determined largely by the environmental conditions of the fermentation process. As a consequence, controlling these environmental conditions is crucial for directing the process towards the desired products (Agler et al., 2011).

1.5. VFA Toxicity and product inhibition

VFA are toxic to micro-organisms, more specifically the undissociated form of the acids. The neutral undissociated acid can migrate through the lipophilic membrane of the micro-organisms. The cytoplasmic pH of micro-organisms is usually between 6.5 and 7.5 (Roos & Boron, 1981), causing the VFA to dissociate. This dissociation releases protons, decreasing the intracellular pH. If the concentration of undissociated VFA is high enough, this pH-decrease can continue out of control of the micro-organisms' homeostatic mechanisms, causing their deaths (Harris et al., 2001).

1.5.1. Influence of pH

An important parameter for VFA toxicity is the pH. This is due to the protonation of VFA at decreasing pH which, as stated before, increases the concentration of undissociated - toxic - VFA. Crucial in this story is the pKa of different VFA, since this is the pH at which half of the VFA are protonated. Table 2 shows the pKa of some VFA relevant for the carboxylate platform.

As can be seen in Figure 3, constructed based on the Henderson-Hasselbalch equation, the fraction of protonated VFA starts to increase rapidly when pH drops below 5.5. For this reason, many applications of the carboxylate platform operate at this pH to inhibit methanogens without inhibiting acidogens (Andersen et al., 2014; Agler et al., 2011).

Table 2. pKa of VFA relevant for carboxylate platform (Source: Jencks and Regenstein (2010))

VFA	pKa
Acetic acid (Ac)	4.76
Propionic acid (Pr)	4.88
Iso-butyric acid (iBut)	4.86
Butyric acid (But)	4.82
Iso-valeric acid (iVal)	4.78
Valeric acid (Val)	4.86
Iso-caproic acid (iCap)	4.85
Caproic acid (Cap)	4.88
Heptanoic acid (Hep)	4.89
Octanoic acid (Oct)	4.89

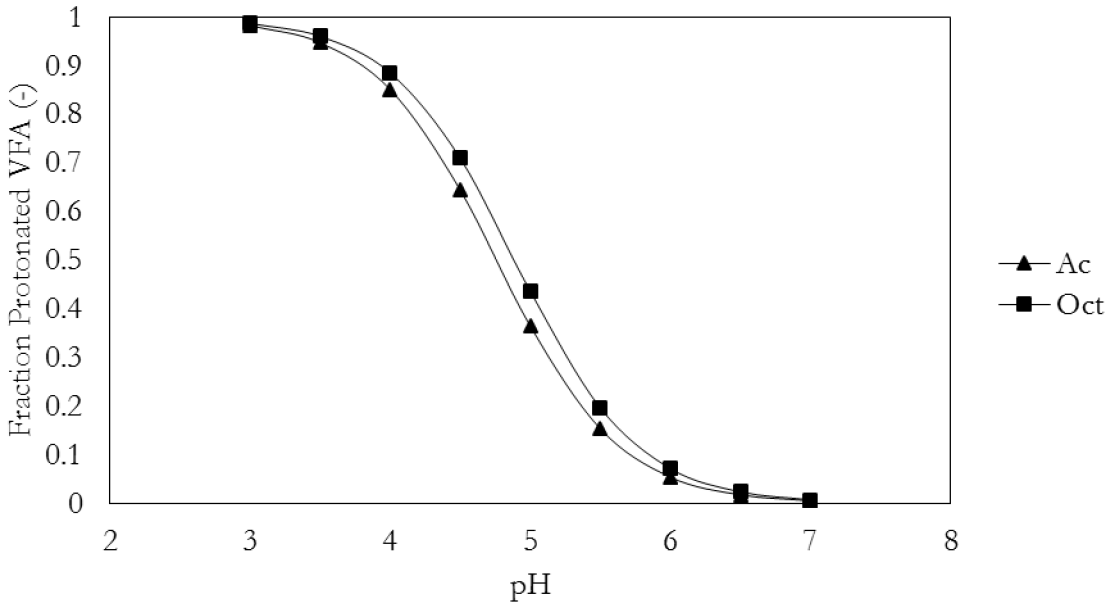

Figure 3. Fraction protonated VFA as a function of pH for Ac and Oct, which have respectively the lowest and highest pKa of the relevant VFA listed in Table 2.

1.5.2. Mechanisms of toxicity and inhibition

As discussed previously, toxicity is thought to be due to migration of undissociated VFA into the cell, causing disruption of proton balances (Harris et al., 2001). However, not all VFA have the same effect. Pratt et al. (2012) found that product inhibition due to Ac has less impact than other VFA, in this case Pr, iBut, But, iVal, Val and Cap. It was also found that inhibition only starts from a certain threshold concentration, due to thermodynamical limitations. It is possible that high VFA concentrations cause certain critical reactions to become thermodynamically limited, however this would not explain the diminished effect of acetate. This could be explained by acetate not being an end product but a by-product of fermentation.

The exact pathway of toxicity is still unclear. It is assumed that the undissociated forms of VFA can penetrate the cell through the lipid membrane, subsequently dissociating and causing toxicity.

However, it appears that both the protons and the anions contribute to the toxicity. Palmqvist and Hahn-Hägerdal (2000) elucidate two mechanisms of toxicity for weak organic acids, such as fatty acids. The first mechanism, called the uncoupling theory, states that inhibition is caused by a drop in internal pH due to the inward migration and subsequent dissociation of acids, which releases protons. As a response to this pH-drop the plasma membrane ATPase pumps protons out of the cell, neutralising intracellular pH. This neutralisation comes at a cost, in this case the hydrolysis of ATP, which causes less energy to be available for growth. At high concentrations of undissociated VFA the ATP is depleted, causing the dissipation of the proton motive force across the membrane. Because the protons can no longer be pumped out of the cell, the cytoplasm starts to acidify. One problem with this theory, however, is the limited capacity of fatty acids to acidify the cytoplasm, since they can only carry one proton into the cell.

Another mechanism, the anion accumulation theory, states that the accumulation of anions after dissociation causes toxicity. The anions accumulate because of their lipophobic behaviour after dissociation. In this way diffusion of the undissociated form will continue until an equilibrium is reached. This equilibrium is a function of the external pH because this is related to the amount of undissociated VFA present externally (Palmqvist & Hahn-Hägerdal, 2000). Resistance to organic acids could then be due to the capacity of these resistant micro-organisms to drop their internal pH. Other effects can also contribute to inhibition such as membrane damage causing leakage and changing membrane fluidity (Ricke, 2003). This could be caused by the ability of undissociated VFA to dissolve at least partially into the membrane, resulting in higher fluidity and less hydrophobic interactions (Royce et al., 2013).

These inhibitory effects, despite being troublesome to the carboxylate platform, have applications as well. An application with large potential is the use of VFA as an antimicrobial to prevent the spread of foodborne diseases in farms. VFA could substitute traditional antibiotics in these applications since many foodborne diseases have developed resistance to these antibiotics. Some form of resistance could also be developed against VFA by these pathogens, but only if they were allowed to adjust to these VFA at sublethal levels (Ricke, 2003). Especially Oct has shown great potential as antimicrobial; Cap has exhibited some potential as well, but to a lesser extent (Skrivanova & Marounek, 2007).

1.5.3. Strategies for reduction of VFA toxicity

To allow for increased VFA concentrations and accompanying increased production rates it can be useful to reduce the toxicity of VFA to micro-organisms. Several strategies can be employed for this purpose.

A first strategy is an increase of pH to reduce the fraction of protonated VFA. This should allow for higher total VFA concentrations, since a higher total concentration of VFA is necessary to achieve the same level of toxicity. However, as Appels et al. (2008) stated, methanogens can survive at pH between 6.5 and 7.2. If the pH is set at 7, this would allow methanogens to grow and break down the VFA to CH_4.

A second strategy that can be implemented to reduce the VFA toxicity is to extract VFA from the reactor broth. This reduces the total concentration of VFA present in the broth and lowers toxicity for the micro-organisms. Besides lowering toxicity for micro-organisms, the separation of VFA is also necessary in order to purify and valorise these VFA. Several techniques are available for separation of VFA from reactor broth. A classical technique for separation of VFA is liquid-liquid extraction by using an organic solvent (e.g. alcohols, hydrocarbons,…) to dissolve VFA. However, micro-organisms can often be very sensitive to these solvents. The use of chemical solvents also has a high environmental impact and should be avoided (Singhania et al., 2013).

One of the most promising methods for extraction is the use of membrane separation techniques (Agler et al., 2011; Singhania et al., 2013), which can be applied as an electrochemical system. VFA present as anions can migrate through a membrane as a response to a current applied over the membrane. An example of this is the Membrane Electrolysis (ME) process, which consists of a two-chamber electrochemical system. The fermentation broth is circulated through the cathode compartment while a clean, saline and low pH-solution is circulated through the anode compartment. Electrons flow from the anode to the cathode under the influence of an external power source. This charge difference needs to be compensated by ion migration over the membrane, which can be achieved either by migration of cations from anode to cathode compartment or migration of anions from cathode to anode compartment. Since the goal is to extract VFA, present as anions in the fermentation broth, it is desirable to avoid cation migration. This can be achieved by using Anion Exchange Membranes (AEM) which prevents cation migration due to its positively charged surface. With this ME-process, VFA produced during fermentation can be extracted in a clean solution useable for production of other chemicals (Andersen et al., 2014).

One possible disadvantage of this technique is the low Coulombic Efficiency (CE), especially at low VFA concentrations in the catholyte. CE in this application is the ratio of target molecule anions passed over the membranes to the electrons used during this extraction. Andersen et al. (2014) showed that as the concentration of Ac increased, both the flux of Ac over the membrane and CE increased significantly. In a catholyte solution with 10 $g.L^{-1}$ Ac, a CE of 99.4±0.1% could be achieved at a current density of 20 $A.m^{-2}$. On the other hand, at lower Ac concentrations in the catholyte, CE decreased. This can be explained by Le Châtelier's principle: The compartments separated by the membrane are in disequilibrium due to a combination of the Ac concentration gradient and the charge gradient due to the applied current. This disequilibrium is larger when the Ac concentration in the catholyte increases, resulting in more Ac anions to be transferred across the AEM.

1.6. Electrochemical Systems & Membrane Extraction

In an electrochemical system, a simultaneous reduction and oxidation is coupled through a power source or electrical application. The reduction occurs at the cathode while the oxidation takes place at the anode. In some cases a third electrode can be used as a reference electrode, which is used to set the potentials of the working electrode and the counter electrode in the system. In applications without this reference electrode, only the potential difference between the two electrodes can be controlled, which is often the case in systems applying electrolysis (Thrash & Coates, 2008). Electrolysis is the splitting of water in H_2 and O_2, as given by the reactions in Table 3. This takes place at a minimum potential difference of 2.058 V, without taking overpotential and other inefficiencies into account.

Table 3. Reactions occurring in electrolytical system (Source: Thrash and Coates, 2008)

	Reaction	ΔE^0(V)
Oxidation	$2\ H_2O \rightleftharpoons O_2 + 4\ H^+ + 4e^-$	1.230
Reduction	$2\ H_2O + 2\ e^- \rightleftharpoons H_2 + 2\ OH^-$	-0.828

Assuming an anaerobic fermentation system with an electrolytical cell, the fermentation broth can be put into contact with the cathode where the water in the broth is reduced. The anode can then be placed in a separate solution, separated from the cathode compartment by a membrane. At the cathode H_2 and OH^- are produced while O_2 and H^+ are produced at the anode. As discussed in Section 1.5.3, the electrical circuit can then be closed by migration of anions from the fermentation broth in the cathode compartment (catholyte) to the solution in the anode compartment (anolyte) or by a reverse cation migration (Andersen et al., 2014). The electrolysis products have some influence on the fermentation process (e.g. homoacetogenic reduction of CO_2 with H_2 - produced by electrolytic H_2O-reduction at the cathode – to Ac (Spirito et al., 2014)). This combination of electrolysis with fermentation has been termed Electro-Fermentation (EF) and literature confirms that reduction of H_2O at the cathode influences products obtained during fermentation (Redwood et al., 2012). Not only can target products be extracted using EF, acidification of the fermentation broth by acidogenic fermentation can also be countered by production of OH^- at the cathode (cf. Table 3). This effectively creates a system capable of controlling pH without input of chemicals.

As mentioned before: to close the electrical circuit of the electrochemical cell, migration of ions between anolyte and catholyte is necessary. Since the focus of this thesis is on the production of VFA, mainly the migration of anions from catholyte to anolyte is relevant. As previously discussed in Sections 1.5.1 and 1.5.3, VFA are chiefly present in anionic form in the fermentation broth, which allows migration through the AEM. Andersen et al. (2014) used an anolyte solution at pH 2 in which extracted VFA are protonated and can no longer migrate through the AEM. The protonation of these VFA consumes protons in the low-pH anolyte solution. This consumption is then compensated by production of protons in the oxidation of water as shown in Table 3.

2. Materials and methods

2.1. Alco BioFuel thin stillage

Thin stillage is, as mentioned in Section 1.2, a side-stream in the production of EtOH from biomass. The bottom stream (stillage) resulting from distillation of the fermented biomass is centrifuged, resulting in a supernatant - thin stillage - and solids - the wet cake. Thin stillage contains large amounts of organics, nitrogen, etc. Some of the principal physicochemical properties of thin stillage are listed in Table 4.

Table 4. Physicochemical properties of thin stillage in first 50 days of storage at 4°C (n=7)

pH (-)	4.86±0.15
Conductivity (mS.cm^{-1})	3.81±0.75
Total COD (tCOD) (g.L^{-1})	53.85±7.99
Soluble COD (sCOD) (g.L^{-1})	36.42±3.2
Alkalinity (meq.L^{-1})	7.78±3.36
Total Suspended Solids (TSS) (g.L^{-1})	19.45±1.38
VSS (g.L^{-1})	19.00±1.31
Total Solids (TS) (%)	4.37±0.26
VS (%)	90.50±0.47

2.2. Semi-continuous batch reactor experiments

Several experiments were performed in a semi-continuous batch reactor system in a 34 °C temperature-controlled room. These reactors, with a volume of 900 mL, were fed manually every 2 days. 300 mL of reactor broth was removed and 300 mL of fresh thin stillage was added at each feeding. This resulted in an equal SRT and HRT of 6 days, chosen to allow build-up of VFA-concentrations while at the same time previous research indicated this HRT was long enough for adaptation of organisms to changing conditions (e.g. pH hysteresis – see Section 2.2.2). Another reason for this short HRT was a reduced chance for methanogenic activity due to slow growth of methanogens (Appels et al., 2008). For continuous tests with thin stillage (Sections 2.2.1, 2.2.2 and 2.2.3) no inoculum was added while for methanogen tests (Section 2.2.4) thin stillage was inoculated (10%vol) with effluent from a mesophilic CH_4-producing digester.

2.2.1. Fermentation at fixed pH

pH was fixed at 5.5 using a Consort pH probe, Consort pH controller – maintaining pH above 5.4 - and a Prominent pump for dosing of 2M NaOH. The reactor was operated for 42 days (6 X HRT), mixed by a magnetic stirrer. Gas produced during fermentation was collected in a gas tube, as shown in Figure 4, with an acidic solution of HCl at pH 3 to prevent CO_2-dissolution in the solution. pH in this solution was monitored using methyl orange as an indicator. Another experiment was run for 48 days at pH 7 in the same set-up. These fermentations will act as control experiments for the experiments described in the following sections.

2.2.2. Fermentation with pH Hysteresis

An identical experiment was performed with pH shifting every HRT, i.e. one HRT at pH 7, the next HRT at pH 5.5, the next returned to pH 7, etc. The hypothesis behind this experiment is that pH hysteresis can select for micro-organisms that are tolerant of high VFA-concentrations at a low pH while simultaneously inhibiting methanogens in the fermenter. pH was controlled using a Prominent pH controller, pH probe and pump. Gas produced during fermentation was trapped in a gas tube with an acidic solution – using methyl orange as indicator - to prevent dissolution of CO_2. In a first phase of 72 days (6 oscillations – 12 x HRT), pH hysteresis between pH 7 and 5.5 was applied. In a second phase of 24 days (2 oscillations – 4 x HRT), the lower pH of the hysteresis was decreased to 5. The bottom threshold of 2M NaOH dosing by the pH controller for pH maintenance was 6.9, 5.4 and 4.95 for periods at pH 7, 5.5 and 5 respectively.

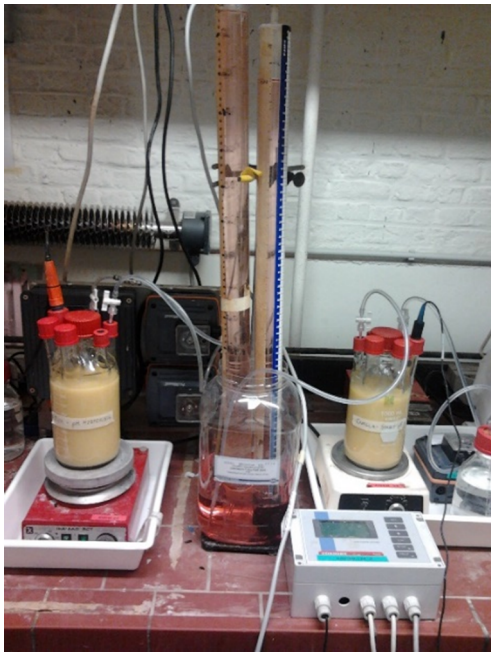

Figure 4. Experimental set-up of the semi-continuous batch reactor. Gas tubes with acidic solution can be seen in the middle. Prominent pH controller visible in the back, Consort pH controller in front.

2.2.3. Fermentation at fixed pH with electrochemical extraction

A semi-continuous batch reactor as shown in Figure 4 was expanded with an electrochemical cell with internal dimensions of 200 mm × 50 mm × 20 mm per chamber, resulting in a total reactor volume of 1200 mL. This set-up aims to bring into practice the first part of the process pipeline described in Andersen et al. (2014) and in this way tests whether the ME-process influences the fermentation process. This expanded set-up is shown in Figure 5. The electrochemical extraction cell can be seen centrally while the fermenter itself is on the left. The recirculation pump, pumping broth from fermenter to electrochemical cell as well as anolyte from the reservoir to the electrochemical cell, is outside the picture frame to the left.

Figure 5. Set-up of fermenter with electrochemical extraction cell, pH controller with bottles and gas trap

The fermentation broth was recirculated from the reactor to the cathode compartment of the electrochemical cell using a peristaltic pump at a rate of 6 $L.h^{-1}$. The anode compartment was separated from the cathode compartment by a 200 mm × 50 mm AEM (fumasep FAB, FumaTech GmbH, Germany). The anolyte solution, a 0.05M Na_2SO_4/0.05M H_2SO_4-solution in deionized water, was recirculated from a reservoir using the same peristaltic pump. The total volume of anolyte solution was equal to that of the catholyte. An AISI Type 316L Stainless steel wire mesh with 564 μm mesh size, 140 μm wire thickness (Solana nv, Belgium) was used as cathode. The choice for stainless steel as a cathode material was made because of its cost effectiveness as well as its capability for efficient H_2-production through water electrolysis (Zhang et al., 2010). The anode was an Ir MMO coated titanium electrode (IrO2/TaO2: 0.65/0.35), 200 mm x 50 mm, with a centrally attached, perpendicular current collector (Magneto Special Anodes BV, The Netherlands). The stability of Ir MMO as anode material for electrolytic oxidation of H_2O is the main reason for its choice in this application (Morimitsu et al., 2004). A potentiostat (VSP, Biologic, Fance) in chrono-potentiometry mode was used to apply a fixed current in the electrochemical cell.

In the first operation phase of 24 days (4 x HRT) 400 mL of the broth was replaced with fresh thin stillage every 2 days to maintain the 6 day HRT. Similarly, one third of the anolyte solution was replaced every 2 days to prevent pH of the anolyte solution from falling and causing membrane damage. As mentioned before in Section 1.6, the cathodic reduction of the fermentation broth produces OH^-, eliminating the need for NaOH-dosing to maintain a pH above 5.4. When pH increased beyond 5.7, due to electrolytic reduction of the fermentation broth, acidic extractant from the previous period was dosed by the pH controller. To minimize dosing of anolyte solution, a current of 100 mA (approx. 10 $A.m^{-2}$) was only applied either for the first 20 to 24 hours or until a pH of 5.7 was reached, after which the current was lowered to 10 to 20 mA to allow for a continuous supply of hydrogen to the broth.

The second phase of 18 days (3 x HRT) had similar operating conditions as phase 1, with no applied current in the electrochemical cell to test whether the effect of the current was reversible. Because

no current was applied, the anolyte was refreshed with demineralised water instead of the acidic solution.

A third phase of operation saw the HRT extended to 12 days by replacing one third of the broth with fresh thin stillage every 4 days for a period of 1 HRT. This increase in HRT tested whether the longer fermentation time would increase hydrolysis of the solids in the thin stillage.

A fourth and final phase of reactor operation saw a decreased HRT of 3 days by replacing one third of the broth every day for a period of 9 days (3 HRT). In this phase, a constant current of 100 mA (10 $A.m^{-2}$) was applied. 600 mL of anolyte was operated in batch mode (i.e. no replacement of the solution when feeding the reactor) to allow accumulation of VFA in the anode compartment.

2.2.4. Methanogen control by pH-shocking

To test the hypothesis that decreasing pH for a short period of time – a "pH-shock" - can control methanogenic activity in a fermenter, an experiment was carried out using the experimental set-up described in Section 2.2.3. The reactor was operated at pH 7 with an HRT of 6 days. Thin stillage was inoculated with mesophilic digester effluent (10 vol%) at the start of the experiment and in feed to ensure methanogenesis in the reactor. Initially an OLR of 7.9±2.9 g $sCOD.L^{-1}.d^{-1}$ and a current of 100 mA (10 $A.m^{-2}$) was applied. However, due to the continued absence of methanogens, this loading rate was lowered after 10 days by diluting thin stillage with water, resulting in an OLR of 3.3±0.6 g $sCOD.L^{-1}.d^{-1}$. On top of this, current density was lowered to 5 $A.m^{-2}$ on day 16. After these adaptations took place, methanogenesis was initiated and between day 19 and 21 a pH shock of 2 days at pH 5.5 was applied by fermentative acidification combined with dosing of anolyte acid and 2M HCl. After 2 days, pH was increased back to pH 7 by application of an increased current of 10 $A.m^{-2}$. Subsequent shocks were applied in much the same way: when CH_4 was detected in the headspace after at least 2 days at pH 7, reactor pH was lowered to 5.5 for the first two shocks, followed by 2 shocks at pH 5.

2.3. Chemical analyses

2.3.1. VFA Analysis

VFA were analysed in accordance with Andersen et al. (2014). C2-C8 fatty acids (including isoforms C4-C6) were measured by gas chromatography (GC-2014, Shimadzu®, The Netherlands) with DB-FFAP 123-3232 column (30m x 0.32 mm x 0.25 μm; Agilent, Belgium) and a flame ionization detector (FID). Liquid samples were conditioned with sulfuric acid and sodium chloride and 2-methyl hexanoic acid as internal standard for quantification of further extraction with diethyl ether. Prepared sample (1 μL) was injected at 200ºC with a split ratio of 60 and a purge flow of 3 mL min^{-1}. The oven temperature increased by 6ºC.min^{-1} from 110ºC to 165ºC where it was kept for 2 min. FID had a temperature of 220ºC. The carrier gas was nitrogen at a flow rate of 2.49 mL min^{-1}.

2.3.2. Solids Analysis

TSS and VSS-analyses were performed following Standard Methods 2540D and E (APHA, 2005). TS and VS were analysed by weighing the difference between the initial sample weight and weight after drying at 105°C (TS) and incinerating at 550°C (VS)

2.3.3. Headspace Gas Analysis

The gas phase composition was analysed with a Compact GC (Global Analyser Solutions, Breda, The Netherlands), equipped with a Molsieve 5A pre-column and Porabond column (CH_4, O_2, H_2 and N_2) and a Rt-Q-bond pre-column and column (CO_2, N_2O and H_2S). Concentrations of gases were determined by means of a thermal conductivity detector.

2.3.4. Stream Characterisation

Four random samples from effluent and feed of both the control reactor at pH 5.5 (Section 2.2.1) and reactor with electrolytic extraction (Section 2.2.3) were selected at random as well as four samples of the anolyte solution from the reactor with electrolytic extraction for deeper stream characterisation. Concentrations in the soluble phase were measured after filtering the sample with 0.45 μm non-sterile syringe filters. Total and soluble COD was analysed with Nanocolor® kits (CODE; Macherey-Nagel). Total Kjeldahl Nitrogen (TKN) of these randomly selected samples (filtered and unfiltered) was analysed according to Standard methods (4500-Norg B; APHA, 2005) and used as an indication of total and soluble protein content of the samples. Quantification of soluble carboyhydrates was performed according to the methods described in Sluiter et al. (2006) and subsequent HPLC-analysis. Chloride, nitrite, nitrate, sulphate and phosphate were determined by conductivity detection after inorganic anion chromatography on a 761 Compact Ion Chromatograph (Metrohm, Switzerland). Organic ion chromatography (Dionex DX 500) was used to quantify lactate, glycerol, 1,3-propanediol, EtOH, propanol and butanol concentrations in the randomly selected samples.

2.4. Community Analysis

Because of its origin in the biological EtOH-fermentation, thin stillage is a complex, non-sterile broth containing organics such as proteins, cellulosic fragments and volatile fatty acids as well as an inherent community. As no analysis of the community present in thin stillage, it is necessary to perform a community analysis to obtain data and gather knowledge on this community. The community analysis was performed on both the fresh thin stillage and the pH 5.5 controlled reactors with and without ME, to evaluate the influence of ME on the microbial community present in a fermenter.

Effluent and feed samples were centrifuged in sterile 2 mL Micrewtubes® (Simport, Canada) for 1 minute at 20238g and supernatant was removed before storage at -21°C. 10 feed samples and 11 effluent samples of the control reactor at pH 5.5 (Section 2.2.1) as well as 10 feed samples and 12 effluent samples of the experimental reactor with electrolytic extraction (Section 2.2.3) were selected for DNA extraction. DNA extraction was performed using the unmodified PowerSoil® DNA Isolation Kit protocol (MoBio, USA), resulting in 100 μL of isolated DNA in 10 mM Tris. The choice for this DNA isolation kit was made because of its ability to isolate DNA when large amounts of phenolic compounds (i.e. lignin) are present in the broth. Concentrations of double-stranded DNA (dsDNA) in the extractant were quantified using the QuantiFluor® dsDNA system and measured with a GloMax® 96 Microplate Luminometer (Promega GmbH, Germany).

A quality control of the extracted DNA was performed by PCR with primers used for Illumina sequencing after which genomic 16s-DNA was sequenced using PCR with one-channel paired read at 300 bp (LGC Genomics GmbH, Germany).

2.5. Batch Tests

2.5.1. VFA-inhibition

The reactors described in Sections 2.2.1 and 2.2.2 reached steady-state conditions - defined as the period during which concentrations vary less than 15% from the average concentration – after resp. 1 and 2 HRT. To test the hypothesis that high VFA-concentrations inhibited production of VFA, a batch test was conducted. Fresh thin stillage was inoculated (10 % of total volume) with effluent from the reactor operated at pH 5.5, as described in Section 2.2.1. In a first experimental test – named Medium hereafter – total VFA-concentration in the inoculated thin stillage was increased with 12.5 g VFA.l^{-1}, to reach approximately the steady-state concentration in the reactor at pH 5.5. The second experimental test – named High hereafter – increased total VFA-concentrations of the thin stillage with 20 g VFA.l^{-1}, to reach a concentration well above the steady-state concentration. This allows testing whether VFA-inhibition increased with concentration. In a control test, no VFA were added. The experimental tests used a VFA-mixture consisting of 22% Ac, 49% Pr, 9% But and 21% Val to increase concentrations artificially. The proportions of these VFA were chosen to emulate the proportions found in the steady-state reactor without ME (Section 2.2.1). All experiments were performed in triplicate, keeping 60 mL of broth in a 120 mL penicillin bottle at 34°C on a shaker. Samples were taken on day 0, day 2 and day 4.

To test the influence of VFA inhibition on the community itself, the triplicates were put together after 4 days and centrifuged at 8500g for 7 minutes. Supernatant was removed and supernatant of thin stillage, also centrifuged at 8500g for 7 minutes, was added. Solids were resuspended and the broth was distributed over triplicate experiments, consisting of 60 mL of broth in a 120 mL penicillin bottle. A second control experiment, in duplicate, was added at this point, where supernatant of fresh thin stillage was kept in identical conditions as the other experiments to measure the baseline VFA-production. Samples were taken at the beginning of the second part of the experiment and after 3 days at 34°C (day 7 of the batch experiment).

2.5.2. Methanogen control by application of pH-shock

A hypothesis was put forward that a pH-shock could be applied to remove methanogens from fermenting or digesting reactors. To test this hypothesis, effluent from a mesophilic digester producing CH_4 was used in a batch test in which a pH-shock was applied. 80 mL of digester effluent, containing 16.7±2.6 g VS.L^{-1} was put in a penicillin bottle after which 0.25 mL of glycerol was added as a carbon source to reach a dosing of approx. 0.25 g COD.g VS^{-1}. A magnetic stirrer was used to mix the broth in the penicillin bottle during the experiment. Gas production and composition as well as VFA-production wer monitored over time. After 6 days the penicillin bottle was opened and a solution of 2M HCl was added to decrease pH to approximately pH 5.5 as well as another 0.25 mL of glycerol as carbon source. After 2 days, pH was increased to 7 by addition of 2M NaOH. The penicillin bottles were stored at 34°C for another 8 days. An identical

experiment was set up as a control where the 2M NaCl was added to achieve the same increase in Electrical Conductivity (EC). To test how short the pH shock could be, another identical experiment was set up where the pH was increased back to 7 after 1 day at pH 5.5. All experiments were performed in triplicate in a temperature-controlled room at 34°C.

2.5.3. Shocking community with pH and VFA to influence production

A similar experiment as described in Section 2.5.1 was performed to test the hypothesis that shocking a community with a specific VFA can influence the product outcome after restoring pH. Fresh thin stillage at pH 7 was shaken for 1 day at 120 rpm at 34°C to allow growth of a fermenting community. Fresh thin stillage was then inoculated with the acclimated thin stillage. In a first experimental test 15 $g.L^{-1}$ Ac was added to the inoculated thin stillage, while in a second experiment 15 $g.L^{-1}$ of Pr was added. A control experiment consisted of adding an equal volume of demineralised water to the inoculated broth. All experiments were corrected to pH 5.5. Penicillin bottles of 120 mL were filled with 60 mL of broth and kept at 34°C for 2 days, sampling on day 0. pH was corrected to 5.5 on day 1. On day 2, samples were taken, the broth was centrifuged for 7 min at 8500g and supernatant from the broth was removed and replaced with supernatant from centrifuged fresh thin stillage. The centrifuged solids were then resuspended and pH was corrected to 7. The resuspended broth (approx. 50 mL) was put in 120 mL penicillin bottles and kept at 34°C. An additional control experiment was added to provide information about baseline auto-fermentation of the supernatant of centrifuged thin stillage by keeping 30 mL of thin stillage supernatant (pH 7) at 34°C in duplicate. pH was corrected to pH 7 on day 3 and 6.

3. Results

3.1. Stream characterisation

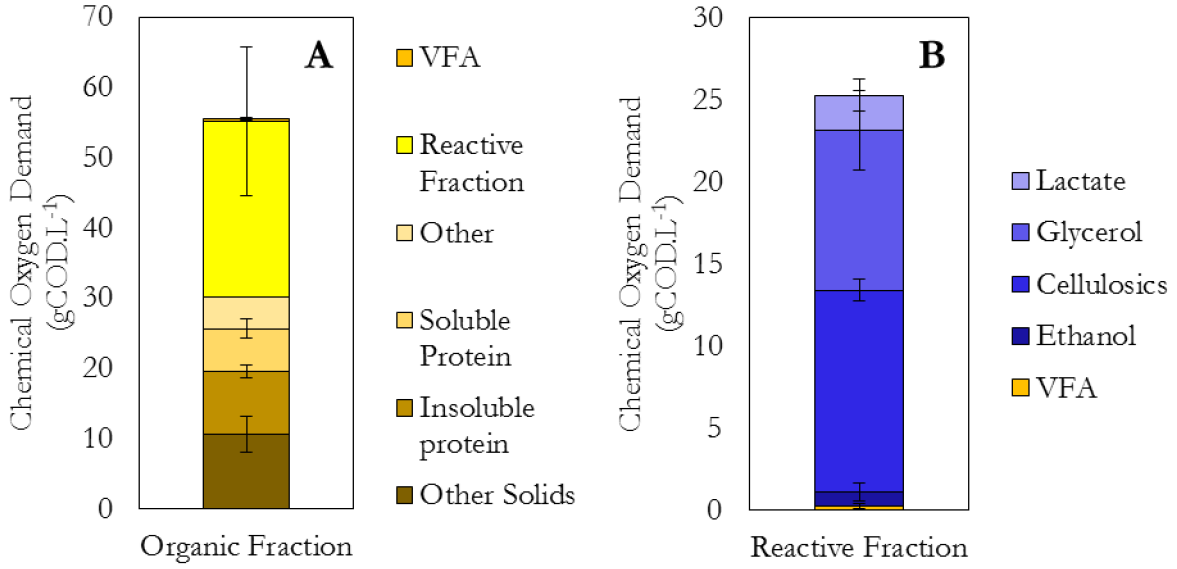

Figure 6. Stream characterisation of thin stillage. Figure A shows composition of the total organic fraction. Figure B shows the composition of the reactive fraction in detail. Note the difference in range on the axis of both figures.

The results of the detailed stream characterisation of thin stillage can be seen in Figure 6. Figure 6A shows the composition of the total organic fraction in thin stillage. Solids comprise approximately one third of the total COD in thin stillage (55.5 g total COD.L^{-1} with 19.5 g solid COD.L^{-1}). Even though these solids have not been wholly identified, nearly half of the solids may consist of insoluble proteins (8.9±0.9 gCOD.L^{-1}) assuming that all the nitrogen measured in the TKN analysis accounts for proteins or protein fragments. Previous work by Y. Kim et al. (2008) puts dry, crude protein content at approximately 0.8 g.L^{-1} or 1.6 gCOD.L^{-1} in thin stillage. Some nitrogen will be present as lignin fragments. The lignin content of corn is about 2 to 4% and may account for up to around 5 gCOD.L^{-1} in this thin stillage, considering that lignin may pass through the EtOH fermentation step unreacted. We were not able to accurately quantify the nitrogenous lignin fragments in this study. Other potential candidates for the remainder of the solids ('Other Solids' in Figure 6) are phenolic fragments, which could include ferulic, humic and fulvic acids, and lignocellulosic fragments. A one-point analysis of phenolics in thin stillage determined a concentration of 1.24 g.L^{-1}, which could account for 2.96 gCOD.L^{-1}, if this fraction was entirely made up of phenol. This data is not included in Figure 6A because no distinction could be made between the soluble and solid phenolics. Analysis of the carboyhydrate oligomers in these lignocellulosic compounds performed in literature shows that the main oligosaccharides present are glucan and xylan (oligomers containing resp. glucose and xylose) while arabinan (arabinose oligomer) is present in lower concentrations (Kim et al., 2008).

Soluble organics account for a total of 36.6±2.5 gCOD.L^{-1}. A large part of this soluble organics fraction has been aggregated in the 'Reactive Fraction'. These are the organic compounds that are

easily consumed by fermenting organisms and converted to VFA. As Figure 6B shows, the main part of this fraction is glycerol (9.8±2.4 $gCOD.L^{-1}$) followed by the cellulosics fraction (12.3±0.7 $gCOD.L^{-1}$). The sugar monomers present in this cellulosic fraction are mainly glucose, xylose and arabinose (respectively 4.7±0.7 $gCOD.L^{-1}$, 4.5±0.5 $gCOD.L^{-1}$ and 3.1±0.4 $gCOD.L^{-1}$). EtOH, Lactate and a VFA-mixture consisting mainly of Ac complete the Reactive Fraction with 0.8±0.6 $gCOD.L^{-1}$, 2.1±1.0 $gCOD.L^{-1}$ and 0.3±0.2 $gCOD.L^{-1}$ respectively. Soluble proteins account for 6.13±1.3 $gCOD.L^{-1}$ of the soluble COD. The remainder of this sCOD (4.6 $gCOD.L^{-1}$) is referred to as 'Other', most likely consisting of fats, oils and biomass.

When comparing the results of the Reactive Fraction analysis with literature analysis of thin stillage, the same compounds are found, but concentrations vary. Differences between production plants in the way corn is treated for production of bio-EtOH as well as variations in the process of a single bio-EtOH plant can contribute to variations in thin stillage composition. Kim et al. (2008) obtained glucose, xylose and arabinose concentrations of respectively 1.0, 0.7 and 0.4 $gCOD.L^{-1}$, (i.e. lower than concentrations determined in this study). On the other hand, glycerol and especially lactic acid concentrations were much higher, 17.5 $g.L^{-1}$ and 17.9 $g.L^{-1}$ respectively. Comparison of other characteristics of the thin stillage used in this study with literature shows a wide variation in thin stillage composition. A literature review of stillage by Wilkie et al. (2000) reports a corn thin stillage pH of 3.6, whereas the pH in this study was 4.86±0.15 (see Table 4). That same review mentions nitrogen concentrations up to 0.880 $mgN.L^{-1}$, whereas the thin stillage used in this study contains 1.50±0.25 $gTKN.L^{-1}$, nearly twice as high while not all nitrogen species (e.g. nitrate, nitrite) are accounted for with TKN. Total COD concentrations correspond rather well with Wilkie et al. (2000), reporting COD concentrations between 59.4 and 64.5 $gCOD.L^{-1}$ while the thin stillage used here contained 53.9±8.0 $gCOD.L^{-1}$. Other studies obtained higher concentrations, up to 131 $gCOD.L^{-1}$ (Lee et al., 2011). TS in the thin stillage are lower than most values reported in literature. While TS in this study are 4.37±0.26%, Lee et al. (2011) report values between 65.9 and 74.9 $g.L^{-1}$, Kim et al. (2008) obtain a value of 7.7% dry matter.

3.2. Electro-Fermentation

In the fermentation process, ME can be applied to separate and recover the products obtained during fermentation. An applied current across the electrochemical cell results in the extraction of VFA by migration of the anionic dissociated form over the AEM. Not only are VFA extracted, but H_2O in the thin stillage is being reduced at the cathode resulting in production of H_2 and OH^-. These products in turn may influence the fermentation process by changing the thermodynamic balances of the reactions taking place.

3.2.1. Influence of EF on VFA-production

The practical set-up used for the fermentation experiments performed with application of ME was shown in Figure 5. As feeding was performed manually every two days, no feeding pump was necessary. This reduced the complexity of the practical set-up (e.g. a fridge nearby was not necessary for feed storage), although it resulted in a less continuous reactor as fresh substrate was dosed in pulses. Experiments were performed in a temperature-controlled room, in this case at

34°C, which again resulted in a less complex set-up as no heating of the fermentation broth was necessary (e.g. with a heating mantel requiring a warm water bath).

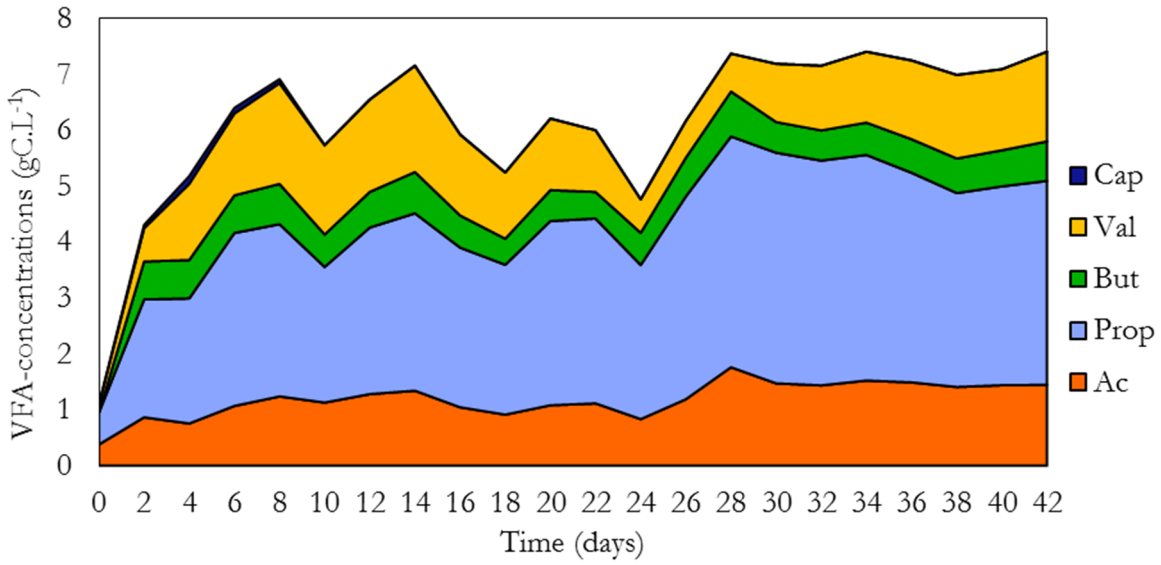

Figure 7. VFA-concentrations in the control experiment at pH 5.5

Figure 7 shows the VFA-concentration in the fermentation broth over the course of the control experiment (i.e. without ME). After start-up over the first HRT, the VFA-profile reached a relatively stable concentration very quickly. Especially the period from day 28 onwards displayed very stable VFA-concentrations.

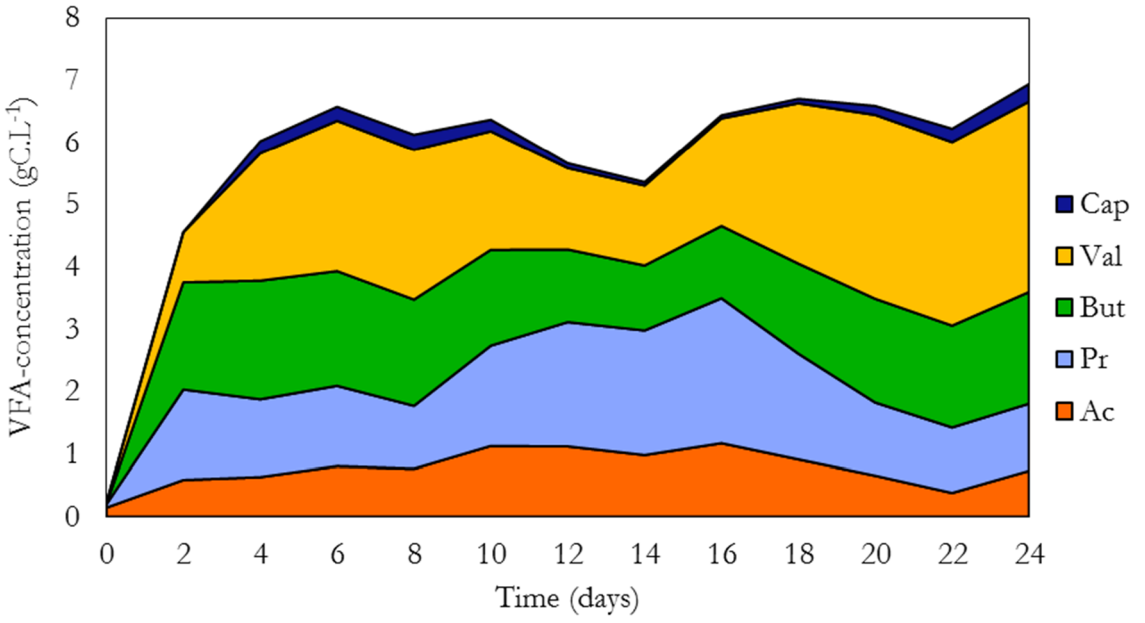

Figure 8. VFA-concentrations in experiment with electrolytic extraction at pH 5.5. VFA-concentrations are the sum of the concentrations in catholyte (fermenter broth) and anolyte.

The figure above shows the VFA-profile in the case of EF. This is the sum of the VFA present both in the fermentation broth and the anolyte. The focus of this experiment on the influence of electrolysis on the fermentation as opposed to the optimisation of VFA-extraction is important to

bear in mind here. The suboptimal extraction performance of the system is reflected in an extraction efficiency of 28.2±3.8% and a CE (i.e. how much of the current applied goes towards migration of VFA, of 15.1±11.0%). While the start-up time and maximum VFA concentrations are approximately the same, this graph already shows a clear shift in the type of VFA produced. This will be discussed further on in this section and in greater detail in Section 3.2.6. For comparison of VFA produced in both experiments, the mass of VFA present in fermentation broth and anolyte solution are summed and normalised over the volume of fermentation broth. As a consequence, the concentrations in Figure 8 are not observed concentrations but theoretical concentrations in the fermenter if VFA would not migrate to the anolyte.

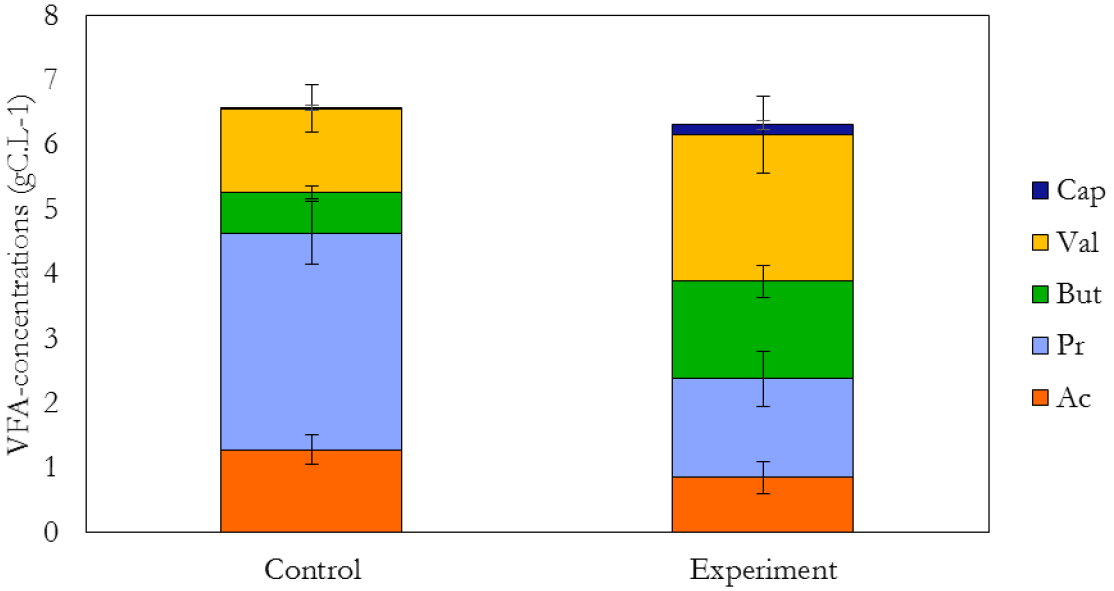

Figure 9. Comparison of average VFA-profile in control and experimental reactor

As can be seen in Figure 9, VFA-profiles are significantly different when comparing the case with and without ME. More specifically, EF significantly lowers Pr-concentrations (from 3.4±0.5 $gC.L^{-1}$ without ME to 1.5±0.4 $gC.L^{-1}$ with ME) while a significant increase of But (0.6±0.1 $gC.L^{-1}$ to 1.5±0.2 $gC.L^{-1}$), Val (1.3±0.4 $gC.L^{-1}$ to 2.3±0.6 $gC.L^{-1}$) and Cap (0.01±0.04 $gC.L^{-1}$ to 0.16±0.08 $gC.L^{-1}$) has been established. This shift towards longer chain VFA, especially But and Val, implies an elongation of Ac and Pr with EtOH-consumption, which will be elaborated on in Section 3.2.6. Despite this shift in the type of VFA produced there is no significant difference in the total product concentration obtained (6.6±0.7 $gC.L^{-1}$ without ME; 6.3±0.4 $gC.L^{-1}$ with ME) nor in the production rate (1.2±0.6 $gC.L^{-1}.d^{-1}$ without ME; 1.4±0.6 $gC.L^{-1}.d^{-1}$ with ME). The equal production rates and obtained concentrations imply that ME cannot be used as a means of accessing the unutilized COD in the thin stillage, at least under these conditions. When linking this to the stream characterisation, this means that electrolysis does not influence the reactive fraction of the thin stillage; none of the "Other Solids" in Figure 6 are solubilized to usable organic compounds.

3.2.2. Solids decrease and ME

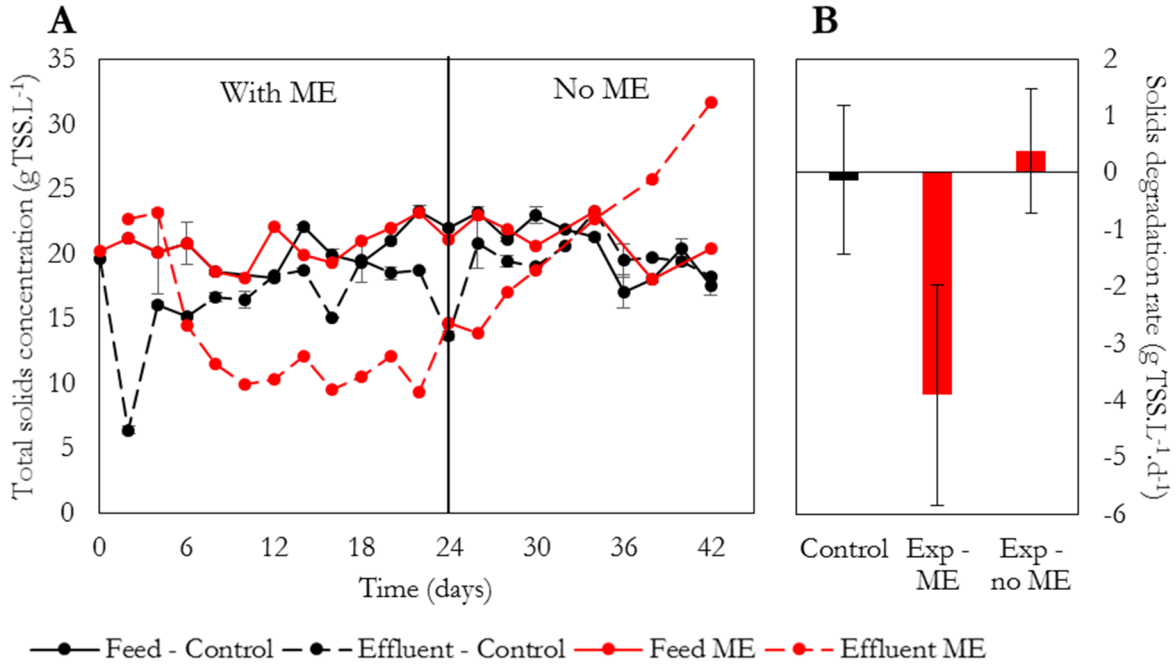

Figure 10. Solids behaviour in the control reactor and reactor with ME. Figure A shows TSS concentrations over time in the reactors. Black lines indicate case without ME, red lines case with ME. Full lines correspond with feed, dashed lines with effluent. Figure B shows average TSS-degradation rate in the control and experimental reactor (for both periods with and without ME). The vertical line indicates stopping of electrolytic extraction to test reversibility of ME.

Even though solid organics were not made accessible by ME, for instance by increased hydrolysis of solids at the cathode, it appears a decrease in solids content did take place when ME was applied. This can be seen in Figure 10A. The average solids concentration in the reactor with ME (11.5±1.7 $gTSS.L^{-1}$) was much lower than both the feed of the reactor (20.9±1.7 $gTSS.L^{-1}$) and the effluent of the reactor without ME (18.5±2.2 $gTSS.L^{-1}$). This results in a higher average rate of solids decrease in the reactor with ME (3.9±1.9 $gTSS.L^{-1}.d^{-1}$) compared to the case without ME (0.1±1.3 $gTSS.L^{-1}.d^{-1}$) as shown in Figure 10B. Neither VFA-production nor the COD-balance are affected by this decrease in solids, so electrolysis does not appear to hydrolyse nor degrade solids. The mechanism for this removal has not yet been explained but it seems likely that some form of electro-coagulation is taking place.

Electro-coagulation is a 3 step process that shows many similarities to the conventional coagulation process. In a first step an electrode is oxidised to produce a coagulant, usually a multivalent metal ion such as Al^{3+} or Fe^{3+}. This coagulant can destabilise the contaminant present in the water. Contaminants treated by electro-coagulation usually have a negative charge, as is the case for phosphates, suspended solids such as clay particles, etc. This negative charge also means a diffuse double layer of positively charged counter ions is present around the contaminant. The multivalent ions produced by the electrode oxidation can reduce the thickness of this double layer and neutralise the charge of the contaminants. At this point contaminant particles or molecules are no longer electrostatically repelled by one another and coagulation can take place under the influence of the Van Der Waals attraction. Since the particles or contaminants have been neutralised, this

does not result in a build-up of charge allowing continued coagulation and eventually results in floc formation. Any colloidal particles that still remain at this point can be trapped in the sludge blanket formed by the coagulation. As this process can remove contaminants with little to no chemical usage, its main application has been as a water treatment technology (Mollah et al., 2004). This process can precipitate soluble contaminants through coagulation, while in EF the opposite appears to take place (i.e. solubilisation of solids after destabilisation). Future research is necessary to establish whether this reversal of the electro-coagulation process is possible and its detailed mechanism causing the decrease in solids as well as the potential of using electro-coagulation to stabilise the colloids in thin stillage. Further experiments into this phenomenon are outside the bounds of this study.

After the 3 HRT of fermentation with ME, applied current was reduced to 0 A for a period of 2 HRT. The first being 6 days while the second was 12 days, cf. Section 2.2.3. This experiment was performed to investigate whether the effect of ME on the solids decrease was reversible. The main observation over this period was a rise in solids concentration as shown in Figure 10A, returning to that of the control reactor. As shown in Figure 10B, the rate of solids increase was 0.4±1.1 $gTSS.L^{-1}.d^{-1}$, which is significantly higher than the 3.9±1.9 $gTSS.L^{-1}.d^{-1}$ decrease when ME was applied and at the same time being insignificantly different from the degradation rate in the control reactor (-0.1±1.3 $gTSS.L^{-1}.d^{-1}$). An increase in solids concentrations and solids degradation rates similar to those found in a reactor without ME seems to imply that the effects of ME on the solids decrease are reversible. Electro-coagulation can again be used to explain this reversibility, as this would have no lasting effects after removing the current.

Considering the decrease of total suspended solids with ME, the extended HRT (12 days, cf. Section 2.2.3) experiment was executed to observe if a decrease in solids would occur at a longer HRT, implicating a hastening of some inherent solids hydrolysis by adding current. As mentioned before, solids concentration actually increased over this period in the reactor. VFA-concentrations also increased in this 12-day HRT period to 7.8±0.3 $gC.L^{-1}$ as opposed to 6.6±0.8 $gC.L^{-1}$, which is in accordance with a study on the influence of extended HRT and VFA-production (Colmenarejo et al., 2004). That same study also concluded that hydrolysis of solids increases at longer HRT, due to a larger decrease in solids, which seems to contradict the increase in solids over the 12-day HRT period observed here. These contradictory conclusions could be the result of using a new batch of thin stillage during the 12 day HRT period, influencing the fermentation in the reactor.

3.2.3. Substitution of NaOH by Membrane Electrolysis

The reduction of the fermentation broth in the electrochemical cell generates hydroxide ions which can replace caustic dosing used for pH control in a conventional reactor. The control reactor at pH 5.5 required an average of 25.0±17.1 mL $NaOH.d^{-1}$ to maintain the pH, while the experimental reactor required no NaOH dosing to maintain a pH of 5.5 when electrolytic extraction was applied. pH would actually increase if a current of 100 mA was maintained for longer than approximately 24 hours so acidic anolyte from the previous period was used to control pH at a maximum of 5.7. After 20 to 24 hours current was manually decreased from 100 mA to 10-20 mA to reduce anolyte dosing. This is done because overdosing of anolyte solution implies an unwanted return of

extracted VFA to the fermentation broth, resulting in energy for extraction being wasted, as continued extraction at high current requires both more energy and increased dosing of anolyte. In a fully realised system, an electronic control system could be used to control current to maintain pH at a desired setpoint, removing the necessity for dosing of anolyte.

This shows that NaOH-dosing can be substituted by electrolytic OH^- production without any significant change in total VFA production. The applied current could be coupled to the measured pH in the reactor to obtain a more efficient pH-control, which in turn can result in a system that does not require the dosing of anolytic acid.

3.2.4. Influence of HRT reduction

Apparently, much of the fermentation took place in the first 24 hours after feeding due to the observation that applied current needed to be reduced to avoid a pH-overshoot, as discussed in the previous section. Therefore HRT was reduced to 3 days for a 3 × HRT experiment. A logical consequence of this halved HRT is a doubling of the OLR into the reactor. The anolyte solution did not follow the reduction to a 3-day HRT but VFA were accumulated in 600 mL of anolyte solution to monitor the behaviour of extraction at increased VFA-concentrations in the anolyte. Current was constantly maintained at 100 mA during this phase of the reactor operation. For comparison with other experimental results (see Section 3.2.1), concentrations in the reactor were calculated by summing the mass of VFA in the fermentation broth with the increase of mass in the anolyte solution and dividing this by the reactor volume (1.2 L). As a consequence, concentrations reported below are not actually observed concentrations, but concentrations that would be found in the reactor without extraction of VFA.

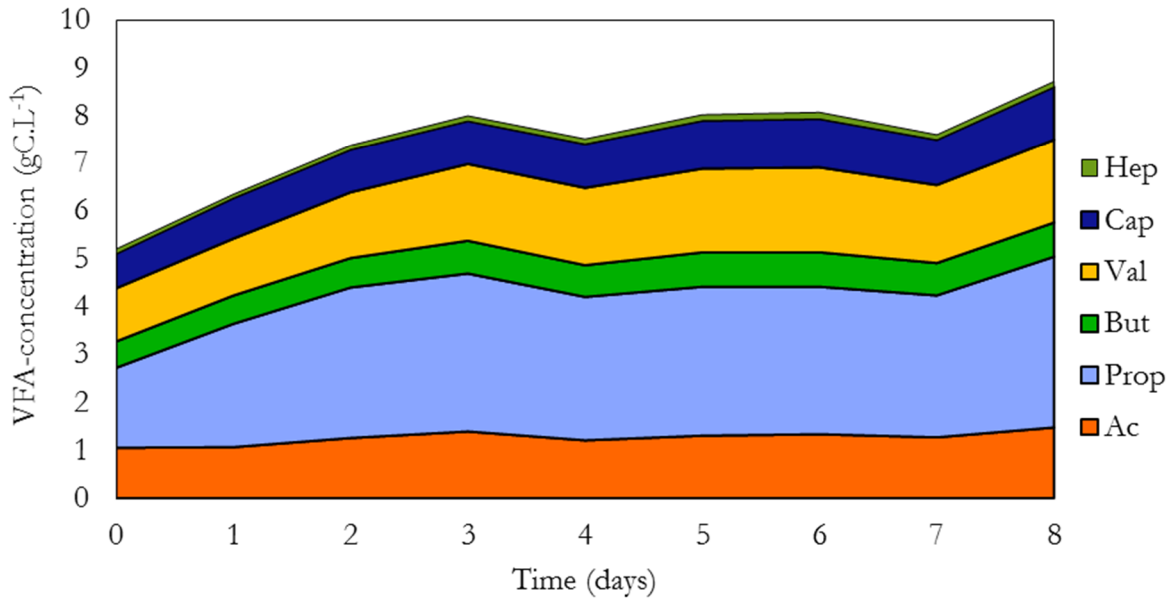

Figure 11. VFA-profile in the catholyte over the 9 days of the 3-day HRT experiment.

Figure 11 shows the concentrations of all VFA over the course of the 3 HRT. During the first HRT, VFA are built-up in the fermentation broth, while the following HRTs show a fairly constant VFA-profile. From this graph it is already visible that much of the increase in VFA is due to an

increased Pr concentration. The total VFA-production rate increased to 2.7±1.0 $gC.L^{-1}.d^{-1}$, which is nearly twice the production rate when ME was applied with an HRT of 6 days. This doubling of the production rate when OLR doubles suggests that it is not the reaction rate which limits VFA-production but OLR itself. Consequently, increasing OLR even further by decreasing HRT might further increase the production rate.

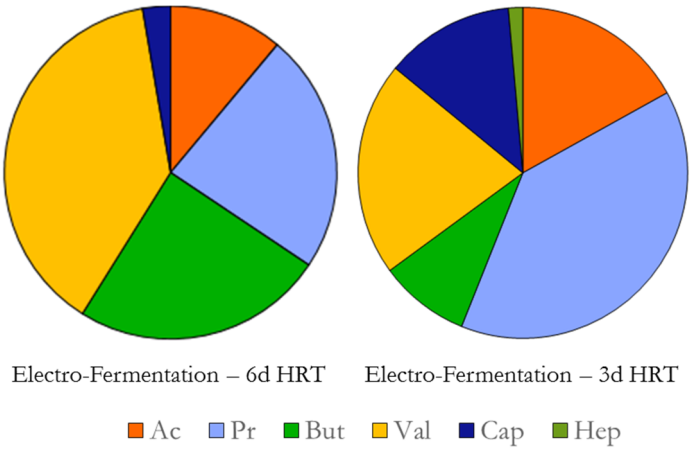

Figure 12. Comparison of the VFA-profile with a 6-day and 3-day HRT when ME is applied.

When comparing the VFA-profile in the case for the 3-day HRT and 6-day HRT, as shown in Figure 12, it becomes clear that But and Val decrease (23.8±3.4% to 8.9±0.6 % for But; 35.6±7.9% to 20.9±1.5% for Val), while Pr, Cap and Hep increase significantly (24.5±7.5% to 39.0±0.3% for Pr; 2.4±1.1% to 12.8±1.5% for Cap; 0% to 1.4±0.3% for Hep). These changes indicate a trend for decreased elongation as the fraction of Ac and Pr increases significantly (38.1±11.3% to 55.9±3.0%). The elongation that does take place in the fermenter is continued to the end products (Cap and Hep) much more at a 3-day HRT than it is at a 6-day HRT.

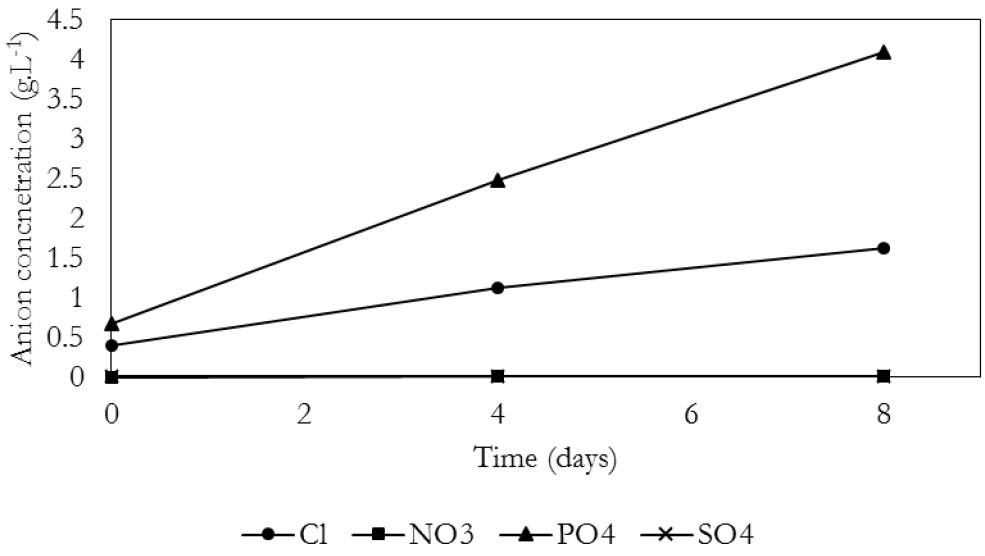

Figure 13. Accumulation of anions in the anolyte solution during the 3-day HRT experiment.

The membrane used for separation of the anolyte and catholyte is an AEM. As this is not selective for anionic VFA only, other anionic species can also migrate from catholyte to fermenter. An anion

analysis of the anolyte solution revealed a clear increasing trend of anion concentrations over the 3-day HRT experiment, see Figure 13. For comparison, the anion concentrations in the effluent over this period were 0.20±0.04 $gCl^{-}.L^{-1}$, 0.01±0.01 $gNO_3^{-}.L^{-1}$, 3.0±0.1 $gPO_4^{3-}.L^{-1}$ and 0.07±0.02 $gSO_4^{2-}.L^{-1}$ while these accumulated to 1.6 $gCl^{-}.L^{-1}$, 0.01 $gNO_3^{-}.L^{-1}$, 4.1 $gPO_4^{3-}.L^{-1}$ and 0.02 $gSO_4^{2-}.L^{-1}$ respectively. This figure also shows that Cl^{-} migrates through the membrane very easily, accumulating up to 7.9 times higher concentrations than those in the effluent after 9 days. This accumulation of Cl^{-} in the oxidative environment of the anode can produce dangerous by-products such as haloacetic acids, trihalomethane and chlorate (ClO_3^{-}) (Bagastyo et al., 2011). These compounds have been linked with increased risk of cancer (haloacetic acids and trihalomethane) and impairment of neurological development (chlorate) (Righi et al., 2014). The accumulation of PO_4^{3-} on the other hand could be very promising for nutrient recovery (Maaß et al., 2014).

3.2.5. Product inhibition of the fermenting community

Carboxylate platform processes are often limited by product inhibition (Agler et al., 2011). This is the phenomenon where production rates are decreased due to concentration of the product in the environment of the micro-organisms. This product can act on one or more enzymes in the production pathway of the product. Usually the effect of the product increases with increasing product concentrations, causing a decrease in production rate (Siegert & Banks, 2005). VFA-concentrations in thin stillage were artificially increased and the influence of high VFA-concentrations on the community (e.g. production rates and obtained products) was investigated.

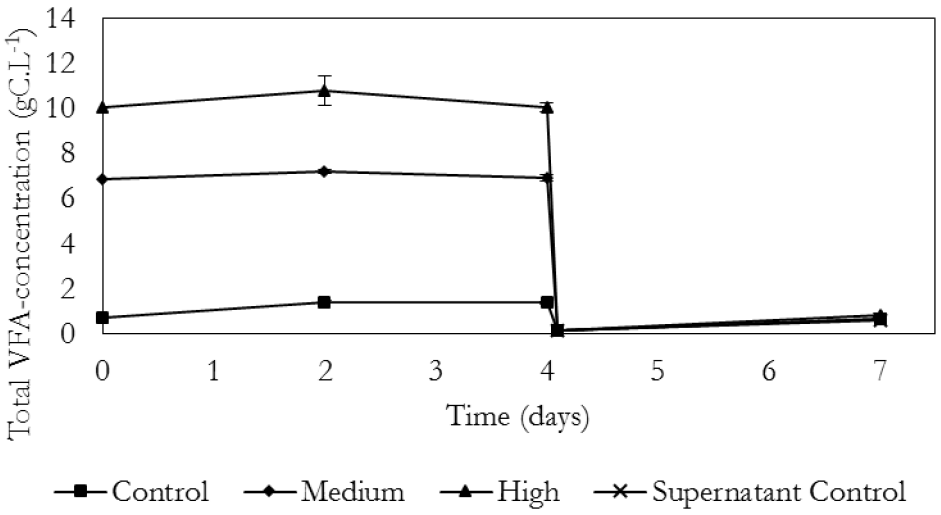

Figure 14. Total VFA-concentrations for each experiment. Error bars may be obscured by medallions

Figure 14 shows the total VFA-concentration in each experiment. On Day 0 the initial concentrations were 0.8 $gC.L^{-1}$ (1.6 g $VFA.L^{-1}$), 6.9 $gC.L^{-1}$ (14.1 $gVFA.L^{-1}$) and 10.0 $gC.L^{-1}$ (20.5 $gVFA.L^{-1}$) for the Control, Medium and High experiments. Proportions of VFA in these experiments were identical: 20% Ac, 50% Pr, 10% But and 20% Val on gC basis. This proportion was derived from the proportions present in the fermenter at pH 5.5 without ME. As can be seen in Figure 14, VFA-concentrations increase in all experiments between Day 0 and Day 2, indicating fermentation proceeded in all cases. Between Day 2 and Day 4 concentrations of VFA decreased

significantly for the Medium experiment (7.20±0.07 $gC.L^{-1}$ to 6.93±0.13 $gC.L^{-1}$) while no significant change was noted in the Control experiment and the High experiment. On Day 4 all triplicates were put together and centrifuged. The supernatant was removed and replaced with an equal volume of supernatant from centrifugation of fresh thin stillage after which solids were resuspended. These resuspended broths were split in triplicates and then fermented for 3 additional days at 34°C. As a control experiment, a duplicate with thin stillage supernatant was also added to the experiment. Due to a lack of samples after resuspension on Day 4, the assumption is made that VFA-concentrations in the resuspended broths are equal to the VFA-concentration of the fresh thin stillage supernatant. On Day 7 only the concentration in the High experiment is significantly different from the concentration in the Supernatant Control (0.82±0.08 $gC.L^{-1}$ for High; 0.64±0.04 $gC.L^{-1}$ for Supernatant Control).

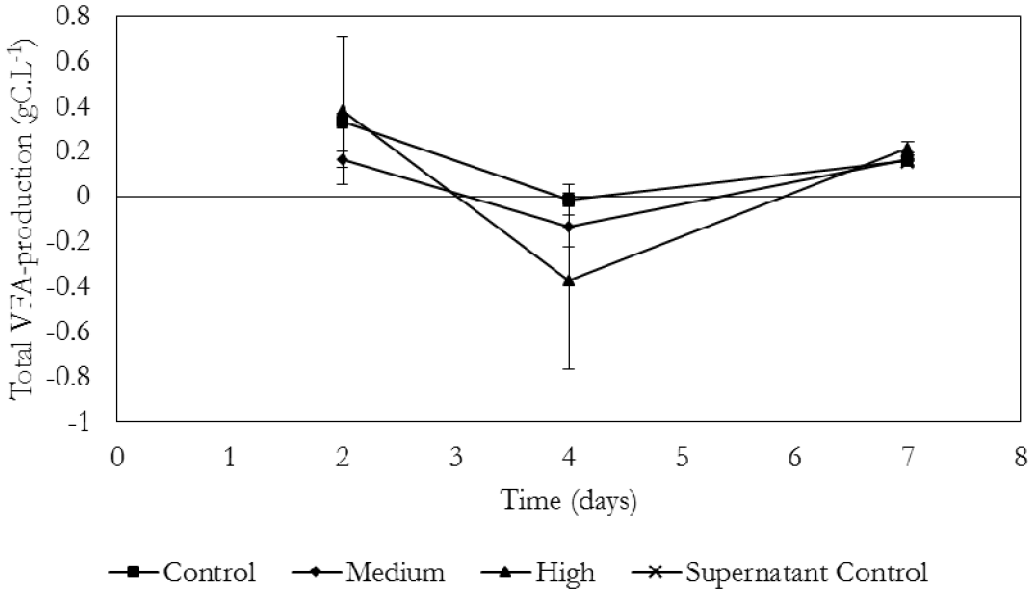

Figure 15. Total VFA-production rates for all experiments. Note that 'Supernatant Control' has only 1 datapoint (Day 7).

When studying the influence of product inhibition, the best comparison can be made by using production rates as a measure for activity. Production rates are shown in Figure 15. On Day 2 production rates in the Medium experiment were significantly lower than in the control case (0.16±0.03 $gC.L^{-1}.d^{-1}$ for Medium; 0.33±0.03 $gC.L^{-1}.d^{-1}$ for Control), while the High experiment was not significantly different from either (0.38±0.33 $gC.L^{-1}.d^{-1}$). On Day 4 all production rates were insignificantly different, although only the Medium case showed a significantly negative production (-0.02±0.07 $gC.L^{-1}.d^{-1}$ for Control; -0.13±0.09 $gC.L^{-1}.d^{-1}$ for Medium; -0.37±0.39 $gC.L^{-1}.d^{-1}$ for High). Similar to the total VFA-concentrations, only the production rate for the High case was significantly higher on Day 7 than the control case (0.21±0.03 $gC.L^{-1}.d^{-1}$ for High;0.16±0.00 for Control).

Several causes might have led to this difference on Day 7. pH was not controlled in the batch tests which means pH could decrease by acidogenic fermentation to a stable pH. Since VFA have a pKa around 4.8 their buffering capacity is the largest between approximately pH 4 and pH 5.5 (see

Section 1.5.1). The same VFA-production results in a smaller pH-decrease when VFA-concentrations are higher in the broth. As a result, pH on Day 4 was significantly higher in the Medium and High experiments compared to the control case (Control: 4.01±0.01; Medium 4.83±0.00; High: 4.99±0.02). The higher pH in the High experiment might be the reason for the slightly increased activity after centrifuging as the lower pH might be inhibitory for the acidogenic fermenters. The increased production rate might also be a result of the assumption that the VFA-concentration in the broth was equal to the VFA-concentration in the fresh thin stillage supernatant. If the solids portion of the fermentation broth after centrifugation still contained some VFA, concentrations might have been higher than in the thin stillage supernatant. This increased concentration at the start would then result in an increased concentration at the end of the experiment, resulting in an apparent higher production rate.

3.2.6. Biochemical pathway analysis

Although no specific experiments were carried out to test the biochemical pathways catalysed during this fermentation, the substrate (cf. stream characterisation) and the obtained products can give some insight into the biochemical reactions in the fermenter with and without ME.

3.2.6.1. Speculation on biochemical pathways present

The three main substrates for fermentation present in the thin stillage (i.e. cellulosics, glycerol and lactate) each have specific biochemical pathways for fermentation resulting in different products.

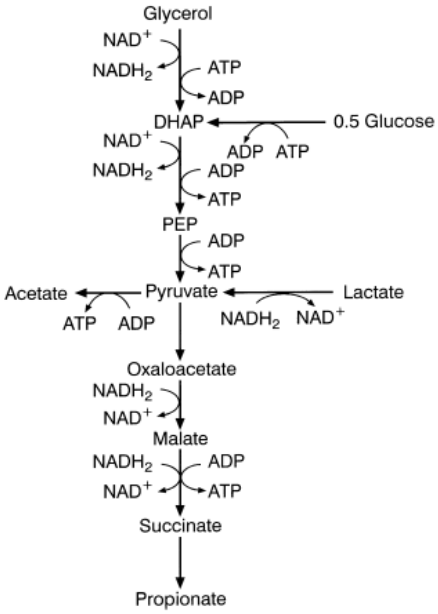

Figure 16. Simplified representation of the fermentation pathways leading from glycerol, glucose and lactate to Pr or Ac (Barbirato et al., 1997)

The glycerol present in the fed thin stillage is most likely converted into Pr, as can be seen in Figure 16 (Barbirato et al., 1997). The pathway towards Pr production can also be used to ferment glucose and lactate, both present in the broth, towards Pr. For reasons that will be elaborated on further (Section 3.2.6.2) it seems likely that lactate is also fermented at least partly to Pr, while glucose appears to result mainly in other products such as Ac and But.

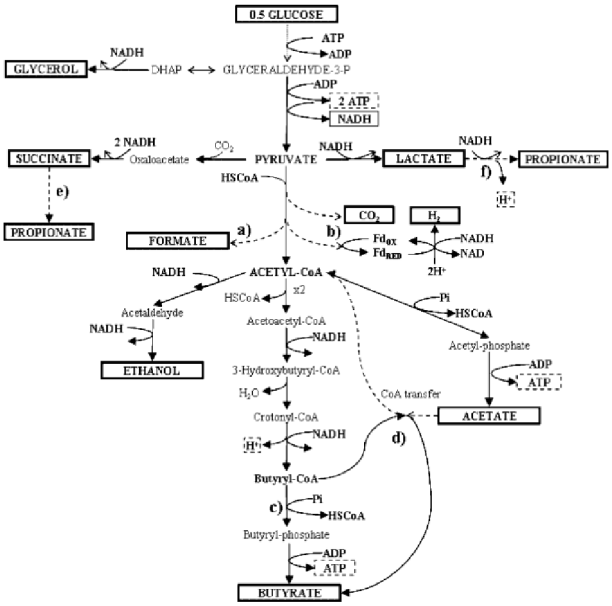

Figure 17. Fermentation pathways starting from glucose, resulting in a mix of glycerol, lactate, Pr, EtOH (Temudo et al., 2007)

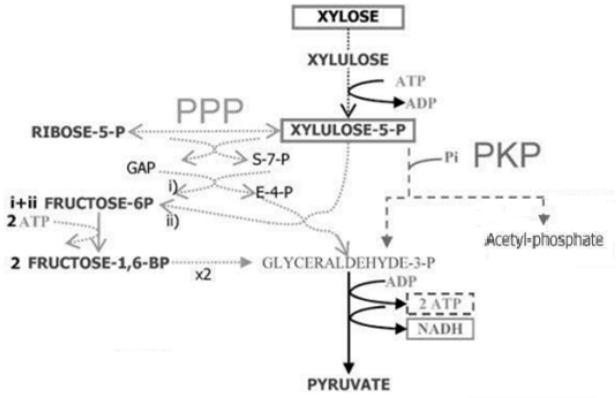

Figure 18. Fermentation pathway for the initial conversion of xylose to pyruvate. After this conversion the pathway proceeds as shown in Figure 17 (adapted from Temudo et al., 2009)

Cellulosics fermentation depends on the type of sugar as can be seen in Figure 17Figure 18. Anaerobic fermentation of glucose and xylose monomeric sugars takes place through pyruvate as an intermediate. The reactions taking place to reach this pyruvate are dependent on the type of monomer being processed. While glucose is converted into glyceraldehyde-3-phosphate and subsequently to pyruvate by glycolysis (Temudo et al., 2007), xylose, which is a five carbon (C5) sugar as opposed to the six carbon (C6) glucose, is first converted into xylulose-5-phosphate before conversion into glyceraldehyde-3-phosphate. Because xylose is a C5-sugar, conventional glycolysis through the Embden-Mayerhof-Parnas Pathway is not possible. Two alternative pathways can then be applied, namely the Pentose Phosphate Pathway (PPP) or Phosphoketolase Pathway (PKP). Both result in the conversion from xylulose-5-phosphate to glyceraldehyde-3-phosphate, which can then be converted into pyruvate cf. regular glycolysis. According to Rodríguez et al. (2006) fermentation of glucose predominantly yields Ac, But or EtOH depending on pH, H_2-pressure and substrate concentration. For instance, an increase in H_2-pressure (e.g. by cathodic reduction of H_2O) results in an increased butyrate concentration. The other possible outcomes of the glucose fermentation pathway such as glycerol, succinate, formate and lactate are usually present in lower concentrations (Rodríguez et al., 2006; Temudo et al., 2007). Due to xylose fermentation through different pathways (PPP or PKP), the resulting product profile contains more But and less EtOH compared to glucose fermentation (Temudo et al., 2009).

The lactate present in the thin stillage can be converted into Ac and Pr (Seeliger et al., 2002; Barbirato et al., 1997), while lactate can also be used to elongate Ac to butyrate in the presence of H^+ (Spirito et al., 2014). Lactate is not the conventional substrate used for chain elongation of VFA as typically EtOH is used (Bornstein & Barker, 1948). This elongation results in transformation of Ac to But and But to Cap or Pr to Val which can in turn be elongated to Hep. Multiple sources of

EtOH can be used for this elongation, for instance the EtOH inherently present in the fed thin stillage, although concentrations are usually low, or EtOH resulting from fermentation of glucose as mentioned before.

The most direct influence of ME on the biochemical reactions being catalysed in the fermenter is the production of H_2 by cathodic H_2O-reduction. This H_2 along with CO_2 produced by fermentation can be converted to Ac through the Wood-Ljungdahl pathway by acetogenic organisms. Ac can then be reduced to EtOH in the presence of H_2 with consumption of H^+ in bio-electrochemical systems (Marshall et al., 2013). It has already been shown that this reduction of carboxylates can take place in syngas (H_2/CO_2) fermentations (Perez et al., 2013), of which the conditions are rather similar to the fermentation performed here with ME.

3.2.6.2. Linking potential pathways to fermenter output

When considering all the reactions performed in this fermentation, one would expect the products of fermentation to be a mix of different VFA. Using the results of the stream characterisation (i.e. concentrations of glucose, lactate and glycerol) a simplified stoichiometric model (equations shown in Table 5) was built in an attempt to allocate each carbon source to a fermentation product. No attempt was made to close the carbon balance, so CO_2 was not taken into account in these reactions. Glycerol was assumed to be completely converted to Pr (Barbirato et al., 1997) while lactate was converted to both Ac and Pr. The inherent multi-product outcome of cellulosics fermentation makes it difficult to provide a closed stoichiometric equation containing all products. As a solution, experimental product yields found in literature were used to make a stoichiometric equation. Glucose, xylose and arabinose were aggregated, taking into account the number of carbons, and assumed to ferment as glucose in spite of the distinct biochemical pathways for C5 and C6 sugars. Note that this is a very crude simplification of the biochemically catalysed reactions. Few fermentations of lactate actually result in a 2:1-ratio of Pr (Piveteau, 1999) nor do fermentations of glycerol actually yield pure Pr (Barbirato et al., 1997). Chain elongation, homoacetogenesis and other reactions speculated to take place (see Section 3.2.6.1) are not taken into account. The results of this simplified model, as well as comparison with the experimental outcomes, are shown in Figure 19.

Table 5. Stoichiometric reactions taken into consideration in the substrate to product allocation calculations.

Reaction	Source
Glucose → 0.4 Ac + 0.05 Pr +0.6 But + 0.1 EtOH	(Temudo et al., 2007)
3 Lactate → 1 Ac + 2 Pr	(Piveteau, 1999)
1 Glycerol → 1 Pr	(Barbirato et al., 1997)

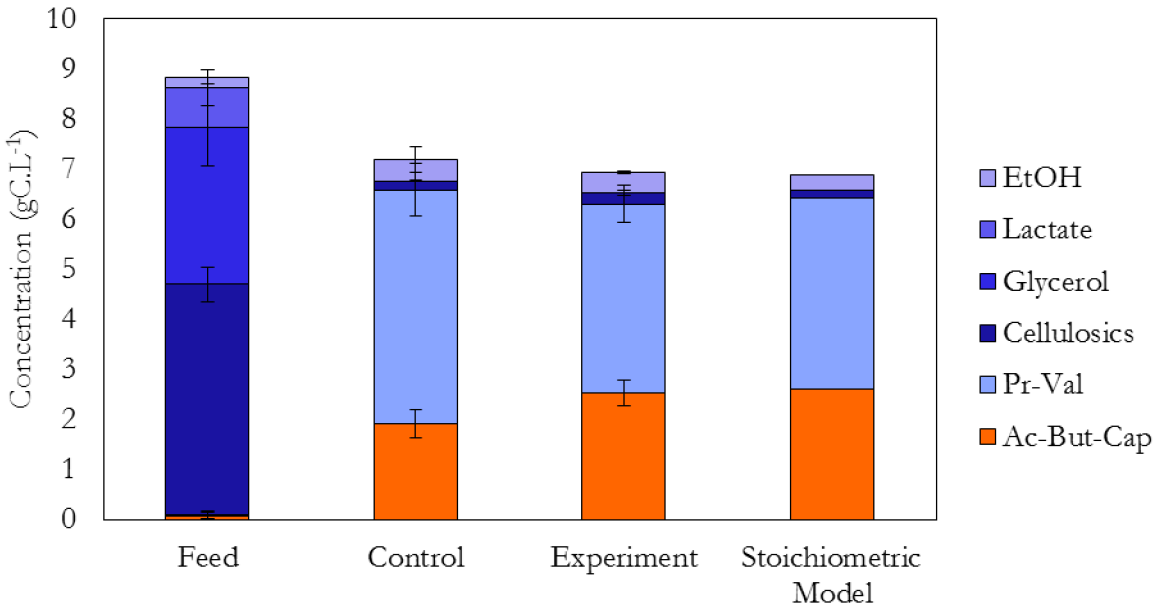

Figure 19. Comparison of product profiles obtained in Control and Experiment reactors and the outcome of a simplified stoichiometric reaction model based on stream characterisation of thin stillage.

The figure above shows that the stoichiometric model closely mimics the actual fermentation process, despite the assumptions. The total concentration of the substrates and products according to these conversion reactions is 6.9 $gC.L^{-1}$ - which closely fits the 7.2±0.8 $gC.L^{-1}$ and 6.9±0.4 $gC.L^{-1}$ of the control and electro-fermentation experiment respectively. Comparison between the model and the experiments is made by studying concentrations of Ac-But-Cap and Pr-Val instead of focusing on the specific obtained VFA, due to chain elongation not being integrated in the model. Comparing these figures, a rather similar structure of the fermentation outcome is obtained. The calculated concentrations of Ac-But-Cap are outside the uncertainty range of the control experiment, although no uncertainty was calculated in the product allocation. The calculated concentrations do fall in the range of the EF experiment (Ac-But-Cap: 2.6 $gC.L^{-1}$ calculated; 1.9±0.3 $gC.L^{-1}$ for Control; 2.5±0.3 $gC.L^{-1}$ for Experiment). The same is true for Pr-Val concentrations; calculated concentrations are outside the uncertainty range of the control experiment but are inside the range of concentrations obtained during EF (Pr-Val: 3.8 $gC.L^{-1}$ calculated; 4.7±0.5 $gC.L^{-1}$ for Control; 3.8±0.4 $gC.L^{-1}$ for Experiment). Further, the fermentation of cellulosics does not appear to follow the stoichiometrics obtained from glucose fermentation in literature, possibly due to the C5-sugars or the use of a non-synthetic medium for the fermentation experiments, contrary to the experiments found in literature. Modeling of the fermentation was, however, not the purpose of this research and the similarity obtained between a theoretical and experimental fermentation are striking enough to conclude that these reactions might account for at least some of the fermentative conversions.

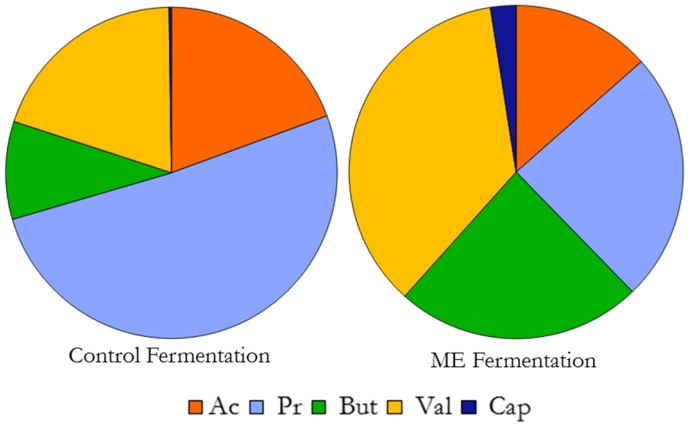

Figure 20. Comparison of VFA-output from fermentation with and without ME

When ME is applied, a shift can be seen in the reactions occurring. The proportion of Pr drops while Val increases, as can be seen in Figure 8 and Figure 20, indicating chain elongation of Pr to Val. As mentioned in Section 0 these differences are significant, as are the increase in But and Cap concentration.

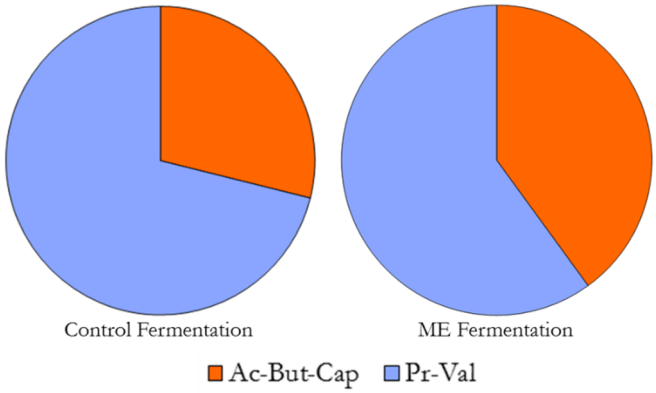

Figure 21. Comparison of relative proportions of Ac, But and Cap compared to Pr and Val

Figure 21 shows total carbon in the form of Pr and Val has decreased by EF from 4.6±0.5 $gC.L^{-1}$ to 3.8±0.4 $gC.L^{-1}$. While this decrease is not significant, the increase of total carbon in the form of Ac, But and Cap is, as this rises from 1.9±0.3 $gC.L^{-1}$ to 2.5±0.3 $gC.L^{-1}$. This is the result of a shift in VFA production from Pr and Val to Ac, But and Cap. Because of the equal end concentrations, the increase in Ac-But-Cap and decrease in Pr-Val match nearly completely, implying that the shift could be the result of increased Pr-degradation by acetogenic processes to Ac, H_2 and CO_2. The CO_2 coming from this process could subsequently be used by homoacetogenic organisms to produce Ac. Another explanation of this shift might be otherwise changed thermodynamic equilibria due to increased H_2-presence resulting in a shift from Pr to Ac, e.g. decreased conversion of pyruvate to Pr and an increase towards Ac as shown in Figure 16.

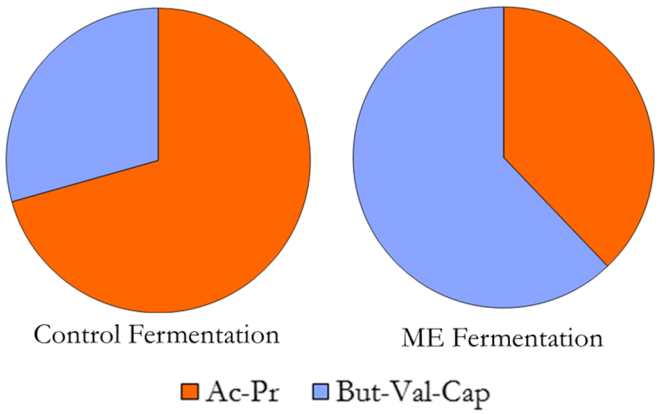

Figure 22. Comparison of proportions of short VFA (Ac, Pr) and longer VFA (But, Val, Cap)

As mentioned before in this section, applying ME results in more longer-chain VFA. Figure 22 confirms this result once more showing a clear shift from predominantly short-chain VFA (defined as Ac and Pr in this study) without ME to a profile dominated by longer-chain VFA (defined as But, Val and Cap in this study) with ME. Concentrations of the longer-chain VFA approximately double from 1.9±0.4 $gC.L^{-1}$ to 3.9±0.9 $gC.L^{-1}$, a result of the significant increase in But, Val and Cap concentrations. This strongly suggests that ME provides EtOH in some way to the reactor. The most likely mechanism is the reduction of Ac in the presence of H_2 to EtOH (Marshall et al., 2013) especially considering the theoretical H_2-production of 0.31 L H_2.L $reactor^{-1}.d^{-1}$. EtOH produced in this way can cause a shift from Pr to Val, Ac to But and But to Cap by chain elongation. It should be noted that although elongation of Ac and Pr to But and Val respectively are taking place, the elongation of But to Cap is not taking place at the same rate resulting in rather low Cap concentrations. A possibility is that all the EtOH produced by Ac-reduction and glucose fermentation is already used in the elongation now taking place, although this is unlikely as there is still 1.61±0.09 $gC.L^{-1}$ EtOH left. Another explanation might be that the But concentrations do not rise high enough for elongation to Cap to start up (Agler et al., 2014).

The shift from Ac to But has been attributed to EtOH earlier in this section, although it is unsure whether this rise in But and decrease of Ac is actually due to chain elongation or if it is the result of a changed thermodynamic situation related to the increased H_2-pressure as predicted by Rodríguez et al. (2006).

The result of Electro-Fermentation is then a more reduced VFA-output. The application of ME provides reductive force for the fermentation by H_2-production resulting from cathodic H_2O-reduction. This H_2 can drive the reduction of CO_2 and Ac to Ac and EtOH respectively, the latter of which can reduce the short-chain VFA (Ac and Pr) to longer-chain VFA (But, Val, Cap), but a deeper analysis of the community is required to confirm this.

3.2.7. Community Analysis

While the results of the Illumina-analysis are still pending, some speculations can be made regarding the composition of the community. We assume the thin stillage has a microbial community present in the broth as it is a complex, non-sterile organic broth, the waste fraction of the biological fermentation to EtOH. Distillation around the water-EtOH azeotrope (approx. 80°C) is used to

separate the EtOH from the beer. This elevated temperature should eliminate most heat-sensitive organisms, such as yeast (Banat et al., 1998), present in the beer. Some organisms can form heat-resistant endospores which can survive the distillation process. Typical examples of such species closely related with anaerobic (fermentation) processes are species belonging to the *Clostridia* and *Bacilli* classes.

The wide range of substrates and accompanying conversion reactions allows the growth of a wide range of micro-organisms, which implies a diverse community. Hollister et al. (2012) analysed the metagenome of a mixed culture fermenting lignocellulosic waste to VFA. This analysis confirmed that mesophilic fermenting communities are very diverse. The communities consisted mostly of several *Clostridium*- and *Bacillus*-species while the most important other groups were *Bacteroidia*, such as *Bacteroides sp.,* and *γ-Proteobacteria*, such as *Klebsiella pneumonia*. The feed used in this fermentation process was pre-treated sorghum, which is vastly different from the thin stillage. No stream characterisation data were available from the pre-treated sorghum, which makes it hard to extrapolate the results to thin stillage fermentation. The apparent presence of some major biochemical pathways as described in Section 3.2.6 can be used to link these pathways to micro-organisms present in the fermenting community.

Glycerol fermentation to propionate for instance can be performed by some *Propionibacterium* species or *Clostridium propionicum* (Barbirato et al., 1997). Some *Propionibacterium* species, such as *P. acnes,* are sensitive to increased temperatures as less than 0.1% of cells are viable after a heat treatment of 20 minutes at 80°C (Lyte et al., 2009). Their presence in fed thin stillage will then most likely be negligible and glycerol fermentation to Pr could be performed by *C. propionicum*.

The Wood-Ljungdahl pathway to produce Ac from H_2 and CO_2 can be performed by several genera such as *Acetobacterium* or *Sporomusa*. *Acetobacteria* do not form endospores so it is again unlikely that they will be present in the community. *Sporomusa* and *Clostridium ljungdahlii* on the other hand do possess the capacity to perform the Wood-Ljungdahl pathway (Leang et al., 2013) and form heat-resistant endospores (Yutin & Galperin, 2013). As a consequence these are the most likely acetogens to be present in the community. Besides the Wood-Ljungdahl pathway, *C. ljungdahlii* also possesses the capability of reducing carboxylates to their corresponding alcohols, as do *C. ragsdalei*, *C. coskatii* and *C. autoethanogenum* (Perez et al., 2013). While the carboxylate reduction is not a major outcome of the performed experiments, it is the most likely mechanism producing EtOH from Ac when ME is applied. This is especially likely since the experiments conducted by Perez et al. (2013) used syngas (H_2/CO_2) fermentation while the headspace of the thin stillage fermentation with ME also consisted mainly of H_2 and CO_2.

Several species can perform the fermentation of lactate to Ac and Pr such as *Propionicum freudenreichii* and *Pelobacter propionicus* although these species are not capable of forming endospores. Therefore, members of the *Clostridia* class are once again most likely to be present in the community to perform this fermentation, i.e. *C. homopropionicum*, *C. neopropionicum* and *Veillonella parvula* (Seeliger et al., 2002; Yutin & Galperin, 2013). Elongation of Ac to But with Lac has been shown possible by *Megasphaera elsdenii* which is also capable of forming spores and thus may be present in the thin stillage (Marshall et al., 2013; Yutin & Galperin, 2013). The presence of lactate in the thin stillage might indicate the

presence of *Lactobacillae* before distillation. Due to their inability to form endospores (Madigan et al., 2012), it appears unlikely these will still be active in thin stillage.

A last key species that is likely to be present is *Clostridium kluyveri* which is capable of performing chain elongation of VFA with EtOH (Bornstein & Barker, 1948). It has been established that this species is often present in mixed microbial communities when chain elongation of VFA is the focus (Van Eerten-Jansen et al., 2013; Steinbusch et al., 2011; Agler et al., 2012). As it appears very likely - based on the VFA-profile - that chain elongation is taking place in EF, it is expected that *C. kluyveri* will be present in the fermenting community.

These experiments demonstrate that ME can influence the VFA-profile from the fermentation process by providing reducing power to the fermentation broth. This influence is limited to a slight elongation of the produced VFA, while there is no significant effect on the total VFA output of the process. There is no significant improvement of the fermentation process due to ME, although its application allows for separation and recovery of the products obtained through fermentation. ME also has the capability of substituting the input of chemicals by electricity usage for the pH control of thin stillage fermentation.

3.3. Methanogen control in a fermenting reactor

3.3.1. Fermenting reactor at pH 7

Fermentation at pH 7 should theoretically result in more efficient fermentation due to reduced stress related to pH and VFA-toxicity. A major downside to fermentation at a high pH, however, is the possibility of methanogen incursion, resulting in the loss of VFA to CH_4.

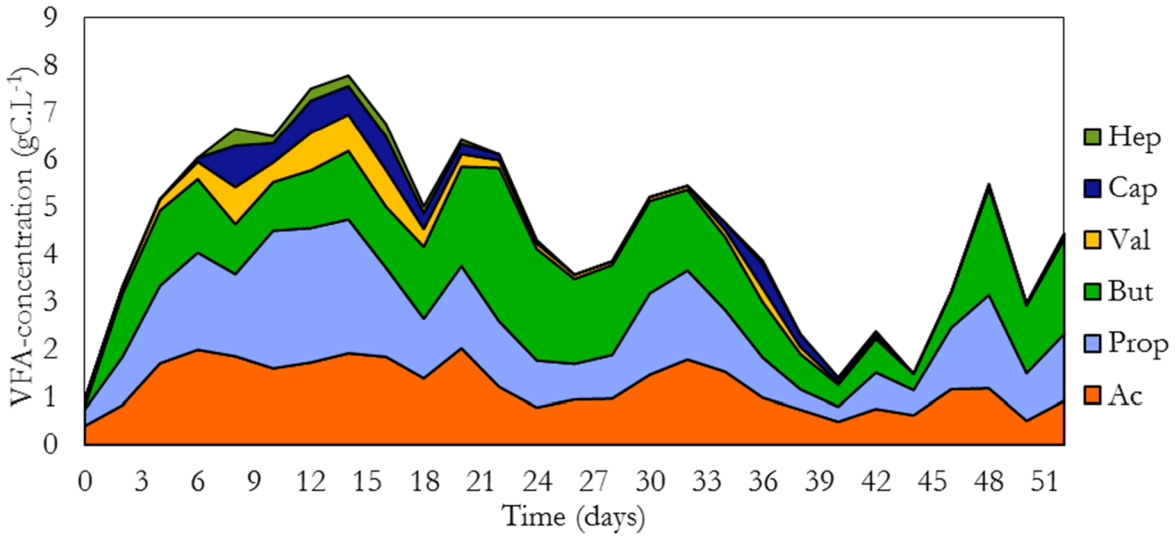

Figure 23. VFA-profile over time in a fermenting reactor operated at pH 7

Figure 23 shows the VFA-output of the fermenter operated at pH 7 for just over 8 × HRT . Due to some operational instability, linked with problems in pH control and feed, this reactor was less stable than the reactor operated at pH 5.5. As a result, a pseudo steady-state was only reached for a short time-frame between Day 6 and Day 15, for approximately 1.5 × HRT. As this is too short to make statistical statements on the fermentation process, steady-state concentrations used further

on are calculated from the data from Day 6 to the end of the experiment. The operational instability also caused a decreased methanogenic activity in the reactor. Examples can be found in literature of fermentation processes at pH 7 resulting in higher CH_4-production rates (Lindner et al., 2015) or processes choosing a lower pH to avoid methanogenic activity (Agler et al., 2014). If acidogenic fermentation did take place at pH 7, other methods were used to inhibit methanogens such as thermal pre-treatment (Steinbusch et al., 2009) or addition of 2-bromoethanesulfonate (Chae et al., 2010; Steinbusch et al., 2011). Apparently, it is not realistic to design a process aimed at VFA production at a pH of 7 without some form of methanogen control. If a strategy can be developed to effectively remove methanogens in case of an incursion, the process would have the benefits of a fermentation process at pH 7 (i.e. decreased VFA toxicity and pH stress) without the disadvantage of undesirable CH_4-production.

3.3.2. pH shocking as a strategy to remedy methanogen incursions

Due to the fact that CH_4 was never detected in the reactor at pH 5.5 and the pH-sensitivity of methanogens (Chen et al., 2008; Appels et al., 2008) a strategy was developed to control methanogenic activity in a reactor operated at pH 7. By dropping the reactor to an acidic pH 5.5, or "pH shocking," the more sensitive methanogens can be eliminated while fermenters, being more acid-tolerant, remain active.

3.3.2.1. pH-shock batch test

This concept was first tested in a batch experiment using effluent from a CH_4-producing mesophilic digester as explained in Section 2.5.2.

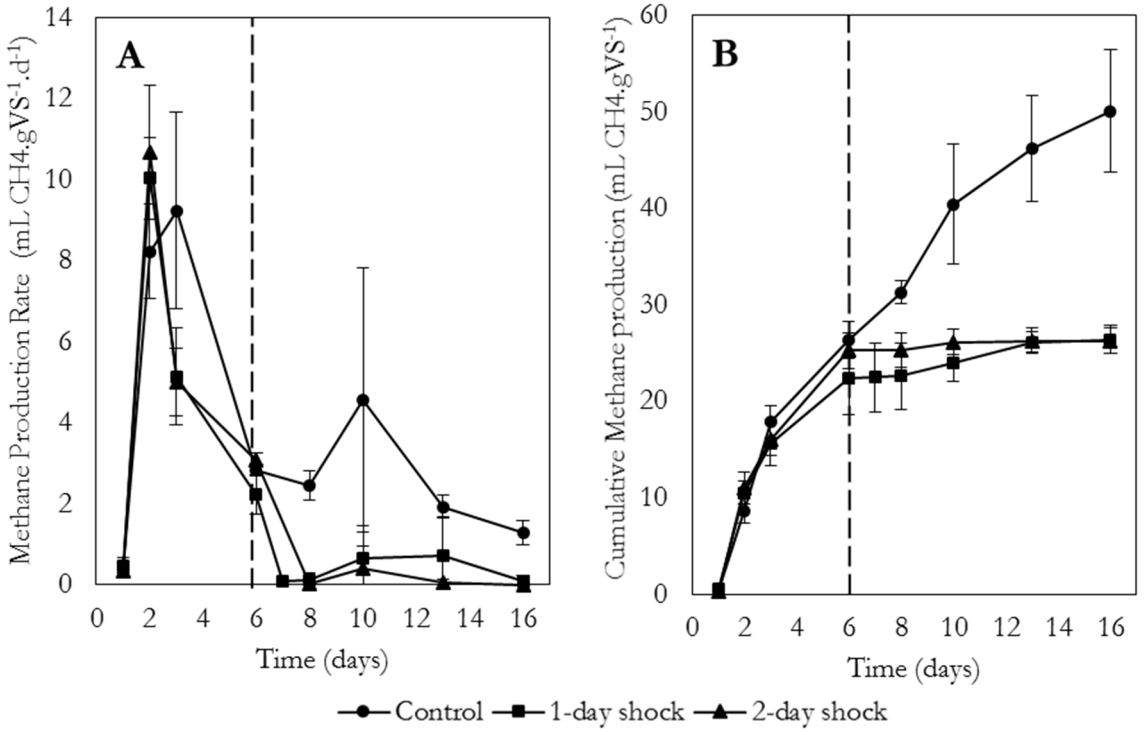

Figure 24. CH_4-production in the batch experiments. Figure A shows CH_4-production rate per mass of initial VS. Figure B shows cumulative CH_4-production per mass of initial VS over the course of the experiment. Dashed line indicates start of pH-shock.

Figure 24A and Figure **24**B respectively show the CH_4-production rate and cumulative CH_4-production over time during the experiment. The pH shock was applied to the digester effluent on Day 6 of the experiment as indicated by the dashed line. Despite the variation on the production rate being rather large in Figure 24A, a clear distinction can be made between the case with pH-shock and the one without pH-shock. Production rates of CH_4 are much lower after pH-shock (regardless of a one or two day shock) compared to the control case. In the control experiment the methane production between Day 8 and Day 16 was 2.6±2.0 $gCH_4.gVS^{-1}.d^{-1}$ whereas it was 0.4±0.7 $gCH_4.gVS^{-1}.d^{-1}$ for a one day shock and 0.1±0.4 $gCH_4.gVS^{-1}.d^{-1}$ for a two day shock; only the two-day shock results in a significant difference compared to the control experiment.

The influence of the pH shock is more clear when the cumulative CH_4-production is compared, cf. Figure 24B. During and after the pH-shock nearly no CH_4 is produced, while the cumulative production of the control experiment continues to rise. This means that the failing of the methanogenesis due to a pH shock is not caused by the rise in EC, as this was corrected for in the control experiment. At the end of the experiment, digester effluent not receiving a pH-shock produced 50.0±6.4 mL CH_4 per initial g VS, while this was only 26.4±1.5 mL $CH_4.gVS^{-1}$ and 26.3±1.3 mL $CH_4.gVS^{-1}$ for a 1 and 2-day pH shock respectively and cumulative productions were not significantly different before pH-shock (Control: 26.4±1.9 mL $CH_4.gVS^{-1}$; 1-day shock: 22.4±3.7 mL $CH_4.gVS^{-1}$; 2-day shock: 25.3±1.9 mL $CH_4.gVS^{-1}$). These numbers clearly show that in the case of mesophilic digester effluent a 1-day pH shock is enough to inhibit the methanogenic community.

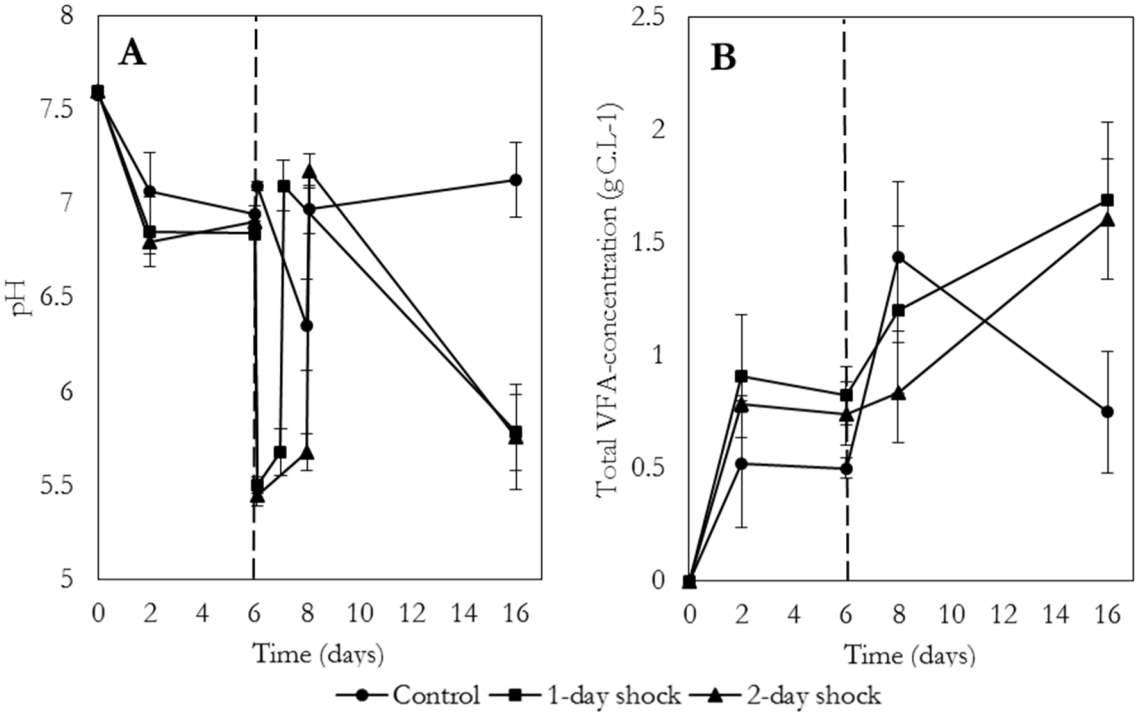

Figure 25. Acidification of the fermentation broth after pH shock. Figure A shows evolution of pH over the course of the experiment. Figure B shows VFA-concentrations over the course of the experiment. Dashed line indicates start of pH shock.

Figure 25A shows the pH over the course of the experiment. As all experiments started from the same broth and received the same amount of glycerol as a carbon source, there are no significant differences up to the pH shock. Between Day 6 and Day 8 instantaneous pH changes occurred due to addition of HCl or NaOH. The addition of NaCl to the control experiment increased pH slightly. During the pH shock, pH increased slightly but significantly (from 5.50±0.04 to 5.68±0.12 for a one-day shock; 5.45±0.06 to 5.68±0.10 for a two-day shock), while the pH in the control experiments decreased from 7.09±0.02 to 6.35±0.24, possibly the result of increased salt concentrations in the Control experiment as a control for the change in EC in the pH shock experiments. After the shock, pH was increased to approximately 7 in the control and both pH-shock experiments and digestion were continued at 34°C. At the end of the experiment (Day 16), a clear decrease in pH was noted in the pH shock experiments (from 7.09±0.14 to 5.78±0.20 for a one-day shock; 7.17±0.09 5.76±0.28 for a two-day shock) while pH in the control experiments remained stable (6.97±0.13 to 7.13±0.20).

Figure 25B shows how VFA-production is influenced by the pH-shocks. During the pH shock, which was accompanied with glycerol dosing, VFA-concentration did not significantly rise. For the control experiment the concentration of VFA present did rise significantly upon increasing the electrical conductivity (from 0.50±0.05 $gC.L^{-1}$ to 1.44±0.33 $gC.L^{-1}$), resulting in a significant difference in VFA-concentrations in comparison to the two-day pH shock (0.84±0.22 $gC.L^{-1}$ on Day 8). An important note here is that even before the pH shock a statistically significant difference in VFA-concentrations was established between the control case and the shock reactors (on Day 6 0.50±0.05 $gC.L^{-1}$, 0.82±0.13 $gC.L^{-1}$ and 0.74±0.14 $gC.L^{-1}$ for the control, 1-day shock and 2-day shock respectively). This indicates that a larger natural variability of the VFA-concentrations is present than the one observed in the triplicate experiments.

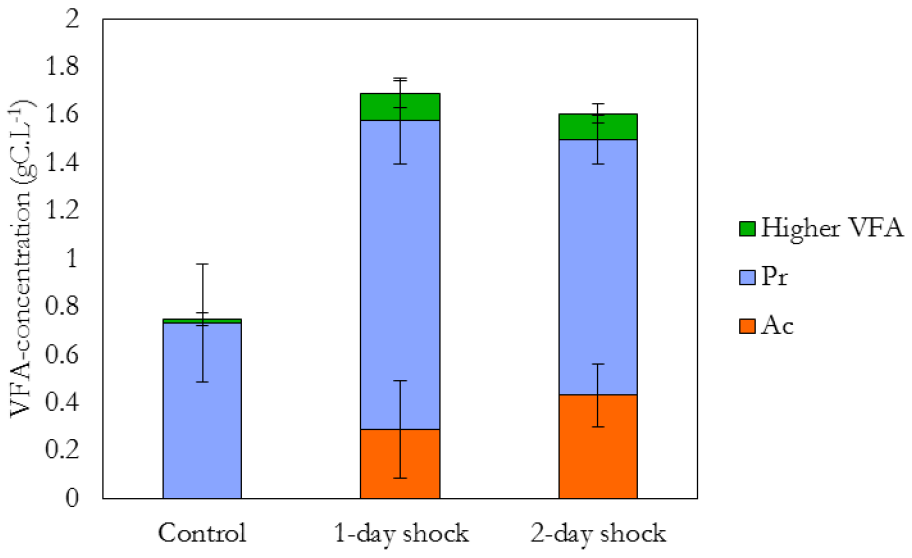

Figure 26. Average VFA-profiles at the end of the batch test

The VFA-concentrations at the end of the experiments, shown in Figure 26, can explain what happens in a digester after methanogenesis inhibition. Total concentrations at the end of the experiment are significantly higher when a shock was applied (0.75±0.27 $gC.L^{-1}$ for control;

1.69±0.35 for a one-day shock; 1.60±0.27 gC.L^{-1} for a two-day shock). The VFA-profile also changes resulting in more Ac and more Higher VFA – these are mainly But and iVal. The build-up of Ac is a result of the failing of acetoclastic methanogenesis, as this process would consume any Ac present in the fermentation broth (Liu & Whitman, 2008). The build-up of But and iVal are most likely the result of amino acid fermentation. Since glycerol was used as a carbon source the pathways detailed in Section 3.2.6 would mainly produce Pr, with some Ac. Degradation of dead micro-organisms (e.g. methanogens) would release proteins which could then be fermented to VFA. The VFA produced after the shock (Ac, Pr, iBut, But, iVal and Val) are all potential outcomes of the fermentation of proteins at pH 7 (Liu et al., 2012).

This batch test shows that a pH-shock can be used to inhibit methanogens in a digester while the acidogenic fermentation appears to be unaffected by the pH-shock. The loss of methanogenic activity, especially Ac-consuming acetoclastic methanogenesis, results in the accumulation of VFA in the broth. This accumulation then results in the acidification of the digester, which in this case prevented any possible recovery of the methanogenic community. These results are a first indication of the possibility of using the pH-shock concept for methanogen control in a fermenter with potentially minimal impact on the fermenting community.

3.3.2.2. Semi-continuous batch reactor experiments with pH shocks

The strategy developed in the previous sections to control methanogenesis was put to practice in a semi-continuous batch reactor as described in Section 2.2.4

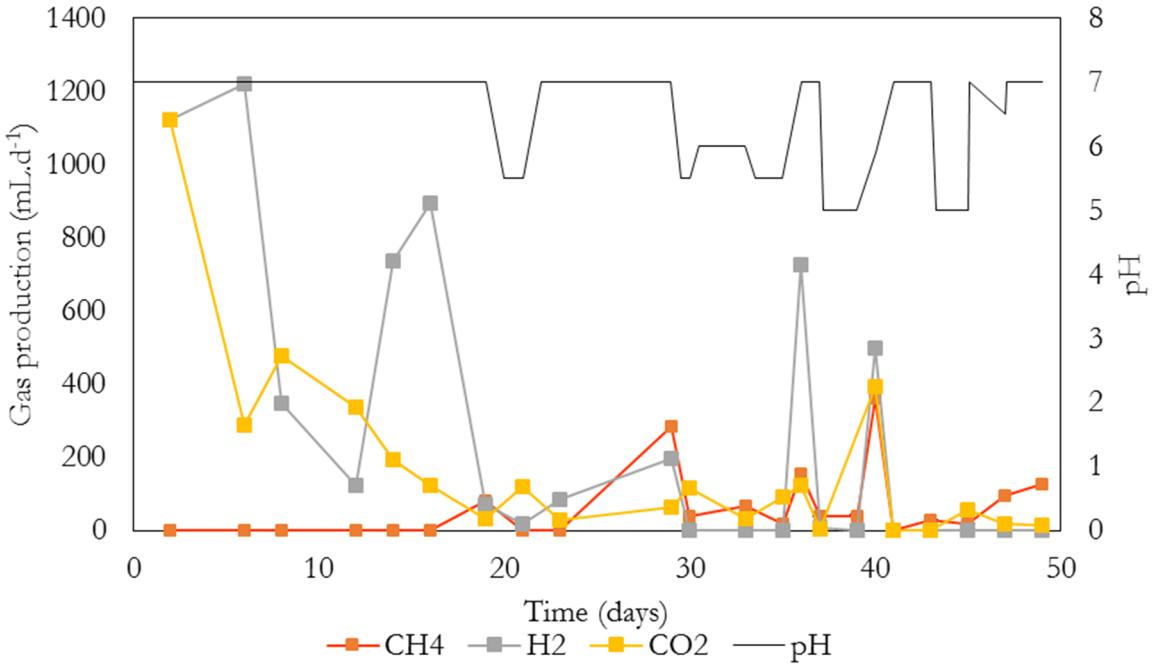

Figure 27. Production of CH4, H2 and CO2 and pH over time in the semi-continuous batch reactor employing pH shocks to control methanogenic activity. Error bars on gas measurements per sampling point are obscured by medallions.

Figure 27 shows the production rates of different gases as well as the pH in the fermenter. The first 10 days the reactor was operated with a high OLR (5.9±2.2 g sCOD.L^{-1}.d^{-1}) and high current

(100 mA or 10 $A.m^{-2}$ of membrane). This resulted in initially high production rates of H_2, a result of electrolysis of H_2O, and CO_2, which is a by-product of the acetogenic fermentation. Despite inoculation of thin stillage with effluent from a mesophilic digester producing CH_4, no methanogenic activity was detected. For this reason OLR was lowered, reducing VFA stress, from day 10 onwards by dilution of fed thin stillage, resulting in an OLR of 3.1±1.7 g $sCOD.L^{-1}.d^{-1}$. This lower OLR did not result in methanogenic activity, despite continued inoculation of the fed thin stillage with mesophilic digester effluent. On Day 16 the applied current was lowered to 50 mA (5 $A.m^{-2}$) because a continuous current of 100 mA increased pH too much resulting in overdosing of acid (2M HCl), possibly causing stress. At this point methanogenic activity started up, after which a first pH shock at pH 5.5 was applied between Day 19 and 21 by a combination of fermentative acidification and acid dosing. After the shock pH returned to 7 by application of a high current (100 mA). As can be seen in Figure 27 this resulted in effective inhibition of the methanogens present in the fermenter as at least 2 days after the shock no CH_4 was produced. The following 4 days headspace gas composition could not be analysed due to technical issues. On day 29 CH_4 was again detected after which a pH-shock was applied for 1 day in an attempt to find the shortest necessary length of pH shock. Despite CH_4 still being present in the headspace after 1 day at pH 5.5, pH was increased again by applying a current over the AEM. However, pH only increased to 6.1, which still resulted in methanogenic activity. For this reason a full two-day pH shock at pH 5.5 was applied in an attempt to remove methanogens completely. Even so the methanogenic community remained active in the fermenter, immediately increasing in activity after pH was elevated to 7. For this reason the pH during the shock was decreased to 5 to create harsher conditions for methanogens. Despite two two-day shocks at pH 5, methanogenic activity was never inhibited completely in the fermenter.

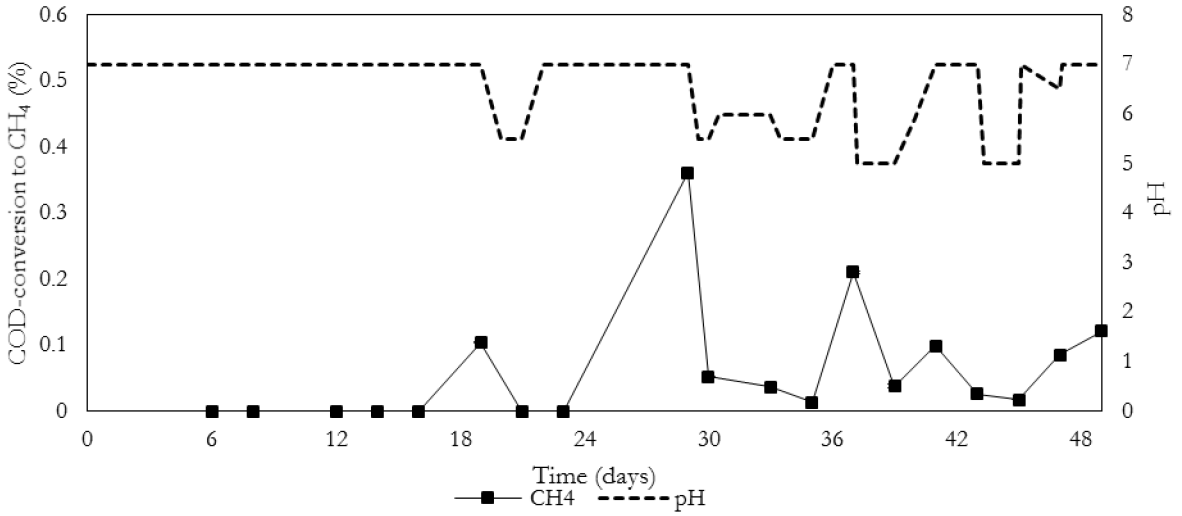

Figure 28. Conversion efficiency of fed soluble COD to CH_4 and pH over time

The figure above shows the efficiency with which the fed soluble COD was converted to CH_4 over time, along with the pH in the reactor. A clear link can be seen with a pH of 7 and high conversion efficiencies (e.g. on Day 19, 29 and 37). At a low pH on the other hand, conversion efficiencies are usually lower (e.g. Day 30 to 35, 39 and 45). The highest conversion efficiency was reached on Day

29 with 36.1±0.3% of COD being converted to CH_4 in 2 days. This means 0.14±0.0 $gC.d^{-1}$ was lost as CH_4 on Day 29. Due to varying OLR over time, the actual highest CH_4-production occurred on Day 40, when 0.17±0.0 $gC.d^{-1}$ was converted to methane.

Despite typically unsuitable conditions for methanogens (i.e. HRT lower than 8 days and periods at decreased pH (Appels et al., 2008)) methanogenic activity continued in the fermenter. It is well established that acetoclastic methanogens are sensitive to a decreased pH (Agler et al., 2014), nonetheless, other methanogenesis pathways are possible. Hydrogenotrophic methanogens, using H_2 and CO_2 to produce CH_4, have been observed in H_2-producing fermenters at a pH down to 4.5 (Kim et al., 2004). Considering the abundance of H_2 in the fermenter due to cathodic H_2O-reduction the presence of hydrogenotrophic methanogens can be expected. Due to the coinciding pH-increase and highest H_2-production rates it is not possible to say whether the peaks in CH_4-production are due to the increase in pH or the increase in H_2-pressure. The presence of hydrogenotrophic methanogens does appear to be confirmed when looking at the gas production on the last days of reactor operation in Figure 27. The expected H_2 production due to electrolysis is absent. This could however be due to production of CH_4 with the available H_2. Although the first shock appears to have inhibited all methanogens, repeated shocks might create a more resistant community (Chae et al., 2010), resulting in the presence of methanogens despite pH as low as 5.

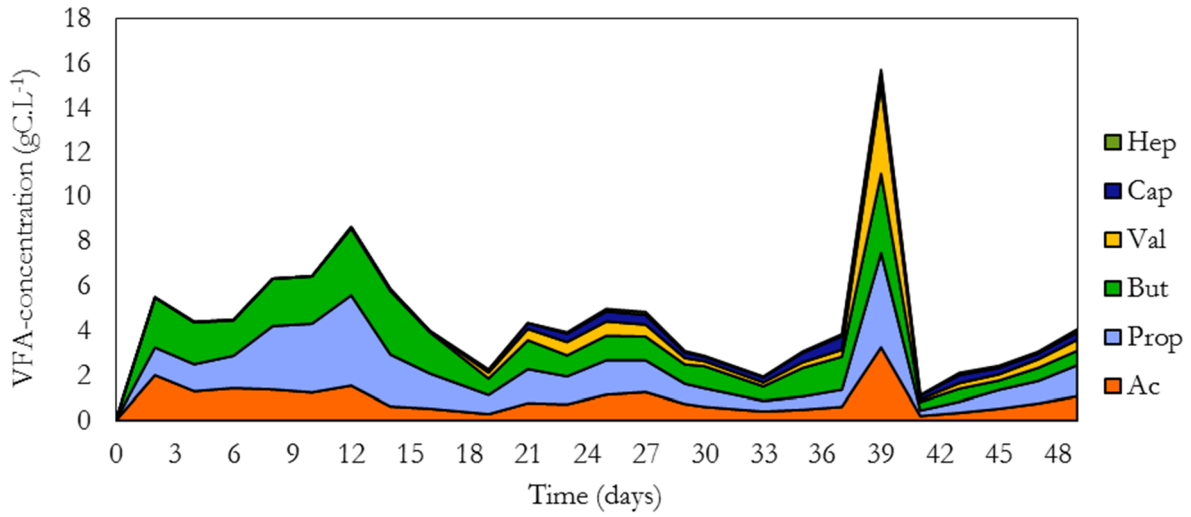

Figure 29. VFA-profile over time in the reactor applying pH shocks for methanogenesis control

Figure 29 shows the composition of VFA present in the fermenter over time. An outlier on Day 39 is immediately apparent, possibly related to issues during feeding. For further calculations this outlier was removed from the dataset. Due to the initial high OLR, high VFA-concentrations were obtained in the first 10 days of operation. The profile corresponds closely to fermentation without ME as there were some issues with electrolysis in this period. From Day 10 onwards OLR was lowered. This resulted in a decrease in VFA-concentrations, while ME issues sustained, resulting in a similar VFA-profile. From Day 19 onwards, when current was lowered, an average total VFA-concentration of 2.7±0.6 $gC.L^{-1}$ was obtained. VFA-concentrations on Day 41 were 1.9 $gC.L^{-1}$, which might be a result of the increased CH_4-production, see Figure 27. When adding the carbon in the form of CH_4 to the total VFA-concentration, a total of 2.1 $gC.L^{-1}$ is obtained, still significantly

lower than the average VFA-concentrations in the reactor. This indicates more than CH_4-production is causing the low VFA-concentration on Day 41.

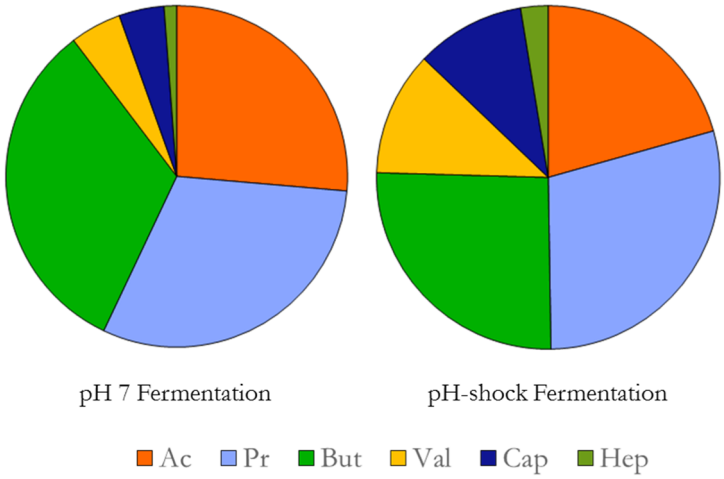

Figure 30. Comparison of VFA-profiles in fermentation at a stable pH of 7 (see Section 3.3.1) and fermentation with pH shocks as methanogen control strategy

The influence of fermentation with pH shocks is shown in Figure 30. Due to feed dilution in this experiment, comparison of VFA-concentrations with the results discussed in Section 3.3.1 is difficult; for this reason it might be better to compare the proportions of each of the VFA. This comparison shows a significantly higher fraction of longer-chain VFA (Val, Cap, Hep) present in the shock reactor (8.9±8.4% for pH 7 fermentation; 24.3±5.0% for pH-shock fermentation). Note that no significant difference is obtained when purely longer-chain VFA, defined earlier as But, Val, Cap and Hep, are taken into consideration (42.1±9.6% for pH 7 fermentation; 50.3±8.6% for pH-shock fermentation). Although no control experiment was performed with ME at pH 7, this apparent increase in elongation might be explained by the production of H_2 by electrolysis, as explained in Section 3.2.

Despite the initial promising results from the both batch test (Section 3.3.2.1) and the first shock in the pH shock fermenter it appears that at low OLR it is possible for a methanogenic community to establish itself in a fermenter operated at pH 7 with intermittent shocks at pH 5.5 and even 5. While this means this strategy is, in this form, not capable of effectively controlling the methanogenic community, it might be possible to use this technique in CH_4-producing digesters to obtain a more robust community. Robust methanogenic communities could be beneficial for CH_4-producing digesters treating a wide range of streams or for digesters treating streams prone to shocks due to unstable OLR, pH, etc.

3.4. Influence of pH shocks on a fermenting community

As established in the previous section, pH shocks have the possibility of controlling methanogenic communities when these communities are not adapted to pH shocks. While the VFA output of the semi-continuous batch reactor, as discussed in the previous section, does not appear to be influenced by the pH shocks, the fermenting community might still be influenced by them. A batch

test, as described in Section 2.5.3**,** was performed to investigate the possible influence of a pH shock when VFA-concentrations are high.

3.4.1. Batch test combining pH shock with high VFA-concentrations

The influence of a pH shock on a fermenting community was investigated by applying a combination of both high VFA-concentrations and low pH for 2 days (Day 0 to Day 2). This shock period was followed by centrifugation and resuspension of the solids in fresh thin stillage supernatant after which fermentation at 34°C was continued.

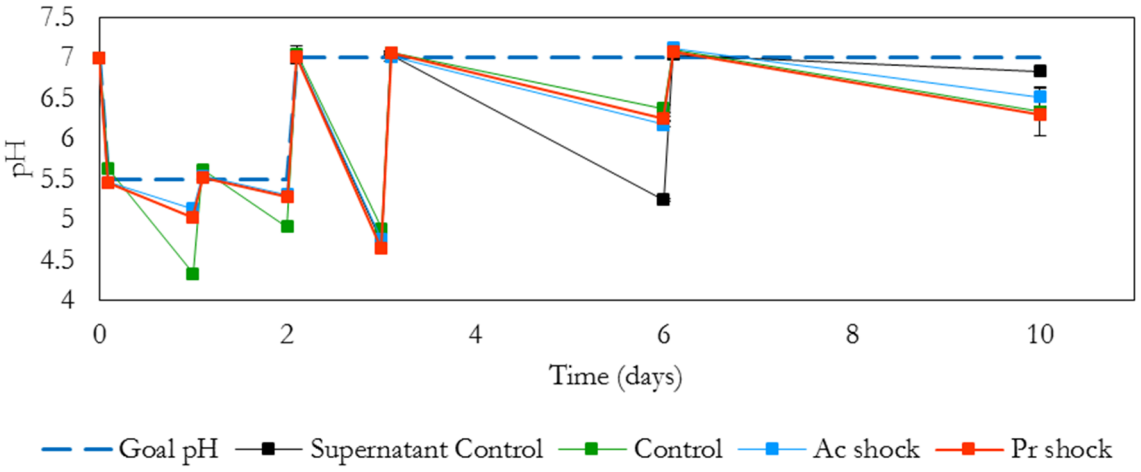

Figure 31. pH for each of the experiments over time along with the goal pH at each stage of the experiment.

Figure 31 shows that despite attempts to obtain a pH of 5.5 during the shock and 7 after the shock, practical pH was lower due to corrections only being performed daily, and further on only every 3 days. During the shock-phase, pH of the experiments shocking with Ac and Pr was higher than pH of the control experiment. This could be due to insignificantly decreased VFA-production during the shock period (0.33±0.05 $gC.L^{-1}.d^{-1}$ for Control; 0.07±0.21 $gC.L^{-1}.d^{-1}$ for Ac-shock; -0.12±0.32 $gC.L^{-1}.d^{-1}$ for Pr-shock) although the increased pH-buffering due to the high VFA-concentrations could be at play as well. This buffering is largest between pH 4 and 5.5 due to the pKa of Ac and Pr (see Section 1.5.1).

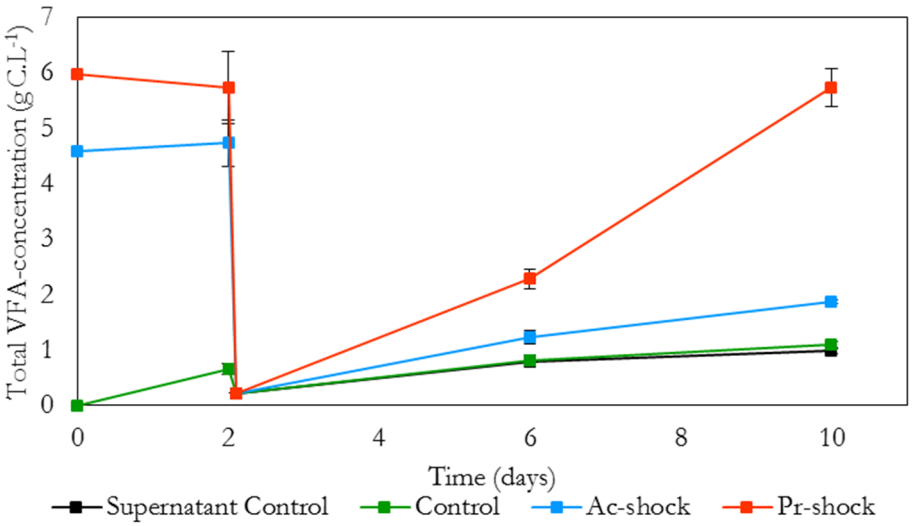

Figure 32. VFA-concentrations in each experiment over time. On day 3 broth was centrifuged, supernatant removed and replaced by supernatant of fresh thin stillage. Some error bars are obscured by medallions.

Total VFA-concentrations over time in each of the experiments are shown in Figure 32. The concentration of Ac and Pr at the start of the shock were 4.6 $gC.L^{-1}$ and 5.6 $gC.L^{-1}$ (or 11.5 g $Ac.L^{-1}$ and 11.6 g $Pr.L^{-1}$) respectively. Figure 32 already indicates a clear influence on the VFA-production as the communities that were shocked produce significantly higher VFA-concentrations by the end of the experiment. On Day 11, total VFA-concentrations in the Supernatant Control (0.98±0.05 $gC.L^{-1}$) were significantly lower than those in the control experiment (1.09±0.06 $gC.L^{-1}$). When an Ac-shock was applied, these concentrations nearly doubled to 1.87±0.03 $gC.L^{-1}$, while the largest increase was noted in the experiment applying a Pr-shock, reaching 5.73±0.34 $gC.L^{-1}$.

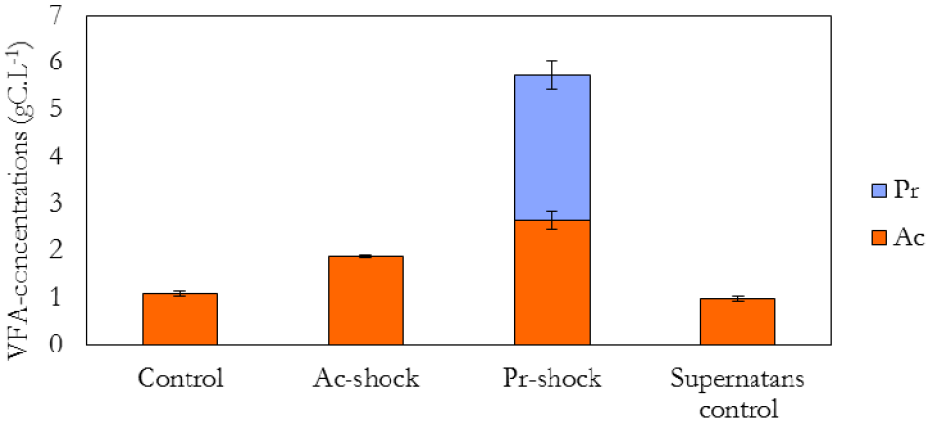

Figure 33. VFA-profiles on Day 10 for each experiments. Error bars signify variation for that VFA.

Not only are the total VFA-concentrations higher in the case of a shocked community, the type of VFA produced can also be changed by the shock. Figure 33 shows that neither the control cases nor the Ac-shock produce Pr, while a community shocked with Pr does show ability of producing Pr. It remains unclear what the exact mechanisms behind this shift in product output are, but a possibility might be that a shocked community becomes more resistant to the VFA it is shocked with. Another proof the fermentative reactions catalysed by the community are changed by the

shock is the presence of H_2 in the headspace in the case of the Pr-shock, while none of the other experiments showed presence of H_2. Because gas production was not quantified it is impossible to quantitatively investigate this fermentation shift.

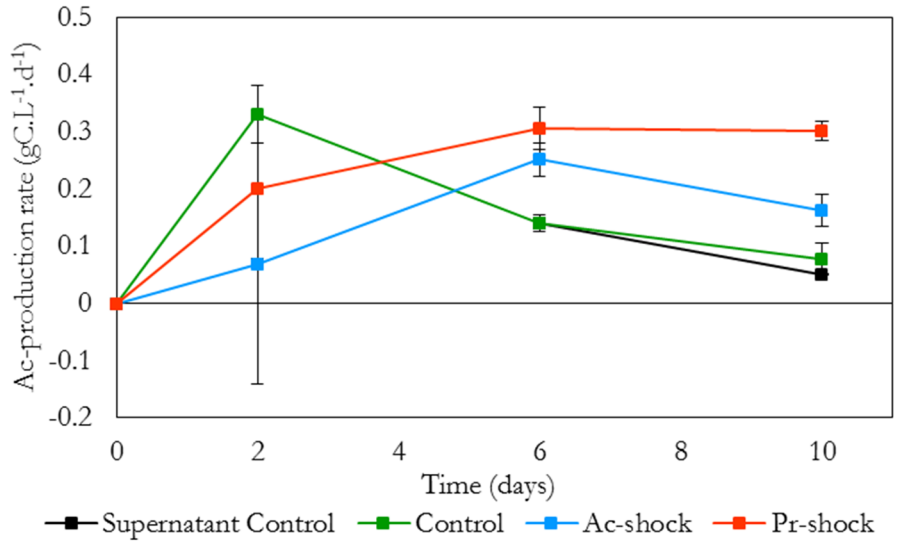

Figure 34. Ac-production rates over time for the combined pH and VFA-shock. Shock applied from Day 0 to Day 2.

Not only is the community shocked with Pr the only community to produce Pr, it also produces Ac at higher concentrations and rates than the control experiment and the experiment shocked with Ac, as can be seen in Figure 34. Between Day 6 and Day 10 the average total production rate in the Pr-shock experiment was 0.86±0.06 $gC.L^{-1}.d^{-1}$, significantly higher than the experiment shocked with Ac (0.16±0.03 $gC.L^{-1}.d^{-1}$) and both control experiments (0.07±0.04 $gC.L^{-1}.d^{-1}$ for 'Control';0.05±0.01 $gC.L^{-1}.d^{-1}$ for 'Supernatant Control').

These experiments demonstrate that pH shock in combination with high VFA-concentrations can influence the VFA-production by a fermenting community. An Ac-shock causes an increased Ac production, while a Pr-shock was shown to stimulate both Ac and Pr-production.

3.4.2. pH Hysteresis Semi-continuous batch reactor

To test the long-term effect of pH shocks on a fermenting community an experiment was performed applying periodic pH changes. In this reactor the pH was shifted from a high pH (7) to a low pH (5.5 in Phase 1, 5 in Phase 2) every HRT (6 days).

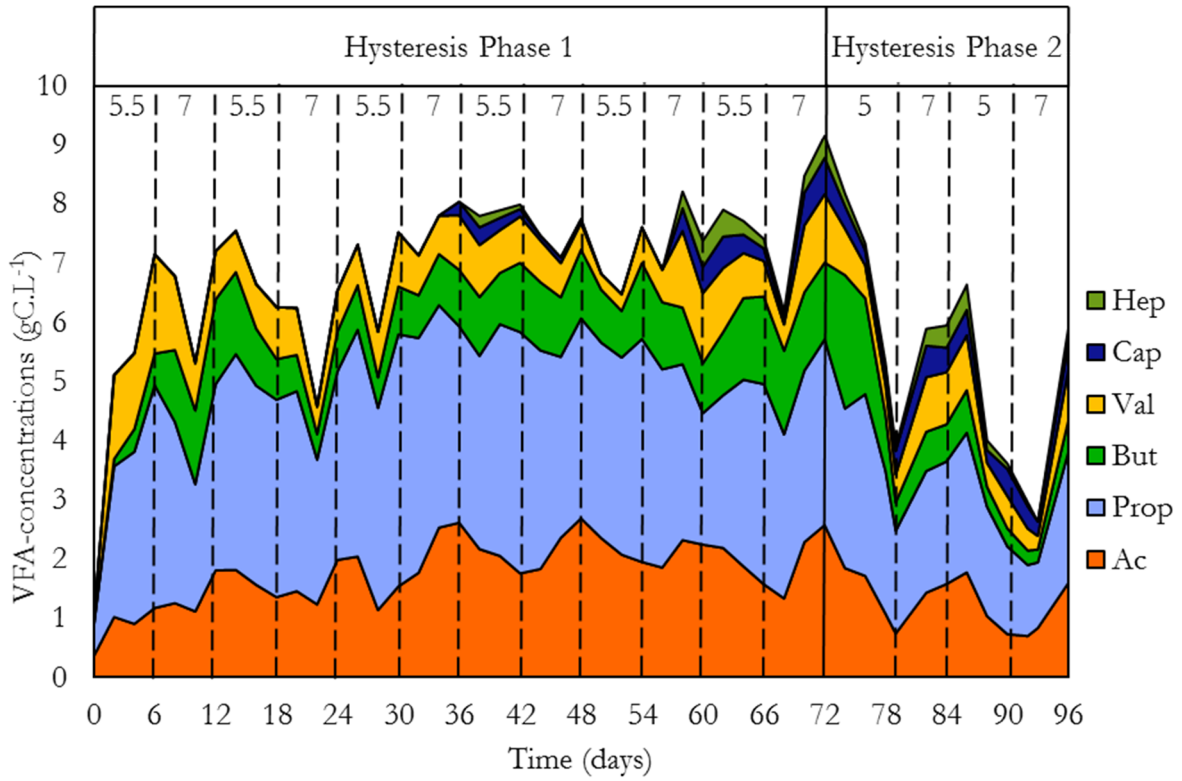

Figure 35. VFA-profile over time for pH Hysteresis fermenter

As can be seen in Figure 35 the VFA-profile, after some initial fluctuations, remained fairly constant during the first phase of hysteresis (from pH 7 to 5.5 and back). This figure shows a fairly similar VFA-profile to a fermenter operated at a stable pH of 5.5, producing predominantly Ac and Pr.

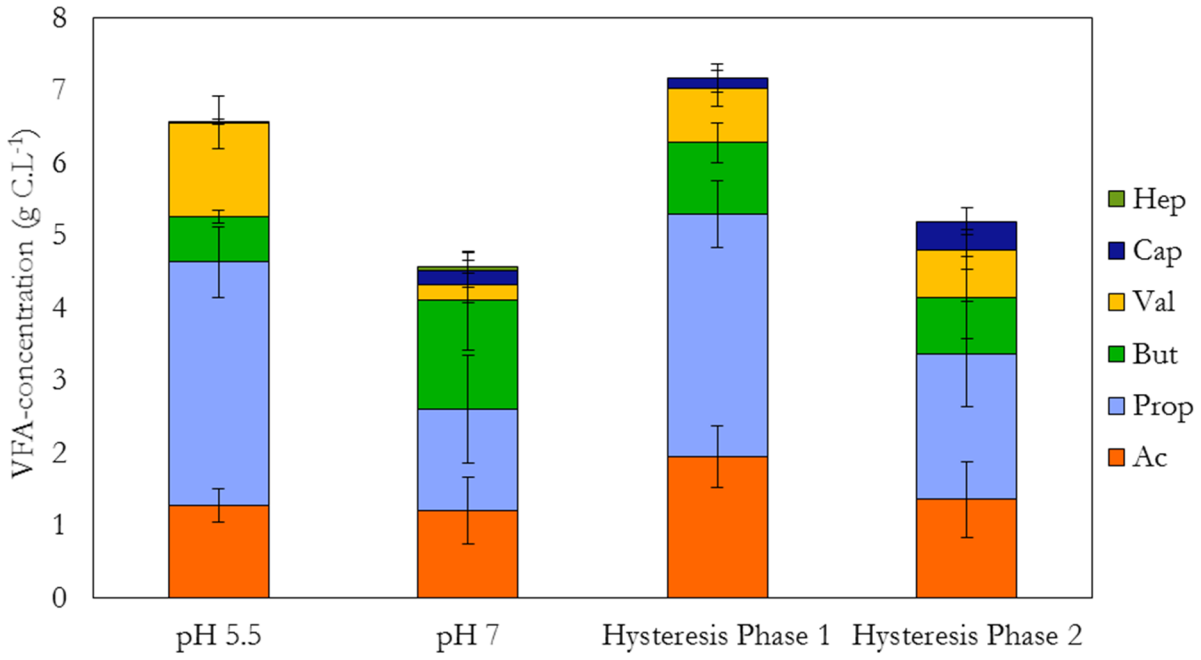

Figure 36. Average VFA-profile during steady-state conditions. Error bars indicate standard deviation on specified VFA.

Figure 36 shows a comparison of the VFA-profiles at steady state for the control fermenters (pH 5.5 and pH 7) and both phases of hysteresis (see Section 2.2.2). Total VFA-concentrations are insignificantly different in pH hysteresis compared to stable pH fermentation (6.6±0.7 $gC.L^{-1}$ at pH 5.5; 4.6±1.8 $gC.L^{-1}$ at pH 7; 7.2±1.0 $gC.L^{-1}$ for Phase 1; 5.2±1.9 $gC.L^{-1}$ for Phase 2). The largest difference between the VFA-profiles is the increased Ac-concentration in the first phase of Hysteresis (2.0±0.4 $gC.L^{-1}$) compared to fermentation at pH 5.5 (1.3±0.2 $gC.L^{-1}$). No significant difference with fermentation at pH 7 was obtained due to the large variation in this experiment (1.2±0.5 $gC.L^{-1}$).

Table 6. Average proportions of each VFA for different fermentation experiments

	Ac (%)	Pr (%)	But (%)	Val (%)	Cap (%)	Hep (%)
pH 5.5	19.3±1.6	51.3±4.8	9.4±1.3	19.9±5.3	0.1±0.4	0
pH 7	28.0±5.9	29.9±7.1	32.8±11.9	4.6±3.8	4.7±4.7	0
Hysteresis Phase I	26.8±4.2	46.5±7.3	13.5±3.3	10.2±2.9	1.8±2.3	1.1±1.8
Hysteresis Phase II	25.6±4.3	37.5±5.8	13.2±5.6	12.3±2.7	7.8±3.8	3.5±1.9

Due to the high variability of the VFA-concentrations it is difficult to compare the VFA profile of the different experiments. The average proportions of different VFA in each experiment as shown in Table 6 can provide a more suitable means of comparison in this case. This data shows that there is no clear shift in ratios of the obtained products if the lower pH in hysteresis is lowered to 5. As there was no significant difference in total concentration either, no distinguishable effect of increasing the pH range of hysteresis could be detected. The differences between fermentation at pH 7 and 5.5 are quite stark. All VFA are significantly different except Cap. Ac and But are lower at pH 5.5, while Pr and Val are higher. A similar influence of pH on fermentation (i.e. an increase

in Ac and decrease in Pr with increasing pH) was found in literature (Yu & Fang, 2002). When comparing the obtained proportions of the different products for the first phase of hysteresis with fermentation at stable pH we observe that the average proportions obtained with pH hysteresis are somewhere between those obtained at pH 5.5 and 7 although not all differences are significant. When comparing with pH 5.5, a significant increase in Ac and decrease in Val can be noted, while comparing to pH 7 shows an increase in Pr and decrease in But.

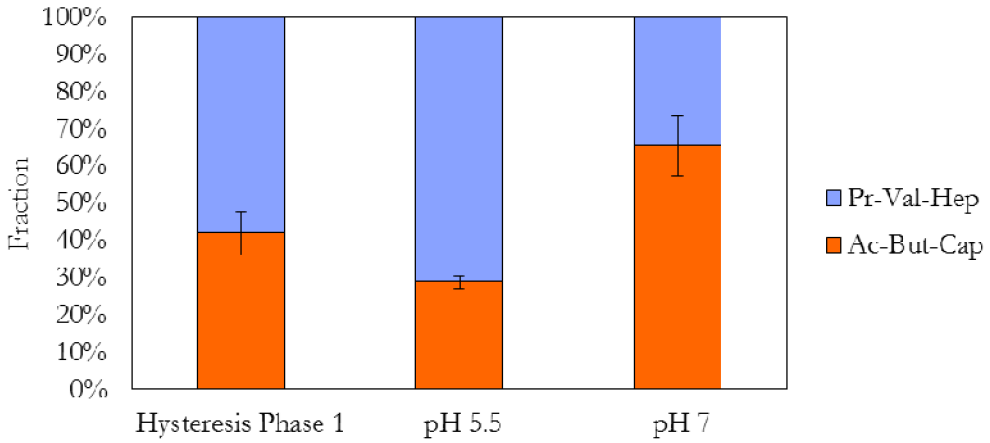

Figure 37. Comparison of fractions of VFA-groups Ac-But-Cap and Pr-Val-Hep for the first phase of hysteresis and both control fermentations

This apparent averaging of product profile when hysteresis is applied seems to be confirmed by Figure 37. This figure, representing the same data as Figure 36, shows the summed fractions of Ac, But and Cap (Ac-But-Cap) and Pr, Val and Hep (Pr-Val-Hep) in the broth. At pH 5.5 71.2±1.8% of VFA are Pr, Val or Hep, while at pH 7 this is only 34.5±8.0%. When hysteresis is applied 57.8±5.8% of VFA are Pr, Val or Hep, approximately the average of the fractions present at pH 7 and pH 5.5. These results indicate that pH has a significant influence on the fermentation profile and the main influence of pH hysteresis on fermentation is a product shift caused by changing pH. Other effects related to the pH shifts (e.g. VFA toxicity, pH stress, etc.) do not appear to affect the fermenting community or the obtained products.

With this idea in mind, it might be interesting to look at temporal variations of VFA-production in the reactor applying a hysteresis regime, as Ac appears to be the predominant product of fermentation at pH 7. As expected, average total VFA production rates are approximately the same (1.2±0.5 $gC.L^{-1}$ during pH 7-period; 1.2±0.3 $gC.L^{-1}$ during pH 5.5-period). When looking at production rates of other VFA, no significant differences could be established. Ac, having a significantly higher concentration in the reactor applying hysteresis, has no significantly higher production rates in a fermenter operating at pH 5.5 (0.23±0.14 $gC.L^{-1}$) or pH 7 (0.17±0.23 $gC.L^{-1}$) compared to a hysteresis fermenter at these pH (0.41±0.17 $gC.L^{-1}$ at pH 7; 0.21±0.12 $gC.L^{-1}$ at pH 5.5). While the shifted product profile appears to indicate a shift in production rate, it is impossible to make statements on this due to the insignificant differences.

Lastly, there is an apparent contradiction between these results and those of the batch test discussed in Section 3.4.1. An important remark to be made here, is that batch tests are not fully

representative for long-term reactor experiments. A distinct difference that can be seen in this case is the lower VFA-concentrations, implying leftover substrate (i.e. Reactive Fraction). Since substrate is not yet limiting in the batch test, the influence of shifting pH might well be very different than when substrate is limiting (e.g. in semi-continuous batch reactors with a 6-day HRT, cf. Section 4.4.2.).

4. Discussion

4.1. Thin Stillage as a biorefinery substrate

4.1.1. Thin Stillage at lab scale

The experiments performed in this study all used thin stillage, a side-stream of EtOH-production from corn. As this is a side-stream of an actual industrial process, the experiments performed here showed that fermentation processes were not confined to streams created in lab environments but could also be applied in real world streams. At the same time, the experiments established the potential of ME for treatment and valorisation of thin stillage. The literature analysis of the potential biochemical pathways in the thin stillage fermentation provided a theoretical basis for the influence of ME on the fermentation process. We can only speculate on the influence of ME on other industrially available substrates such as molasses, wastewaters from (agro)-industrial processes, waste sludge from water treatment, etc., as these have inherently different properties (e.g. pH, alkalinity, solids content, chemical composition, etc.).

Waste activated sludge for instance has a higher solids content, higher pH and much lower sCOD/tCOD-ratio than thin stillage. Because of this, fermentation at short HRT (5 days) yielded low concentrations of VFA (max. 0.21 $gCOD.L^{-1}$), with some minor hydrolysis taking place in a study performed by Ucisik & Henze (2008). This is similar to the conclusions obtained from this study, namely the easier conversion of soluble COD compared to solid COD at short HRT. To maximise VFA-potential a balance should be found with the hydrolysis-rate increase and the increased methanogen performance at increased HRT (Colmenarejo et al., 2004; Appels et al., 2008). Molasses is another stream that has been studied for production of VFA. tCOD can achieve much higher concentrations than those obtained in thin stillage, up to 100 $gCOD.L^{-1}$, and most of this COD is soluble, implicating a high potential for VFA production at short HRT. Solids are higher than for thin stillage, but a key difference between thin stillage and molasses is the much higher conductivity of the latter (up to 10 times higher) (Jiménez et al., 2003). This is the result of a much higher salt concentration and can influence anaerobic processes (Gueguim Kana et al., 2013). Tying this comparison to the carboxylate platform barriers, it would seem likely waste activated sludge would have issues with both ecology barrier (overcoming biologically limiting production rates) and methanogen barrier, while molasses might have issues with the ecology barrier and potentially separation barrier due to its high salt content, as high concentrations of K^+ in molasses can affect digestion (Ryan & Johnson, 2001) while anions (e.g. Cl^-) can migrate very easily across an AEM resulting in a decrease of CE (See Section 3.2.4).

As the experiments performed here were always at lab scale, it is also impossible to make statements on the influence of upscaling on the fermentation process.

4.1.2. Economics of the corn bio-ethanol process

4.1.2.1. Carboxylates production from thin stillage

The USA are one of the main producers of bio-EtOH with an estimated total of $54.1*10^9$ L of EtOH produced in 2014, of which 98% originated from corn (Renewable Fuels Association, 2015).

Taking into account that for a typical dry-grind process 6 to 7 L of thin stillage per L of EtOH is produced (Arora et al., 2011) and the fact that 90% of corn EtOH is produced by means of this process, this equates to a production of up to $334*10^9$ L of thin stillage per year by the US alone. This annual production of thin stillage corresponds with approximately $20*10^6$ tonnes of total COD or $5.8*10^6$ tonnes of reactive fraction COD, representing an abundant source of organics that could potentially be used for production of highly valuable chemicals.

If the entire reactive fraction (See Section 3.1) of this annual thin stillage production would be converted to pure Ac, a total of $5.4*10^6$ tonnes of Ac could be produced, corresponding with 45% of the global Ac demand in 2013 (Le Berre et al., 2014). This could theoretically generate over 2.5 billion USD annually. For a hypothetical U.S. plant with a capacity of 100 million gallons per year this could generate up to 19.2 million USD per year. Alco BioFuel has an annual production capacity of $200*10^6$ L of EtOH and produces of $130*10^6$ tonnes of thin stillage per year, which is rather low comparing with typical dry-grind corn EtOH plants (Arora et al., 2011). For this plant, conversion of the reactive fraction in the thin stillage to VFA could produce 1 million USD worth of Ac annually. It is important to take larger market mechanisms into account when considering these processes. When a theoretical annual production of 45% of the global Ac-market can be obtained by thin stillage fermentation, large-scale market mechanisms come into play making it impossible to predict the reaction of the Ac market. At the same time it is important that there is a market for the obtained product at the plant level. If a process costs too much to implement or if no potential market for the product can be found it is economically nonsensical to invest in this process.

Although Ac has a wide range of uses and a large market is available for the product, it remains unattractive to produce Ac biologically due to the technological immaturity and higher production cost compared to petrochemical Ac-production (Wagemann, 2014). If the entire reactive fraction could be converted to Cap on the other hand, a total of $2.6*10^6$ tonnes of Cap could be produced in the US, generating a theoretical revenue of 13.2 billion USD. The hypothetical US plant could produce 92.8 million USD worth of Cap, while the ALCO BioFuel plant could generate over 5 million USD per year with sales of Cap. This shows that shifting the production towards longer-chain VFA can make the fermentation of thin stillage – and the carboxylate platform in general – economically more interesting.

Approximately 2.6 million USD worth of VFA could be produced if ALCO BioFuel were to implement a thin stillage fermentation process that produces the same VFA-profile as the experiments performed in this study. When only the extracted fraction of the VFA is taken into account this would decrease to 0.7 million USD. This scenario is obviously unrealistic, as the experiments performed here were not focussed on the upscaling of the fermentation process, nor on the optimisation of VFA-extraction. The VFA produced in these experiments are also worth less due to the impurity of the VFA-mix as well as the presence of some contaminants in the extraction broth such as proteins, haloacetic acids, etc.

This section has already given some economic data on the possibilities of using thin stillage as a substrate for carboxylate production. It is however also interesting to look at the applications of

these products. Ac, for instance, is used in the production of vinyl acetate and acetic anhydride, which are used in the production of polyvinyl acetate and cellulose acetate respectively. The first of these is used to produce vinyl plastics while the latter can be used to produce photographic films. Pr is often used for production of cellulose fibers, herbicides and perfumes; But is often used in food, perfume and polymer industries (Jang et al., 2012). Val is often esterified producing valerate esters, which can be used as fuel (Lange et al., 2010) while Cap can be used as an antimicrobial agent (Skrivanova & Marounek, 2007) as well as an additive to perfumes, food, lubricants, etc. (Cheon et al., 2014). As already mentioned with Val, all of these carboxylates can be used for production of fine chemicals, provided the VFA can be separated. An often employed processing step is esterification of carboxylates to esters (Andersen et al., 2014) which have their own uses, for instance fuels and solvents (Agler et al., 2011). Other possibilities include production of ketones, alcohols and alkanes from the obtained carboxylates.

4.1.2.2. DDGS as coproduct from the corn bio-ethanol process

As explained in Section 1.2, EtOH is not the only product obtained in the corn bio-EtOH process. DDGS are obtained by combining the dried wet cake with the syrup obtained after evaporation of water from thin stillage. This is then further dried and pelletized resulting in DDGS. The livestock industry is one of the main end users of DDGS as animal feed for cattle, pigs, poultry and fish (Renewable Fuels Association, 2015). An estimated total of $39.6*10^6$ tonnes of DDGS were produced in the US between September 1st 2013 and August 31st 2014 (Agricultural Marketing Resource Center, 2015). Due to the increase in corn demand by the emergence of the bio-EtOH industry, prices of corn have risen, making corn less attractive as fodder (Skinner et al., 2012). At the same time DDGS can substitute corn and soy meal fractions in animal feed, due to its increased protein content relative to corn as animal feed. The combination of these factors has allowed DDGS to become increasingly important over the last decade to the livestock industry, especially in North-America (Hoffman & Baker, 2011).

DDGS are of major economic importance as they are currently the main coproduct making the bio-EtOH production from corn economically viable. According to an economical model for a corn EtOH-plant producing 100 million gallons of EtOH per year by Hofstrand (2015), the total revenue per gallon of EtOH was $1.92 in March 2015. $0.53 (27.6%) of this revenue was generated by DDGS sales. EtOH prices in this same period were $1.39 per gallon, causing a net loss of $0.07 per gallon. Ceasing the sales of DDGS would exacerbate the economic situation; the hypothetical plant in this model would only have been profitable for 30% of the time since January 2005, demonstrating the necessity for DDGS-production in the current bio-EtOH plant.

If all thin stillage produced in the ALCO BioFuel plant were to be used solely for production of VFA, a total $5.7*10^3$ tonnes of solids would not end up in DDGS every year, a loss of approximately 3.5% when 1L of EtOH results in 0.8 kg of DDGS (Taheripour et al., 2010). This decreased DDGS production represents just under 1 million USD of lost revenue. Depending on the targeted product this loss could be made up for by production carboxylates. As mentioned before, a total of 2.6 million USD worth of carboxylates could be obtained from thin stillage while 0.7 million USD worth of carboxylates could be extracted with the current state of technology (i.e. lab-scale

equipment). It is however not necessarily true that fermentation of thin stillage would cause a loss of 1 million USD in DDGS sales. As the experiments performed in this study showed (see Section 3.2.2), solids remain largely unaffected by fermentation if no ME was applied. If solids could be recovered from the effluent of the fermenter, these could be returned to the DDGS-production to reduce the loss. Because of the application of DDGS as animal feed it is important to keep track of the influence of these processes on the composition of DDGS. As Kim et al. (2008) pointed out, wet cake has a much higher protein content than DDGS due to the low protein content of the thin stillage syrup that is added to the wet cake. As the stream characterisation of the thin stillage showed, approximately half of the organic solids present in thin stillage are proteins. Fermentation does not alter this, so if only the solids of the thin stillage are returned this might have a profound influence on the composition of the resulting DDGS which would have implications for its use as animal feed.

4.1.2.3. Diversification of coproducts for economic buffering

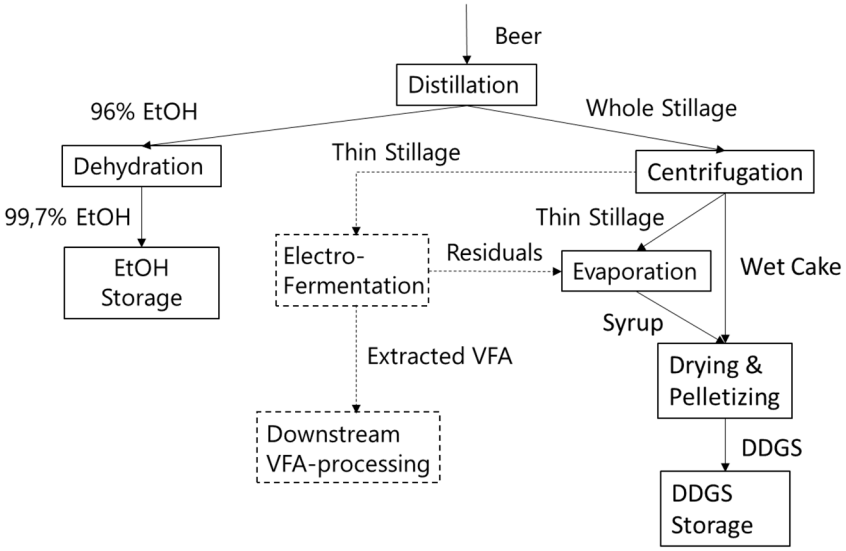

Figure 38. Process diagram showing conventional stillage treatment (full lines) and potential future treatment (dashed lines) for coproduct diversification (adapted from Alco BioFuel, 2014)

The most interesting path for the future of thin stillage as a biorefinery substrate would be the use of the soluble COD – specifically the Reactive Fraction - present for production of VFA, targeting longer chain carboxylates (C5 and up), while the solids can be returned to DDGS production, provided this does not alter feed composition in a negative way. The addition of EF, as shown in Figure 38, provides an additional source of revenue for the bio-EtOH plant. This not only increases their total revenue but can also act as an economic buffer for the bio-EtOH plant. Currently, the corn EtOH-process is dependent on the market prices of corn, EtOH and DDGS. Due to its agricultural nature the price of corn is volatile as it is dependent on factors such as weather and extreme events (Wagemann, 2014). Meanwhile, EtOH-prices are dependent on the evolution of oil prices over the long term, while in the shorter term policy changes can also influence EtOH-prices (Merkusheva & Rapsomanikis, 2014). When production of other fine chemicals could be added to the corn bio-EtOH process, impacts of volatile corn prices as well as price changes in EtOH could be mitigated due to the additional source of revenue. The possibility of producing a

wide range of products, depending on the necessary equipment and possibilities of steering carboxylates production, could further increase the economic resilience of the process. In this way, production of VFAs from thin stillage can be used to further diversify the product range obtained from the corn bio-EtOH process, increasing profitability and economic resilience.

4.2. Zero-chemical input control

The price of NaOH is entirely dependent on the Cl_2-demand and supply, as both are produced simultaneously in the chloralkali process, but this does not mean NaOH has no environmental impact. A Life Cycle Analysis (LCA) of NaOH-production, allocating emissions to Cl_2 and NaOH on a mass basis, obtained a total greenhouse gas emission (both by electricity and fuel) of 1.915 kg CO_2-eq per kg NaOH (Turner & Collins, 2013). In the fermenter operating at pH 5.5 without ME, discussed in Section 0, this would result in an emission of 4.3±2.9 gCO_2-eq per day. For comparison, gaseous emissions from the control reactor were on average 0.22±0.14 g CO_2-eq per day. This shows that CO_2 from chemicals are not negligible in the carbon footprint of the system and substitution of NaOH could alleviate some of the environmental impact of the fermenters. As it has been shown NaOH-dosing could be substituted by applying ME, it is relevant to check whether using electricity to control pH is actually beneficial for the environment. The power necessary for the electrochemical caustic production also has its impact on the environment. An average of 0.21±0.20 W was necessary for this caustic production. With a CO_2 emission of 730 gCO_2-eq.kWh^{-1} from a conventional blend of electricity sources (Granta Design, 2014) this would result in an emission of 3.4±3.2 gCO_2-eq.d^{-1}. The wide variation in this emission is most likely the result of the variation in applied current and accompanying power necessity. Although it appears on a very basic level substitution of NaOH-dosing by ME might reduce CO_2 emissions, differences between the two cases being insignificant, this was not a complete LCA and it is premature to make a definitive conclusion on this aspect of the experiments. For example, a complete LCA would have to incorporate the impact of pumps for NaOH-dosing and recirculation, mixing devices, heating equipment as well as the impact of the reactor vessel, electrochemical cell, etc., which is outside the scope of this study, but would be an interesting exercise for localised electrochemical production of caustic.

The use of renewable energy sources, such as solar energy, could further decrease this carbon footprint. Assuming an average power output by a photovoltaic (PV) panel of 115 $kWh.m^{-2}.yr^{-1}$ (Volcke, 2014) a PV panel surface of 12.7 m^2 would be required per m^3 of reactor, assuming an equal volumetric electricity demand. With a carbon footprint of 29.2 gCO_2-eq.kWh^{-1} (Asdrubali et al., 2015) the use of PV electricity could further reduce the electric carbon footprint to 0.15±0.14 gCO_2-eq.d^{-1}. The use of off-peak electricity (i.e. the surplus generated by centralized plants during periods with low electricity usage) could be beneficial in the reactor set-up described. Planning periods of high applied current during these periods would reduce both cost of electricity and impact on electricity requirements during periods with high electricity demands.

Other disadvantages are associated with the use of NaOH besides its carbon footprint. Because NaOH is usually produced in a 50% solution (European Commission, 2001) it is very corrosive. This corrosiveness entails worker safety issues as well as issues regarding equipment maintenance,

equipment degradation, NaOH-storage, etc. Although these issues can be circumvented, they are another reason why substitution of NaOH by electrochemical caustic production is an interesting solution also being researched for other applications (Pikaar et al., 2011)

4.3. Potential and future development of electrochemical systems for extraction

Substitution of NaOH-dosing by electrochemical reduction of H_2O has its consequences. When this system is applied in larger reactors, larger currents will be necessary to produce the necessary OH^- to prevent acidification of the fermentation broth as well as to extract the produced VFA. To obtain an efficient electrolysis system, key parameters are the surface area of the cathode (Rader & Logan, 2010), the stability of the anode (Hu et al., 2014) and the membrane area (Yee et al., 2012).

In the set-up used in this study, a steel wire mesh cathode was used in a two-chamber electrochemical cell. This material has a specific surface area between 3900 and 6800 $m^2.m^{-3}$ (Zhang et al., 2011), while other materials such as cathode felt can achieve specific surface areas of up to 22700 $m^2.m^{-3}$. As pH-control was achieved, OH^- production is apparently sufficiently efficient in this application as. The electrolytic oxidation of H_2O requires a stable anode, usually achieved by using precious metals, as was the case in this study. These materials are scarce and expensive, so their usage should be limited. Efforts have been made to find substitutes for the commonly used Ir-MMO anodes requiring fewer scarce materials (Hu et al., 2014). Reduction of the anode area per cell could also be useful in reducing the requirement of precious metals. Lastly, membranes are often expensive parts of an electrochemical cell, stimulating research on more cost-effective materials with high efficiencies for ion exchange (Yee et al., 2012). Decreasing the cost of the electrochemical cell can also be obtained by maximisation of the membrane surface area to volume ratio of the cell.

When discussing the size and configuration of an electrochemical cell, the design with the highest achievable membrane area to volume ratio is likely to be a cylindrical electrochemical cell. This type of electrochemical cell would place the anode centrally, surrounded by the membrane which in turn is surrounded by the cathode. This type of construction could result in a 3.17-fold increase in the membrane surface area to volume ratio of the cell while reducing the anode area, assuming total volume and height of the cylindrical and flat-plate electrochemical cell are equal. Other advantages of using cylindrical devices are the reduced pumping costs for recirculation of the fermentation broth through the electrochemical cell, since cylindrical devices can be submerged in the broth, and an – in principle - easier implementation of multiple extraction devices in one reactor, although this has not yet been researched.

A comparison could be made with submerged membrane bioreactors (MBR). While an MBR uses a pressure drop as driving force to obtain a clarified effluent, an electrochemical cell uses current over the membrane as a driving force, causing migration of specific species as opposed to removing water containing solutes to obtain a clarified product stream. MBR are well-known in literature and have been used both aerobically and anaerobically (Ylitervo et al., 2013). No MBR have been reported in literature for VFA-production, so it is difficult to compare performance of an MBR

and ME for extraction. Assuming a thin stillage fermentation broth containing 10 $gAc.L^{-1}$, ME can extract up to 1.05 kg $Ac.m^{-2}.d^{-1}$ (Andersen et al., 2014). An MBR allowing free migration of Ac through the membrane could obtain a flux of 1.03 kg $Ac.m^{-2}.d^{-1}$ at an average permeate flux of 4.3 $l.m^{-2}.h^{-1}$, a value obtained in a pilot plant anaerobically treating corn thin stillage (Dereli et al., 2012). The similarity between the two, despite their fundamental differences, shows their potential.

MBR are typically applied in two configurations: submerged and external-loop. In the first water permeates from the outside inwards in a device submerged in the reactor, while in the latter, water permeates either through a flat membrane or from the inside outwards through a cilindrical membrane in devices placed in a recirculation loop outside the reactor. An often encountered issues with submerged MBR is fouling of the membranes, especially at high concentrations of solids, which is usually countered by purging gas along the membrane (Ylitervo et al., 2013). This fouling could also be an issue when using submerged electrochemical devices. In external-loop MBR fouling is also an issue - usually countered by applying high flow velocities (Ylitervo et al., 2013) - but fouling of the membrane was outside the scope of this study. Fouling of membrane and electrode, as well as the lifetime of these components are expected to be critical for this application. The decrease in solids that was observed as a result of applying ME will also have an impact on fouling of the cell and its components.

For the application of ME on a larger scale it will be crucial to study the application of new anode and membrane materials on the extraction and the cost-effectiveness of the system as well as the consequences of using cylindrical extraction devices for ME. Once a cylindrical ME system has been developed, the possibility of using multiple extraction devices in one reactor can be of great interest. Applied current per extraction device could be reduced while simultaneously increasing membrane surface area and decreasing anode area per volume of reactor.

4.4. ME and Carboxylate Platform Barriers

The experiments performed in this study aimed to tackle all three carboxylate platform barriers defined by Agler et al. (2011). These three barriers are the separation, ecology and methanogen barrier. The first of these is the necessity for the development of technology able to separate and recover the carboxylates produced in carboxylate platform processes. The ecology barrier is the issue of steering and controlling a microbial community towards the production of a target product and overcoming biologically limiting production rates and product inhibition. The last barrier is the methanogen barrier, which pertains to the problem of methanogenesis converting multi-carbon VFA to CH_4. While this conversion is desirable in classic anaerobic digestion it is undesirable for the carboxylate platform as its main goal is the production of industrial precursor chemicals, for which multi-carbon compounds are more attractive.

4.4.1. Separation Barrier

The principle motivation of ME is separation and recovery of anionic VFA (Andersen et al., 2014). 28.1±3.8% of the produced VFA could be extracted by ME in the experiments performed, despite the experimental focus on the influence of ME on fermentation and not optimisation of VFA-extraction. This demonstrates that the VFA produced can be separated from the fermentation

broth using ME after which further downstream processing to other chemicals such as volatile esters (Andersen et al., 2014) is possible. ME cannot selectively extract target VFA which results in a mixture of VFA in the anolyte solution. As a consequence downstream processing would also require development of a system for VFA separation or target an aggregate product where a mix of chemicals with different chain lengths is allowable. An example of such a product could be fuels, where alkenes of varying chain lengths could be used. These products could be obtained by reduction of carboxylates to alcohols with H_2 and subsequent dehydrogenation. At least part of the required H_2 could be obtained from the headspace gas of the fermenter. In the experiment with ME described in Section 3.2 the theoretical molar H_2-production rate was approximately half of the carboxylate production rate, while reduction of carboxylates with H_2 requires 2 moles of H_2 per mole of carboxylate (Holtzapple & Granda, 2009). For this processing pathway, another source of H_2 (e.g. electrolysis of H_2O) would be necessary to complete the reduction.

4.4.2. Ecology Barrier

H_2-production by ME stimulates chain elongation and in that way can steer community towards a larger abundance of chain elongating organisms such as *C. kluyveri* (See Section 3.2.6). Production of EtOH by reduction of Ac is crucial for this stimulation, although this reaction could only be implied from the obtained VFA-profile and could not thoroughly be demonstrated.

With regards to biologically limited production rates and product inhibition, several experiments performed in this study point towards the substrate as the limiting factor for production of VFA from thin stillage. Stream characterisation indicated that the reactive fraction is used for most of the VFA-production in thin stillage fermentation at short HRT (6 days). As stoichiometric equations obtained from literature could match the actual product profile rather closely, assuming complete conversion of the reactive fraction, the main limitation appears to be the availability of readily biodegradable COD present in thin stillage.

Limitation by the substrate was confirmed by both an increase and a decrease in HRT. An increase in HRT did not result in a doubling of the VFA-concentrations, which would be expected if production rates remained the same and substrate was not limited. The argument may be made that this could be the result of product inhibition due to high concentrations. However, halving the HRT from 6 to 3 days, implying a doubling of the OLR, resulted in a doubled production rate. This can be explained by all of the reactive fraction being consumed within 24 hours by the fermenting community, again suggesting limitation of substrate as opposed to product inhibition causing production limitation. A final demonstration of the substrate being limiting as opposed to product inhibition causing limitation of VFA production is the experiment on product inhibition in thin stillage fermentation (see Section 3.2.5). The VFA production rate in this experiment was not affected by an increase in VFA-concentration even if these were higher than the steady-state concentrations obtained in a fermenter at pH 5.5 without ME. To mimic conditions in the reactor, the proportions of VFA added in this batch test were similar to the proportions of the VFA produced by the reactor. As the production rate was not influenced it is clear that the products of fermentation do not affect the fermentation taking place in this community. This, again, leads to

the conclusion that, when thin stillage and its inherent community are used for fermentation, abundance of the substrate determines the production of VFA.

4.4.3. Methanogen Barrier

To retain as much carbon as possible in the form of carboxylates it is crucial to inhibit methanogenesis in a VFA-producing fermenter. An attempt was made in this study to control CH_4-production by means of pH shocks. Initial experimental results proved promising, but it eventually became clear that despite the application of 2-day shocks at pH 5, well below the established pH-range for methanogenesis (Chen et al., 2008; Appels et al., 2008), VFA were still converted to CH_4. Not only did these shocks not work, acidogenic fermentation was insufficient to reach a pH of 5 in the fermenter, meaning that acid (anolyte acid or 2M HCl) had to be used. This was a step back in the attempt to control the fermentation process without input of chemicals. As explained in Section 3.3.2.2, the most likely explanation for CH_4-production was hydrogenotrophic methanogens surviving the pH shocks, possibly becoming more tolerant to pH shocks (Chae et al., 2010). While this might be a negative outcome when VFA-production is the main goal, this strategy increasing tolerance to pH might be useful when CH_4 is the target product and H_2 is abundantly present in the headspace.

Returning to the inhibition of methanogenesis, it could very well be that the low OLR and low VFA-concentrations obtained in the fermenter described in Section 3.3.2.2 were the key parameters allowing presence of methanogens as CH_4-production was only initiated after OLR was lowered. In literature 2-bromoethanesulfonate is often used to prevent CH_4-production through methanogenesis as this selectively inhibits methanogens (Steinbusch et al., 2011; Zinder et al., 1984). This is not realistic in full-scale processes due to the high cost of 2-bromoethanesulfonate. At a concentration of 10 $g.L^{-1}$ for inhibition (Steinbusch et al., 2011) and a cost of up to 0.946 $USD.g^{-1}$ (Sigma Aldrich, 2015) this would cost 9.46 USD per L of reactor. For comparison, the total VFA produced during electro-fermentation at pH 5.5 in this study represents a value of approximately 0.024 USD per L of reactor.

5. Conclusion

In this thesis the properties and potential of thin stillage as a substrate for biorefinery processes, focusing on the production of VFA, were explored. The novel ME technology was applied to study the influence of electrolytic extraction on the fermentation process, a process dubbed electro-fermentation. We can conclude from the performed experiments that due to the inherent community present in thin stillage, a result of its origins in the corn bio-EtOH process, fermentation of part of the organics (i.e. the reactive fraction) in the thin stillage to VFA can take place very quickly with complete conversion of this fraction in as little as 24 hours.

ME can help overcome at least two of the three barriers defined by Agler et al. (2011) as being limiting to the breakthrough of the carboxylate platform as a mainstream platform for chemicals production from biomass. ME was designed as a technique to overcome the separation barrier as its main purpose is the separation of the produced VFA from the fermenting thin stillage to a clean stream available for downstream processing of chemicals. The experiments performed in this thesis were not meant to optimise the extraction of the VFA from the fermentation broth, yet the technique was able to extract VFA produced by fermentation. Optimisation of the extraction, i.e. by using batches of anolyte solutions as opposed to the semi-continuous system used here, could yield even higher extraction efficiencies and product concentrations.

A key focus of the experiments applying ME in a fermenting reactor was the influence of the cathodic reduction of water on the fermentative reactions taking place. It was shown that this reduction results in a product profile containing more long-chain VFA (i.e. But, Val, Cap) than is the case without ME. Although no community analysis results were available at the time of writing, it appears realistic from literature analysis that the increased presence of H_2 in the headspace of the reactor allows reduction of Ac to EtOH, a key substrate for chain elongation. This would imply an increase in the abundance of chain elongating organisms (e.g. *Clostridium kluyveri*), suggesting ME can alter the composition of the fermenting community. This ties into a second barrier defined by Agler et al. (2011), the ecology barrier. Product inhibition, also relating to the ecology barrier, has little influence on the fermentation of thin stillage and biological production rates can be very high. The fastest conversion rates obtained may have been limited by the daily feeding of thin stillage showing biological limitations appear insignificant for thin stillage fermentation.

Although a main goal of this thesis was to obtain zero chemical input control over the entire fermentation process, methanogenic activity could not effectively be controlled using ME. This conversion of multi-carbon VFA to the single carbon CH_4 is undesirable in light of the carboxylate platform. The goal here is to produce industrial precursor chemicals, and while the more reduced state of CH_4 may be energetically more attractive, it is less favourable for production of chemicals. pH shocks, although an attractive strategy for inhibition of acetoclastic methanogens, are unsuitable for methanogenic control in electro-fermentation as the increased H_2-pressure may be beneficial for hydrogenotrophic methanogens. As these are more resilient to low pH, pH shocks are unsuitable as a strategy for inhibition of these organisms. Consequently this study could not yet overcome the methanogen barrier, the third barrier described by Agler et al. (2011).

6. Sustainability

The technology used in a process can have a large influence on the sustainability of the overall process. A large part of this thesis focussed on increasing the sustainability of carboxylate platform processes by reducing the chemicals demand for control of the process. Nonetheless a total of 6.8 L 2M NaOH, 2.5 L 1M H_2SO_4 and 0.4 L 2M HCl was used for pH control over the course of 9 months of experiments. Much of this was used on experiments that ultimately failed to produce relevant data for the thesis, due to experimental issues, irrelevance of the experiments, etc. The carbon footprint of the NaOH used in the experiments amounts to 1.04 kg CO_2-eq. Since the use of ME could cut the carbon footprint of pH-control (NaOH vs. electricity requirement) with 20%, this thesis has provided a step forward in reducing the chemicals usage and associated carbon footprint for future experiments.

Over the course of 9 months of lab work, a total of 712 VFA-analyses were performed. VFA-analyses require 2 mL of diethyl ether for extraction of VFA per sample, which means a total of approximately 1.4 L of diethyl ether was used over the course of the performed experiments. This is a very volatile solvent, implying emissions of volatile organic compounds to the atmosphere. Reducing the number of times the diethyl ether bottle is opened could reduce these volatile emissions. By using a pumping system to pump diethyl ether as opposed to opening the bottle to take ether with a pipette, these emissions could be further reduced. An added advantage of this system would be more accurate dosing of ether, although care should be taken to use materials that do not react with diethyl ether, avoiding both equipment damage and diethyl ether contamination.

7. Future research possibilities

Several interesting research possibilities can be based on the result of this thesis. First of all, research on the potential of increasing OLR for methanogen inhibition could help overcome the methanogen barrier and unlock thin stillage's potential for carboxylate production.

Since ME is necessary for valorisation, all its influences should be investigated. This includes the apparent Ac-reduction producing EtOH, stimulating chain elongation but also the mechanism behind the solids decrease should be elucidated. As explained in Section 3.2.2, a process similar to electro-coagulation could be responsible for this solids decrease, although this remains unclear.

Once (electro)-fermentation of thin stillage is well understood, optimisation of the process - both production of VFA and their extraction - is a next step in future research. Optimisation of the production of VFA would involve studying the lowest possible HRT for stable VFA-production and complete conversion of the reactive fraction. Extraction of the products could be optimised by intermittent extraction of VFA. Intermittent extraction entails a period with no or low applied current to allow VFA-production - and corresponding increase in VFA-concentrations - followed by a period with increased current for extraction, which should be more efficient at these higher concentrations. In theory, one extraction device could be used for multiple reactors with this system. On the other hand, ME could also be coupled to an electronic pH-control system which could increase current when pH drops too low due to acidogenic fermentation. Study on the behaviour of membranes in environments with high VFA-concentrations and low pH is also necessary to allow product concentrations adequate for downstream processing. Besides membrane behaviour, materials research into membranes selectively extracting VFA could also be beneficial to increase extraction efficiencies.

Further on, studies on upscaling, using dedicated experimental reactors, will be necessary for this process to be implemented in industrial settings. Not only the reactor, but also ME - more specifically the configuration and operation of the extraction device(s) - will need to be optimised for this purpose. For further optimisation, the fermentation and extraction processes could be modelled mathematically, in much greater detail than the stoichiometric model presented here. This model could be used to perform virtual experiments, allowing cost-effective identification of optimal operating conditions (e.g. pH, HRT) as well as identification of bottlenecks in the process.

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