Development of a mixed culture chain elongation process based on municipal solid waste and ethanol

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T.I.M. Grootscholten

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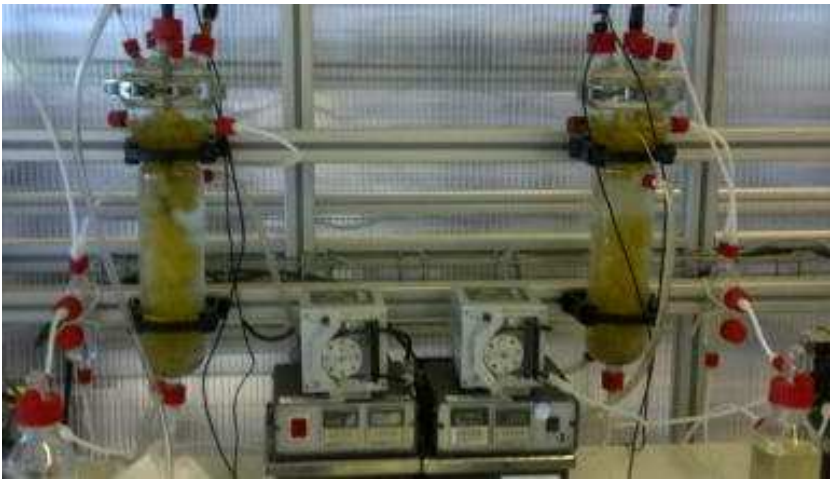
Voor mijn familie,

In het bijzonder voor

mijn grootmoeder, mijn moeder en mijn vrouw.

Table of Contents

1. Introduction



1.1 Driving forces for biofuel and chemical production

Nowadays, we are living with seven billion of people on earth. Due to the increasing population and the increase of consumption per capita, the pressure is elevated on natural resources to enable this consumption. Moreover, this consumption leads to waste production, which can have an additional negative effects on natural resources (Figure 1). This current way of life is well described as linear (from resource to waste via consumption), especially when the negative effect of waste materials on the natural resources are not taken in account. Moreover, most natural resources have become more scarce in the last century.

This is especially true in the case of oil. Most of the easily extractable oil resources have been depleted (Hughes and Rudolph, 2011), resulting in structural higher oil prices (± 100US$ compared to 10 - 30US$ before 2005) and dependence on oil from political instable regions. Moreover, more difficult extractable oil requires application of more complex techniques, of which the adaption is not fool-proof and thus occasionally oil spills occur. Especially spills in environmental sensitive areas, such as the artic and the amazon regions, can have large effects on biodiversity and life conditions of (local) inhabitants.

To avoid negative effects of waste materials, to reduce the pressure on natural resources and to reduce dependence on oil, methods to recycle waste materials into consumable products should be developed. Such a recycling system fits in a circular lifestyle instead of a linear lifestyle (Figure 1).

For materials produced from biological components, this circular system is also known as the biobased economy. In the biobased economy, crops are used for fuel and chemical production to reduce the of fossil resources. Because the biobased economy can interfere with food production, it should be applied carefully to prevent food shortages.

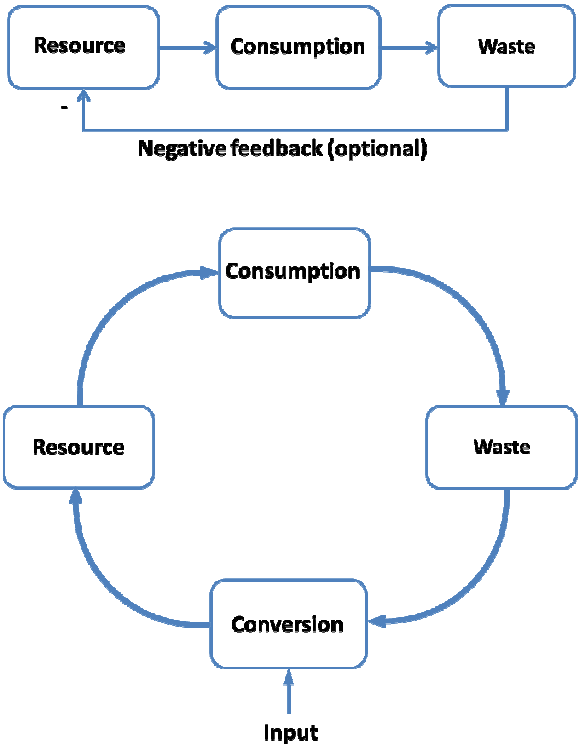


Figure 1. Schematic representation of linear economy (top) and circular economy (bottom).

This thesis focusses on the development of a biobased production system for medium chain fatty acids (MCFAs) from the organic fraction of municipal solid waste (OFMSW) and ethanol using mixed bacterial cultures. By using OFMSW, the MCFA production process, called chain elongation, valorises waste materials into biochemical building blocks. The use of ethanol can be controversial, if the ethanol is produced from food resource (1st generation biofuels). However, current research is on-going to produce ethanol that avoids the food-fuel debate (not-first generation biofuels). Sources for this not-first generation ethanol production can include organic residues (from agriculture, forests, industry or households) and carbon dioxide.

1.2 Perspectives for fuel and chemical production from biomass

Biomass can be used for both fuel and chemical production to replace fossil resources. However, its production is not infinite and therefore it should be determined whether it is more useful to use it for fuel production or for chemical production.

Currently, the world energy demand is 532 Exajoule (EJ = 10^{18}J) per year (IEA, 2011). According to the 450 Scenario[1] , the world energy

[1] 450 Scenario is based on global greenhouse gasses content of 450 ppm CO_2-equivalent and its energy demand is in the lower range of estimations

demand will increase to 623 EJ per year in 2035 (IEA, 2011). Based on the estimated reserves of approximately 40.000 EJ (Shafiee and Topal, 2009), there would be sufficient fossil resources for 64 – 75 years (linear demands). However, about 65% of fossil resources is present in the form of coal, which is the most undesired form and is mainly used for electricity generation. According to Shafiee and Topal (2009), the depletion of oil and gas would proceed much faster, respectively 35 and 37 years.

Biomass supplies roughly 10% of the current world energy demand (53 EJ; IEA, 2012). Based on the calculation of Dornburg *et al*. (2010), biomass could deliver theoretically 1500 EJ per year worldwide, which would be more than sufficient to cover the energy demand. However, due to several limitations, including water availability and competition of land for food production, they concluded that the energy potential of biomass is more likely to be 200-500 EJ per year (in 2050), which is probably not sufficient to completely replace fossil resources. Due to these limited possibilities and the large presence of coal, biomass should not be used to replace coal. Therefore, biomass based processes should be intended to reduce the most expensive fossil resource, namely oil. Although gas reserves are similar in energetic value as oil, it is more energy efficient to make gas rather than oil from coal (Sudiro and Bertucco, 2009). Therefore, it will be more difficult to compete with gas than with oil.

To replace oil successfully, biomass should be converted to products, which derivation from oil is more expensive than from biomass (van Haveren *et al*., 2007). Functionalized chemicals, which contain oxygen and/or

nitrogen, require additional processing from oil, while they can directly be produced from biomass. Fuels, on the other hand, require as little oxygen and/or nitrogen in their composition and therefore they are easily produced from oil. Because biomass contains oxygen and/or nitrogen in its composition, functionalized chemical production from biomass is likely more interesting than fuel production from biomass.

1.3 MCFAs as biobased building block for fuel and chemical production

MCFAs are straight chain carboxylic acids with a carbon chain length of six to twelve carbon atoms. The focus of this thesis will be on the production of caproate (C6), heptanoate (C7) and caprylate (C8). Although caproate, heptanoate and caprylate should scientifically be named carboxylates instead of acids, the name MCFA is used for both forms. In case a distinction is required, the term dissociated (or ionized, for the carboxylate form) of undissociated (or unionized, for the acid form) is added to the abbreviation MCFA.

MCFAs can be applied widely in industry, both directly and indirectly. Its direct applications mainly corresponds its antimicrobial characteristics. For that reason they can be applied as preservative to prevent food and silage spoilage (Marounek *et al*., 2003, Woolford, 1975). Additionally, MCFAs could be used as corrosion inhibitors (Kuznetsov and Ibatullin, 2002). By the direct use of MCFAs, the lifetime of other products (food, feed and metal) is extended and consequently, its pressure of related natural resources

is reduced. Especially, corrosion inhibition can be important, as metal prices have increased dramatically in the last decades. Some of these metals reserves are expected to be depleted within 50 years (Ragnarsdottir, 2008), possibly earlier than the expected depletion of fossil fuel resources.

Indirectly, MCFAs can be applied as pre-cursors for several fuels and chemicals products to replace fossil sources. After esterification, several forms of esters can be produced, depending on the type of alcohol added to form the desired ester. These esters could use for biodiesel production, lubricant production or fragrance production. For lubricant production with low temperature applications (*e.g.* aircraft engines and refrigerators), heptanoate based esters (*e.g.* polyol esters) are desired, because heptanoate has a lower melting point than caproate or caprylate due to its structure (Boyde, 2002).

Instead of esterification, MCFAs could also be used in other chemical upgrading processes such as ketonization or Kolbe-electrolysis. In ketonization, two molecules of MCFAs are converted into one longer chain ketone and a carbon dioxide molecule (Renz, 2005). The carbon chain of this MCFA based ketone can varying between 11 and 15 carbon atoms, depending on the used MCFA form(s). The applications of formed ketone include biodiesel production. Upgrading of MCFAs via the Kolbe electrolysis produces dimers, alkanes, alkenes, alcohols and esters (Levy *et al.*, 1981).

The final discussed application for MCFAs is its use as a precursor for the production of medium chain length polyhydroxyalkanoates (Mcl-PHAs), which could be used as biodegradable plastics (Salehizadeh and van

Loosdrecht, 2004). Compared to the previous applications, PHAs can be produced biologically (inside plants or micro-organisms) instead of chemically (Sudesh *et al*., 2000). Short chain PHAs can also be produced from volatile fatty acids (VFAs), which are the precursors of MCFAs. The main advantage of medium chain length PHAs (based on MCFAs) over short chain length PHAs (based on VFAs) is their reduced brittleness (or increased flexibility), which makes them suitable for higher value applications, including medical plastics (Witholt and Kessler, 1999).

1.4 Chain elongation compared to some other biobased processes

Instead of MCFA production, several other processes can be used to valorise biomass. In this chapter, a short comparison is made of chain elongation with some other biobased chemical processes, respectively ethanol production, gasification combined with the Fisher-Tropsch process and anaerobic digestion for methane production. In thermochemical processes, the water in the biomass is evaporated consuming a large amount of the produced energy. In biochemical processes, the reaction often takes places in the aqueous phase and therefore drying of biomass can be avoided. In general, dry biomass with a low water content (< 30 wt%) are converted more efficiently with thermochemical processes, while conversion of wet biomass with a high water content (> 70 wt%) is preferred with biochemical processes (Figure 2).

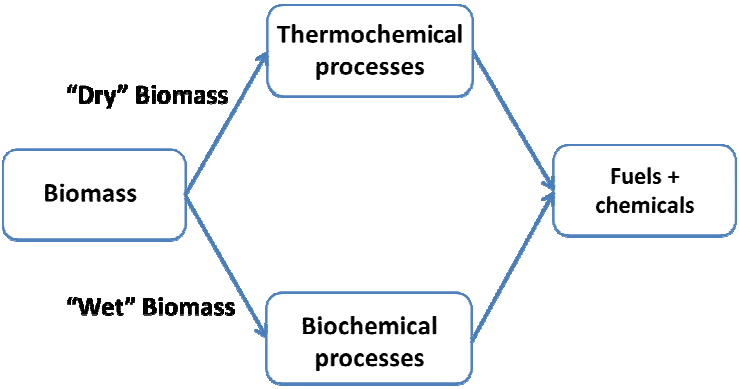


Figure 2. Biomass can be converted by thermochemical processes and biochemical processes to fuels and chemicals. "Dry" biomass refers to biomass with a water content lower than 30 wt%, while "Wet" biomass is defined as biomass with a water content lower than 70 wt%.

Ethanol production from sugars is a well-known process, which can be produced by yeast and bacterial fermentations, often in pure cultures. These pure culture fermentations often require aseptic conditions to avoid contaminations and/or addition of antibiotics. Ethanol production from biomass for biofuel production has also been demonstrated decades ago and currently it is the most applied biofuel production process. Apart from biofuel, ethanol can be used for other applications in the chemical industry, such as ethanol based ester production or as solvent. Compared to ethanol, MCFAs have a wider range of applications than ethanol, including more higher value applications. Consequently, MCFAs are more valuable per carbon atom than ethanol (Agler *et al.*, 2012). Another major disadvantage of ethanol is its cost

to extract it from the fermentation broth, because ethanol is miscible in water and hygroscopic. Due to their hydrophobic tail, MCFAs are not completely miscible in water and therefore easier to separate from water than ethanol.

The gasification process in combination with the Fisher-Tropsch process produces mainly alkanes from biomass. In the gasification process, biomass is thermochemically converted to syngas consisting of carbon mono-oxide and hydrogen. Temperatures in this process are much higher compared to biological processes (T > 700°C) (van Rossum *et al*., 2007), which makes the process less efficient for wet biomass due to energy loss by water evaporation. Nevertheless, especially for dry biomass (water content < 30 wt%), gasification has a large advantage over biochemical processes (including chain elongation), because it can use a much larger fraction of the biomass. After gasification, the syngas can be upgraded to synthetic diesel in the Fischer-Tropsch process. This synthetic diesel contains less impurities than conventional diesel resulting in lower particle matter emissions (Szybist *et al*., 2005). Apart from Fischer-Tropsch diesel, other interesting components can be formed from syngas, including ethanol and methanol. These components could be used to produce MCFAs indirectly from syngas.

In anaerobic digestion (AD), biomass is converted by an anaerobic mixed culture of micro-organisms into biogas, a mixture of methane and carbon. AD is often divided into four separated stage, namely hydrolysis, acidogenesis, acetogenesis and methanogens. In hydrolysis complex organic structures are converted by enzymes into monomeric structures (*e.g*. sugars). Generally, it is the rate limiting step in AD and consequently it requires a lot of

attention. After hydrolysis, the monomeric structures are converted into intermediate products (like VFAs and ethanol), carbon dioxide, protons and hydrogen. Due to the formation of acids and protons, the pH is lowered, which explains the name acidogenesis for this stage. In this thesis, the term acidification is also used, which includes hydrolysis and acidogenesis. The third stage, acetogenesis, is the production of at least acetate, protons and hydrogen from other VFAs than acetate and ethanol. Depending on the VFA species carbon dioxide could also be formed. Finally, methane is produced from acetate, resulting in methane and carbon dioxide, or from carbon dioxide and hydrogen. The first type of methane formation is called acetotrophic methanogenesis, while the second type is called hydrogenotrophic methanogenesis.

The chain elongation process shares several steps with AD, only methanogenesis needs to be limited to prevent substrate consumption by methanogens. Compared to MCFAs, the number of applications of the biogas is smaller (mainly gaseous biofuel) and therefore its value per carbon atom is lower than caproate. However, the separation of methane from water is much easier than the separation of MCFAs, because it is gaseous and poorly soluble in water.

1.5 Status chain elongation at start of investigation

In this thesis we define chain elongation as a mixed culture fermentation that produces MCFAs from VFAs and (non-pure) ethanol. Production of caproate and heptanoate from VFAs and electron donors in

pure culture fermentations has been known for decades. These electron donors could be ethanol in a *Clostridium kluyveri* fermentation (Barker, 1937; Bornstein and Barker, 1948), lactate in a *Megasphaera elsdenii* fermentation (Elsden and Lewis, 1953; Marounek *et al*., 1989), amino acids in a *Eubacterium pyruvatiorans* fermentation (Wallace *et al*., 2003) and methanol in a *Eubacterium limosum* fermentation (only for caproate) (Genthner *et al*., 1981). Apart from VFAs and electrons, caproate can also be produced from glucose in a mixed culture fermentation (*e.g*. Ding *et al*., 2010) or in a pure cultures (*e.g.* with *M. elsdenii* (Roddick and Britz, 1997)). A reason to focus on chain elongation based on VFAs and ethanol was the discovery of caprylate in mixed cultures with acetate, ethanol and hydrogen as substrates (Steinbusch, 2010). Moreover, ethanol was the largest produced biobased chemical at the start of this thesis in 2008.

The main advantage of chain elongation is the increased hydrophobicity of the produced MCFAs compared to ethanol and VFAs. This characteristic makes it easier to separate (undissociated) MCFAs from the fermentation broth than VFAs or ethanol. The solubility of caproic acid in water under standardized conditions is around 10 g l^{-1}, while the solubility decreases to 2.4 g l^{-1} for heptanoic acid and 0.7 g l^{-1} for caprylic acid. These solubilities are much lower than the solubilities of VFAs in water, while ethanol is completely miscible in water (as discussed above).

Because we investigate chain elongation based on ethanol, *C. kluyveri* is an important reference bacterium to the mixed cultures used in this thesis. Moreover, Steinbusch (2010) demonstrated that *C. kluyveri* was the

dominant bacteria in her mixed culture chain elongating reactors. *C. kluyveri* is a mesophilic strictly anaerobic spore-forming bacterium, which can also consume succinate and propanol besides VFAs and ethanol (Kenealy and Waselefsky, 1985). It can be found in fresh water and brackish sediment and it can growth in a pH range from 5.2 to > 8.0 (Kenealy and Waselefsky, 1985).

Its catabolism to obtain energy has been under debate for decades (Thauer *et al.,* 1968; Schoberth and Gottschalk, 1969; Seedorf *et al*., 2008), although recently Li *et al*., 2008 demonstrated that *C.kluyveri* can obtain energy via both ethanol oxidation to acetate and acetyl-CoA elongation to butyryl-CoA. At elevated partial hydrogen pressures (pH_2 > 1 atm), slower growth of the organism was observed (Schoberth and Gottschalk, 1969). Elevated partial hydrogen pressures can make ethanol oxidation to acetate thermodynamically unfeasible. In other words, it becomes more difficult for *C.kluyveri* to obtain energy via ethanol oxidation. However, Steinbusch (2010) was able to growth *C.kluyveri* under elevated hydrogen pressures, so apparently sufficient energy can be produced with the elongation route to sustain growth.

Instead of using one reaction equation, such as proposed by Thauer *et al.*, 1968, for the typical substrates and products of *C.kluyveri* fermentations, a combination of the two main catabolic mechanism could also be used (1,2). Whether these reactions can function completely independent of each other is not clear at the moment. According to Seedorf *et al.* (2008), for each five molecules of ethanol are used for elongation one molecule of ethanol is oxidized to acetate (Figure 3).

$$C_2H_5OH + H_2O \rightarrow CH_3COO^- + H^+ + 2\ H_2 \quad (1)$$

$$C_xH_{2x+1}COO^- + C_2H_5OH \rightarrow C_{(x+2)}H_{(2*(x+2)+1)}COO^- + H_2O \quad (2)$$

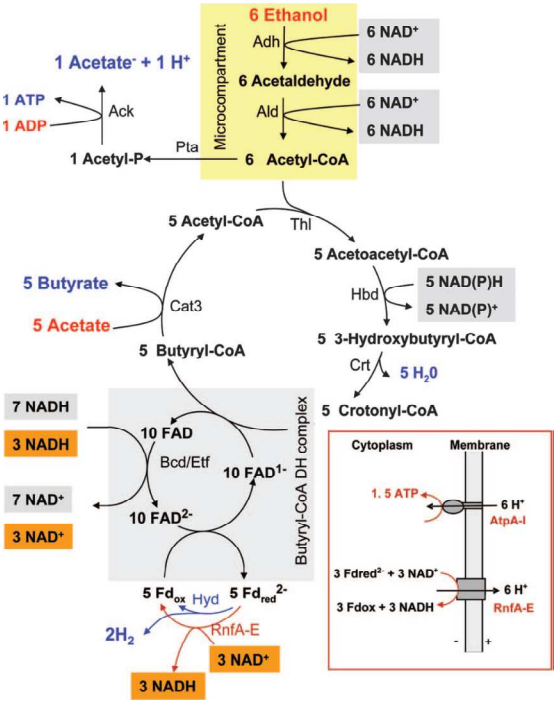


Figure 3. Overview of metabolism of C.kluyveri (from Seedorf et al. (2008)). Ethanol is the main driver for the fermentation, although a VFA (acetate in the picture) is required as electron acceptor.

The first publication about *C. kluyveri* appeared in 1937 (Barker, 1937) and since then the bacterium received quite some attention (> 100 papers). However, most of these works were performed in batch cultures and

were orientated on the bacterium itself. In these tests predominately butyrate was produced and little efforts were made to develop production processes with its products. Kenealy and Waselefsky (1985) were the first to test pure *C. kluyveri* strains in continuous reactors. They indicated that a higher growth rate of *C. kluyveri* can be obtained in continuous reactors than in batch cultures. Smith and McCarty (1989) were the first to publish (temporarily) MCFA production after applying perturbations of ethanol in continuous propionate fed reactors.

Steinbusch (2010) demonstrated continuous production of caproate and caprylate with caproate concentrations above the solubility of caproic acid in water. She achieved a MCFA production rate of 0.5 g MCFA l^{-1} d^{-1} (± 0.1 mol e eq l^{-1} d^{-1}), which is roughly 40 times slower than a fast methane producing reactor. The MCFA selectivity was 76%[2] and a methanogenic inhibitor, 2-bromoethanosulfonate (2-BES), was added to prevent hydrogen and acetate consumption. Finally, the achieved caprylate concentration was below the solubility of caprylic acid.

1.6 Objectives

The objective of this thesis was to develop a MCFA production process based on municipal solid waste and ethanol. If successful, this

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[2] based on consumed electron from ethanol and acetate, consumption of hydrogen excluded

process could become an alternative for anaerobic digestion. To become a serious alternative, the volumetric productivity of the chain elongation process should exceed the maximal volumetric productivity of a methane producing reactor (40 gCOD l^{-1} d^{-1} or 5 mol e eq l^{-1} d^{-1}). Moreover, the consumed electrons (from VFAs and ethanol) should be directed selectively to MCFAs instead of competitive products, like methane. This selectivity should be achieved without the use of chemical agents, like 2-BES. These agents are not only expensive, they require also additional expensive treatment (*e.g.* dehalogenation) before streams, containing these agents, can be disposed into the environment. To easy separation, the concentration of the produced MCFAs should be higher than the solubility of its undissociated form in water under standard conditions (ref). Finally, we would like to investigate whether the difference between MCFA production from municipal solid waste and ethanol using a single phase reactor system and a two phase reactor system.

1.7 Outline of thesis

This thesis consists of two major aspects of the chain elongation process. In the first part (chapters 2, 3 and 4), chain elongation is studied with use of synthetic media. In **chapter 2**, a new reactor concept is discussed to produce MCFAs selectively in mixed cultures based on acetate and ethanol. In **chapter 3**, heptanoate production from propionate and ethanol is studied. In **chapter 4**, chain elongation at reduced the hydraulic retention times is studied to improve the MCFA productivity. After the first part of the thesis, an intermezzo is located to discuss the in-situ inhibition of methanogens during dry anaerobic digestion. This intermezzo is an introduction for the second part

of the thesis (chapters 5 and 6) in which chain elongation based on municipal solid waste and ethanol is discussed. In **chapter 5**, ethanol additions were performed during acidification of municipal solid waste to promote chain elongation (single phase reactor system). In **chapter 6**, chain elongation was performed in a two phase reactor system.

2. Chain elongation of acetate and ethanol in an upflow anaerobic filter for high rate MCFA production

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Abstract

Recently, interest has regained for medium chain fatty acids (MCFAs) as a low cost feedstock for bio-based chemical and fuel production processes. To become cost-effective, the volumetric MCFA production rate by chain elongation should increase to comparable rates of other fermentation processes. We investigate the MCFA production process at a hydraulic retention time of 17 hours in an upflow anaerobic filter to improve the volumetric MCFA production rate. This approach resulted in a MCFA production with a volumetric production rate of 16.6 g l^{-1} d^{-1}, which is more than seven times higher than the current production rate. Moreover the rate is now in the range of other fermentation processes like methane, butanol and ethanol production. Increasing the ethanol load lead to higher volumetric production rates and a high MCFA selectivity of 91 per cent. During operation, methane percentages lower than 0.1 per cent were detected in the headspace of reactor.

2.1 Introduction

In the search for bio-based chemical and fuel production processes, often costs are limiting the transition from fossil to bio-based chemicals and fuels. Fermentations are one the key processes for the production of bio-based chemicals and fuel . However, fermentations have a high water content and their products are often soluble in water. The extraction of miscible fermentation products in water (like ethanol) is a large cost factor, because it is energy intensive. Ethanol requires significant amount of the energy for its distillation from the fermentation broth. As alternative feedstock for fuel production, medium chain fatty acids (MCFA) could be produced. The MCFA fermentation got recently more interest, because its products can be produced in high concentrations close to the solubility of their undissociated form in water (Steinbusch *et al.*, 2011). Furthermore, the MCFA fermentation can use diluted ethanol directly (without its ineffective distillation) as a substrate (Agler *et al.*, 2012). Moreover, MCFAs, straight carboxylic acids with six to eight carbon atoms, can be produced in a non-sterile environment and have more versatile applications than ethanol, including antimicrobials (directly), biofuels or bioplastics (both indirectly) (Levy *et al.,* 1981; Witholt and Kessler, 1999). By producing these biochemical in a non-sterile environment, no costly sterilisation step is required.

MCFAs can be produced from intermediate biochemical produced in the anaerobic digestion process, like acetate, propionate and other volatile fatty acids (VFAs). These intermediate products can be produced from low grade biomass (Agler *et al*., 2011; D'Addario *et al*., 1993). However, they are

mostly completely miscible in water and are difficult to extract. By adding a second fermentation step with addition of diluted ethanol, the carbon chain length of the VFAs is elongated to six, seven or even eight carbon atoms. This elongated carbon chain makes the

MCFAs more hydrophobic than VFAs and drastically decreases their solubility in water. This MCFA production process, which elongates VFAs with ethanol, is called the chain elongation process and is considered to be a breakthrough, because of better separation properties from water than volatile fatty acids (VFAs) and ethanol and promising selectivity (Agler *et al.*, 2011; Angenent and Kleerebezem , 2011).

To become a cost-effective platform technology for the production of biochemicals, several limitations of the non-sterile chain elongation process have to be improved. First, the MCFA volumetric production rate should be improved with high MCFA concentrations for easier separation. Second, the ethanol consumption should lead to selective MCFA production otherwise valuable ethanol is wasted. Because we work in non-sterile conditions, competitive processes like methanogenesis from acetate should be limited to obtain this selective MCFA production. According to Agler *et al.*, 2012, chain elongation and methanogenesis from carbondioxide and hydrogen are not competitive processes. Hence, the need to limit hydrogenotrophic methanogens does not seem to be as essential as the required limitation of acetotrophic methanogens. Finally, the operation of the chain elongation process should be robust and stable without additional chemical agents, like 2-Bromoethanosulfophate (2-BES), for methane inhibition.

Steinbusch *et al.,* 2011 achieved a volumetric caproate rate of 0.5 g l^{-1} d^{-1} (0.1 mol e eq l^{-1} d^{-1}) at a concentration of 8.2 g l^{-1} (71 mM) from acetate and ethanol. Additionally, their maximum volumetric caprylate production rate reached 54 mg l^{-1} d^{-1} (16 mmol e eq l^{-1} d^{-1}) with a concentration of 0.3 g l^{-1}(2 mM). Recently, Agler *et al.*, 2012 achieved a volumetric caproate production rate of 2.1 g l^{-1} d^{-1} (0.6 mol e eq l^{-1} d^{-1}) from dilute ethanol in yeast-fermentation beer. In contrast to Steinbusch *et al.,* 2011, Agler *et al.*, 2012 mentioned no significant amount of caprylate production.

A possible explanation for the lack of significant caprylate production in their study could be the removal of caproate from the fermentation broth by use of in-line extraction, which was not used in Steinbusch *et al.*, 2011. However, Agler *et al.*, 2012 produced caproate without an additional chemical agent for methane inhibition, while Steinbusch *et al.,* 2011 applied 2-BES.

To increase the volumetric productivity of a MCFA production process, better retention of MCFA producing biomass is desired. Although, reactor systems with (internal) settlers retain more MCFA producing biomass, they retain unwanted microbial populations, such as acetotrophic methanogens, as well. Consequently, an ideal MCFA producing system should retain MCFA producing biomass and avoid accumulation of methanogenic biomass.

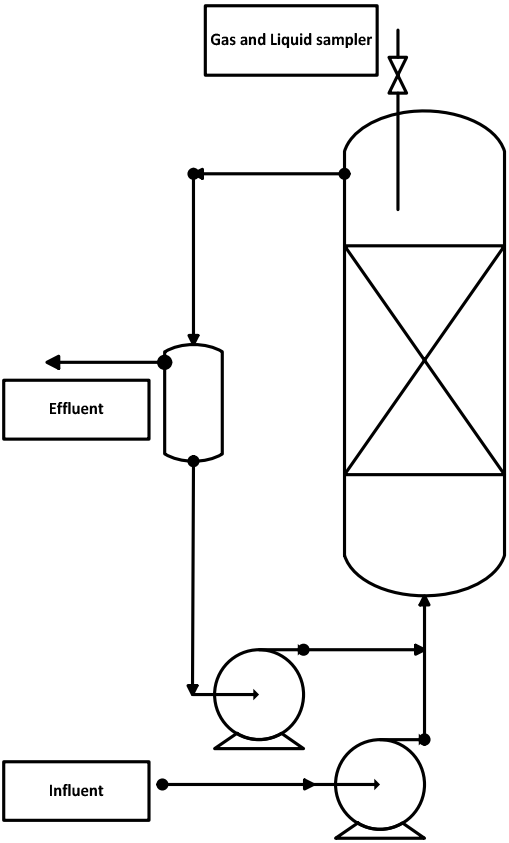

Figure 1. Schematic overview of reactor set-up. Influent is pumped up from the refrigerator (not shown) into the bottom of the upflow anaerobic filter. On the top, the liquid and the gas in the reactor overflow into a small vessel, in which the excess of gas and liquid is removed. The other liquid (and gas) is recycled to the bottom of the reactor. Liquid samples are taken from inside the anaerobic filter, while gas samples are taken in the headspace of reactor (by pulling up the needle to the headspace).

A possible reactor system to increase biomass concentrations selectively is the upflow anaerobic filter (or anaerobic fixed film bioreactor), which has a carrier material for biomass support. These reactors are well-known in mixed culture fermentations and have been used for various applications in environmental biotechnology, including caproate production from glucose (Ding *et al.*, 2010) and methane production systems (Raynal *et al.*, 1998; Hamoda *et al.*, 1996). For methane production, these were not applied as much as reactor systems having internal settlers, like upflow anaerobic sludge blankets (UASBs) and expanded granular sludge beds (EGSBs), because less biomass was retained in the anaerobic filters (*e.g.* Ruiz *et al.*, 1997). Moreover, the additional cost of packing materials in anaerobic filters makes them less favourable for methane production than internal settler systems. An explanation for lower biomass retention in methanogenic anaerobic filters can be found in the type of biomass formation, which cannot resist large shear stress in case the upflow velocity larger than 1 m h^{-1} is applied (Smith *et al.*, 1996; Suraruksa *et al.*, 2003). Once the methanogenic biomass is detached from its carrier, it could be washed-out of the reactor if the hydraulic retention time (HRT) is low enough.

In this research, we investigate an anaerobic filter to improve the volumetric MCFA production rate of the chain elongation process based on acetate. The filter was filled with polyurethane cubes as carrier material. For selective MCFA production, the reactor had a HRT of 17 hours to retain detached MCFA producers and to reduce the impact of free methanogens. Furthermore, an upflow velocity of 1.2 m h^{-1} was used to stress methanogens

that could be attached to the carrier material. Additionally, we want to investigate the effect of the ethanol concentration in the reactor, because Steinbusch *et al.*, 2011 noticed that the volumetric MCFA production rate was low in case of low ethanol concentrations in the reactor.

2.2 Material and methods

2.2.1 Reactor set-up and inoculum

The experiments were executed in one litre glass upflow reactor filled with polyurethane cubes (Recticel, Belgium) to promote biomass retention. A liquid recycle with a recirculation rate of 2.5 litres per hour was applied for mixing the reactor. For both influent and liquid recycle peristaltic pumps were used (Marlon Watson 403 U/R1, U.K.). The temperature of the reactor was controlled at 30.0 ± 0.1 °C with a water bath (Julabo, Germany). The influent was prepared in an anaerobic hood and stored anaerobically in a refrigerator. *Figure 1* shows the schematic overview of the reactor set-up. The inoculum was derived from a mixed culture continuous stirred tank reactor performing chain elongation (Steinbusch, 2010). The reactor contained 2-BES to suppress methanogens, so it is possible the inoculum did not contain any active methanogenic bacteria.

2.2.2 Medium

The synthetic medium was based on the medium of Steinbusch *et al*., 2011 containing acetate and ethanol. Additionally, in our experiments 4.0 g K_2CO_3 per litre medium was applied, as *Clostridium kluyveri*, a well-known MCFA producer, requires it for growth (Tomlinson and Barker, 1954).

Furthermore, two ml of trace elements and B-vitamins per litre medium were used instead of one ml per litre. In contrast to Steinbusch *et al.*, 2011 no 2-BES was supplied. To keep the pH between 6.5 and 7.0, 3.8 g sodium hydroxide per litre medium was added. From day 12 on, we added one gram yeast extract per litre to improve protein synthesis for the MCFA producing bacteria.

2.2.3 Experiment

The experiment consisted of a start-up phase in which the MCFA production was not stable, followed by phases I, II and III that all had stable MCFA production. The total experiment took 80 days. The HRT throughout phases I, II and III was 17 hours. During phases I, II and III, the ethanol load was changed from 13.0 g l^{-1} d^{-1} to 26.0 g l^{-1} d^{-1} by increasing the ethanol concentration in the influent from 9.2 g l^{-1} to 18.4 g l^{-1}. Due to different ethanol loads, the ethanol concentration in the reactor changed and its effect could be investigated. In Table 1, an overview of the experiment is presented.

Table 1: Experimental overview of the investigation in different phases

Phase	Days after start experiment	Acetate load (g l^{-1} d^{-1})	Ethanol load (g l^{-1} d^{-1})	Stable MCFA production?
Start-up	Before day 56	8.5	13.0	No
I	Day 56 to day 63	8.5	13.0	Yes
II	Day 64 to day 70	8.5	19.5	Yes
III	Day 71 to day 80	8.5	26.0	Yes

2.2.4 Measurements

pH was measured automatically by an electrode (type QP108X, QIS, The Netherlands). Five liquid samples a week were taken to determine carboxylic acid (C2 to C8) and ethanol concentrations in the reactor. Samples for carboxylic acids measurements were 40 times diluted with 1.5 % v/v formic acid solution, while samples for ethanol measurements were 25 times diluted with demineralized water. Both carboxylic acids and ethanol samples were measured by gas chromatography (Steinbusch *et al*., 2008). Occasionally, the headspace composition was monitored for methane, oxygen and nitrogen by gas chromatography (Steinbusch *et al*., 2008). Gas samples were taken with a syringe and directly injected in the GC. Free biomass was measured with a modified Hartree-Lowry method in the same way as described by Steinbusch *et al.*, 2011.

2.2.5 Calculations

2.2.5.1 Selectivity

The selectivity is defined as the concentration of electrons in the formed product (butyrate, caproate and caprylate) divided by the net consumed electrons from the fed acetate and ethanol (equation 1). Acetate contains 8 moles of electrons per mole, ethanol 12 moles of electrons per mole, butyrate 20 moles of electrons per mole, caproate 32 moles of electrons per mole and caprylate 44 moles of electrons per mole.

Selectivity = [Product] (mol e eq l^{-1}) / consumed ethanol and acetate (mol e eq l^{-1}) (1)

2.2.5.2 Volumetric production rate

The volumetric production rate of the products (butyrate, caproate and caprylate) is defined by its concentration divided by the hydraulic retention time (equation 2). The unit of the volumetric production rate is g l^{-1} d^{-1} or mol electrons equivalents l^{-1} d^{-1} (mol e eq l^{-1} $d^{-1)}$. The unit mol e eq l^{-1} d^{-1} can be used to compare this process with other biochemical processes.

Volumetric production rate = [Product] (g l^{-1} or mol e eq l^{-1}) / HRT (d) (2)

2.3 Results and discussion

2.3.1 Improved MCFA production occurs in the upflow anaerobic filter

Using the anaerobic filter, continuous stable production was established for 24 days in the upflow anaerobic filter with a HRT of 17h without the use of chemical agents for methane inhibitions. This stable reactor run of 33 times the hydraulic retention time demonstrated that MCFA producers were retained in this system. This retention was expected. Due to the fast maximum growth rate of MCFA producers (> 0.1 h^{-1} for *C.kluyveri* (Kenealy and Waselefsky, 1985)), a HRT of 17 h is long enough to retain MCFA producers in the bioreactor. In contrast to the MCFA producers, the methanogenic population was hardly active in the reactor. During the 80 days of operation, methane percentages lower than 0.1 % were detected in the headspace of reactor.

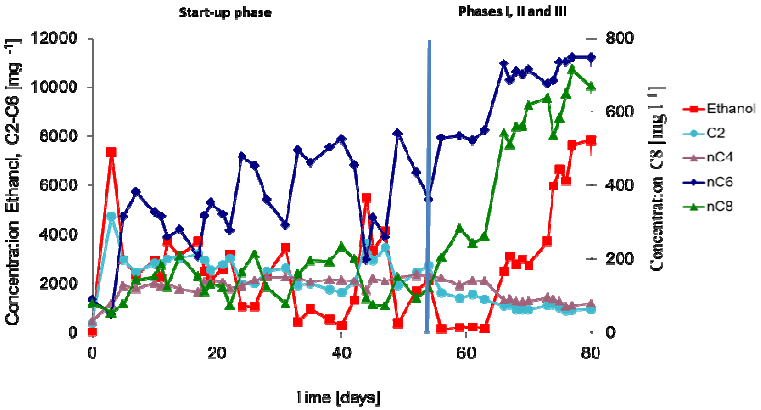

Figure 2. Results of the whole experiment, which can be divided in a non-stable period, the start-up phase, and a stable period including phases I, II, III. The start-up phase could not become stable due to pH variations and clog-ups. From day 55 on stable reactor operation was achieved with high MCFA production. The concentrations of ethanol, acetate (C2), butyrate (nC4) and caproate (nC6) are displayed on the left axis, while the concentration of caprylate (nC8) is displayed on the right axis.

Due to their low maximum growth rate of approximately 0.013 h^{-1} (Gujer and Zehnder, 1983), detached acetotrophic methanogens are likely to be wash-out in an anaerobic filter (thus without (internal) settler) with a HRT of 17 h. However, if detachment of acetotrophic methanogens would not occur, additional actions are required. Among them could be increasing the upflow velocity to increase the shear stress on the attached acetotrophic methanogens or heat shocks, which can be survived by spore-forming

bacteria like *C.kluyveri.* Although Agler *et al.*, 2012 stated that simultaneous chain elongation and hydrogenotrophic methanogenesis is feasible, heat shock could also be used to limit hydrogenotrophic methanogens, in case they have a negative effect on chain elongation. Because the maximum growth rate of hydrogenotrophic methanogens is also high (> 0.1 h^{-1}), wash-out of detached hydrogenotrophic methanogens is much more difficult than wash-out of detached acetotrophic methanogens. Hence, increasing the upflow velocity does not seem to be effective for limiting hydrogenotrophic methanogenesis. Disadvantages of heat shocks could be the costs of heating the reactor and the reduced MCFA production due to the time required for recovery of MCFA producing biomass. Future research should demonstrate whether build-up of acetotrophic methanogens can be prevented, preferably without heat shocks.

The free biomass concentration was measured throughout the experiment and this confirmed the presence of microbial biomass in the reactor (data not shown). The maximum concentration of free biomass achieved in the stable period (day 56 to day 80) was 0.5 g VSS l^{-1} (on day 68). This concentration is higher than the maximum free biomass concentration in the work of Steinbusch *et al.*, 2011 with a fed-batch reactor (0.4 g VSS l^{-1}), indicating that the anaerobic filter retains more biomass. The measured values of free biomass are not representing the total biomass concentrations as part of the biomass adhered to the reactor wall and the polyurethane cubes.

Table 2: Overview of the results of the investigation. The subscript ss denotes steady state

Phase	$Caproate_{ss}$ ($g\ l^{-1}$)	$Caprylate_{ss}$ ($g\ l^{-1}$)	Caproate production rate ($g\ C6\ l^{-1}\ d^{-1}$)	MCFA selectivity ($mol\ e\ mol^{-1}\ e$ * 100%)
Start-up	-	-	-	-
I	8.0	0.3	11.3	78
II	10.7	0.5	15.1	88
III	11.1	0.6	15.7	91

In the start-up phase no stable caproate and caprylate concentrations were achieved.

In the final phase of the experiment, a maximum stable volumetric caproate production rate of 15.7 g l^{-1} d^{-1} (4.3 mol e eq l^{-1} d^{-1}) with a concentration of 11.1 g l^{-1} (96 mM) and a selectivity of 85 % was achieved (Table 2). Besides, the maximum volumetric caprylate production rate reached 0.9 g l^{-1} d^{-1} (0.3 mol e eq l^{-1} d^{-1}) with a concentration of 0.6 g l^{-1}(4 mM) and a selectivity of six per cent.

Compared to the previous results of Steinbusch *et al.*, 2011, the volumetric production rate of caproate was improved by more than 30 times, while volumetric production rate of caprylate was improved by more than 16 times. Additionally, the concentrations of caproate and caprylate were higher as well, although only with respectively 35 and 88%. Compared to the work of Agler *et al.*, 2012, the increase of the volumetric caproate production rate is lower, but still more than seven times higher. Because Agler *et al.*, 2012 used diluted yeast-fermentation beer instead of synthetic medium , their volumetric

caproate production rate could have been limited by a lack of internal VFA production. Possibly, this lack of internal VFA production was limited by the hydrolysis of left-over corn grain biomass, depending on the internal production of VFAs from ethanol, carbon dioxide and hydrogen, sugars and/or yeast biomass. Nevertheless, probably due to the decay of yeasts and/or sugar-degrading bacteria, they did not need to add any additional costly yeast extract, like we added in this investigation. To make the process more cost-effective, future research could be performed to reduce or even eliminate the usage of yeast extract depending on its cost.

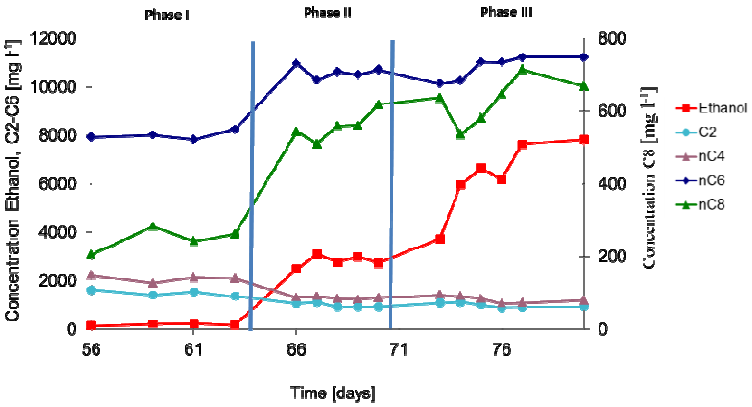

Figure 3. Overview of the concentrations during the stable period. In phase I (ethanol load of 13.0 g $l^{-1}d^{-1}$) MCFA production was clearly limited by low ethanol concentrations, while in phase II (ethanol load 19.5 g $l^{-1}d^{-1}$) and III (ethanol load 26.0 g $l^{-1}d^{-1}$) ethanol was not limiting anymore. The concentrations of ethanol, acetate (C2), butyrate (nC4) and caproate (nC6) are displayed on the left axis, while the concentration of caprylate (nC8) is displayed on the right axis.

2.3.2 Reactor operation during start-up phase (first 55 days)

During the first 55 days of the reactor operation, MCFA production was not stable due to pH deviations (between 5.6 and 8.6) and clogging problems in the feed stream that resulted in fluctuating loads (*Figure 2*). pH can have a direct effect on the growth of the MCFA producers, but it also determines how the added K_2CO_3 is distributed over CO_2 (or even H_2CO_3), HCO_3^- and CO_3^{2-}. It is known that *C. kluyveri* requires carbon dioxide for their protein formation (Tomlinson and Barker, 1954). Possibly, this protein formation might proceed faster with CO_2 than with HCO_3^-. At pH levels above 7.3 less than 10% of the added carbonate is present in the form of CO_2, while at pH levels below 6.3 more than 50% of the added carbonate is present as CO_2. Note that these percentage are based on standard conditions. So alkaline conditions, above 7.0, might limit MCFA producing bacteria and therefore MCFA production, in case of CO_2 absence. Kenealy and Waselefsky (1985) stated that *Clostridium kluyveri* has an optimal pH for growth of 6.4, which is close to the pKa of CO_2 and HCO_3^-. At acid pH levels, below 6.0, the MCFA producing bacteria is likely to invest energy to maintain their internal pH, resulting in a lower biomass production.

On day 54, the pump tube was replaced with a new tube, which had a larger diameter to avoid further clogging problems. That resulted in stable MCFA volumetric production rates with low ethanol concentrations, below 0.3 g l^{-1}, as limiting factor. It is likely that the low ethanol concentrations caused a substrate limitation for MCFA production.

2.3.3 Higher reactor concentrations of ethanol increase selectivity MCFA production process

In the first stable period, phase I (from day 55 to day 63), an acetate load of 8.5 g l^{-1} d^{-1} and an ethanol load of 13.0 g l^{-1} d^{-1} were applied to the reactor resulting in a stable caproate and caprylate production of respectively 8.0 g l^{-1} (69 mM) and 0.3 g l^{-1} (2 mM) (*Figure 3*). Besides MCFAs, 2.1 g butyrate l^{-1} (24 mM) was produced as well. Butyrate is an intermediate product required for the formation of caproate, but should be low to have a high selectivity for MCFA. Besides, butyrate is harder to separate than MCFAs from the fermentation broth due to its higher solubility in water. *Figure 4a* demonstrates the distribution of the formed products in this period. In the first stable period, 78 % of the consumed electrons from acetate and ethanol was converted into MCFAs, while 16 % was converted to butyrate.

In phase II, from day 64 to day 70, the ethanol load was increased to 19.5 g l^{-1} d^{-1}, to remove the ethanol limitation in phase I (*Figure 3*). After increase of the ethanol load, caproate and caprylate concentrations immediately increased to 10.7 g l^{-1} (92 mM) and 0.5 g l^{-1} (4 mM). Moreover, the butyrate concentration decreased to 1.3 g l^{-1} (15 mM). Consequently, the MCFA selectivity increased from 78 % (75% + 3%) to 88 % (83% + 5%), which is shown in *Figure 4b*.

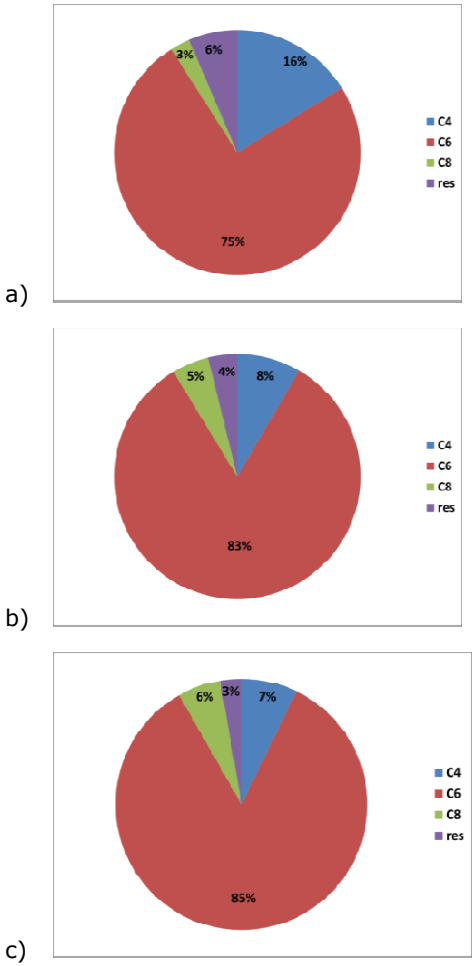

Figure 4. Distribution of product formation during phase I (a), phase II (b) and phase III (c) based on consumed electrons from acetate and ethanol. Increasing the ethanol load leads to more selective MCFA production (C6+C8), while butyrate production (C4) and other residual products (res), including biomass, are reduced.

To determine whether ethanol concentration in the reactor was still limiting the MCFA production process, the ethanol load was increased again to 26.0 g l^{-1} d^{-1} in phase III (day 71 to day 80). Average caproate and caprylate concentrations rose to respectively 11.1 g l^{-1} (96 mM) and 0.6 g l^{-1} (4 mM), although the increases were less spectacular than after the first ethanol increase (*Figure 3*). Also, the butyrate concentration decreased again slightly to 1.1 g l^{-1} (13 mM). Consequently, the MCFA selectivity increased as well to 91 % (85% + 6%) (*Figure 4c*). The MCFA production rate hardly increased after the second increase of the ethanol load (19.5 to 26.0 g l^{-1} d^{-1}), because the ethanol concentration in the reactor during phase II (2.8 g l^{-1}) was not the main limitation for the MCFA production rate anymore.

A downside of increasing the ethanol load could be associated with costs, especially if large part of the ethanol is not converted to MCFAs anymore, which happened in phase III. On the other hand, an ethanol concentration of 18.4 g l^{-1} represents an alcohol percentage of approximately 2.4 % (v/v) in water, which can easily be met with yeast fermentations (like beer and wine fermentations). That confirms that the use of distilled ethanol is not necessarily required in the chain elongation process.

2.4 Conclusions

In this investigation, we demonstrated that selective high rate MCFA production can be performed in a continuous upflow anaerobic filter for a period of 80 days. We improved the chain elongation process to a volumetric MCFA production rate of 16.6 g l^{-1} d^{-1} (4.6 mol e eq l^{-1} d^{-1}), which is the range

of other biochemical processes like anaerobic digestion (methane), butanol and ethanol production. By increasing the ethanol load, the limitation by the low ethanol concentrations in the reactor during phase I was removed and the MCFA production rate increased. Moreover, the increased ethanol load improved MCFA selectivity.

3. High rate heptanoate production from propionate and ethanol using chain elongation

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Abstract

Heptanoate (or enanthate), a saturated mono-carboxylate with seven carbon atoms, is a commercially produced biochemical building block with versatile applications. Currently, heptanoate is mainly derived from the oxidation of heptaldehyde, which can be obtained after pyrolysis of castor oil. The objective of this investigation was to achieve efficient high rate heptanoate production using a mixed culture chain elongation process based on propionate and ethanol. An efficient high rate heptanoate production using chain elongation could offer an alternative for heptanoate production from castor oil. The investigation was performed in an upflow anaerobic filter with a hydraulic retention time of 17 hours. A heptanoate production rate of 4.5 g l^{-1} d^{-1} was achieved with a heptanoate concentration of 3.2 g l^{-1}. These results show sufficient potential to consider this approach as an alternative for heptanoate production from castor oil. Future research should make heptanoate production from propionate and ethanol more cost-effective.

3.1 Introduction

Heptanoate (or enanthate), a saturated mono-carboxylate with seven carbon atoms, is a commercially produced biochemical building block with versatile applications, including biodiesel production (Renz, 2005), antimicrobials (*e.g.* Woolford, 1975) and bioplastic production (*e.g.* Liebergesell *et al.*, 1991). Currently, heptanoate is mainly derived from the oxidation of heptaldehyde, which can be obtained after pyrolysis of castor oil (Das *et al.,*1989). In this thermochemical step, the castor oil is cleaved into undecylenic acid as major product and heptaldehyde as by-product.

However, heptanoate production from castor oil does have some disadvantages. First, the castor oil production itself is inefficient, only the seeds of the plants are used for castor oil production instead of the complete plant[3]. Second, most of the castor oil production is currently located in India, China, Brazil and Thailand (Ogunniyi, 2006). For castor oil importing countries, these limited production areas could lead to high prices due to lack of competition. Finally, the overall heptanoate selectivity from castor oil does not exceed 25% (castor oil as 100% Ricinoleic acid assumed). An alternative heptanoate production process could avoid these disadvantages.

Chain elongation could be such an alternative process. It is a fermentation that produces medium chain fatty acids (MCFAs) from volatile fatty acids (VFAs) and ethanol, in which undistilled ethanol can be used (Agler

[3] The plant (*Ricinus communis)* has other applications that are not discussed here

et al., 2012). Both VFAs and ethanol can be produced from lignocellulosic (waste) materials (Agler *et al.*, 2011; Sarkar *et al.*, 2012), which can contain a larger part of the plant than the seeds. Moreover, chain elongation is an efficient fermentation with a MCFA selectivity of more than 80% (Agler *et al.*, 2012; Grootscholten *et al.*, 2013b). In the studies of Steinbusch *et al.* (2011) and Agler *et al.* (2012), *Clostridium kluyveri* was found as dominant chain elongating micro-organism. However, it is possible that other (unknown) micro-organisms could perform chain elongation as well.

So far, research on the chain elongation process has mainly focussed on caproate (Steinbusch *et al.*, 2011; Agler *et al.*, 2012; Grootscholten *et al.*, 2013b), a straight mono-carboxylate with six carbon atoms, resulting in caproate production rates of 15.7 g caproate per litre per day (4.3 mol e eq l^{-1} d^{-1}). However, it is known that heptanoate can be produced by chain elongation as well, if propionate and ethanol are used (Bornstein and Barker, 1948).

Heptanoate is often found in low concentrations (up to 0.4 g l^{-1}(3 mM)) during acidification of the organic fraction of municipal solid waste (OFMSW) without ethanol additions (*e.g.* Grootscholten *et al.*, 2013a). Bornstein and Barker (1948) were the first to report heptanoate production from propionate and ethanol with a pure culture of *C.kluyveri*. They achieved a heptanoate concentration of 0.3 g l^{-1}(2 mM) in their batch experiments. Smith and McCarty (1989) found temporarily elevated heptanoate concentrations (up to 1.5 g l^{-1}(12 mM)) after perturbations with ethanol in mixed culture propionate fed continuous reactors. Grootscholten *et al.* (2013a) produced

heptanoate from OFMSW after ethanol additions during acidification (up to 1.5 g l^{-1}(12 mM)) in dry anaerobic batch reactors, but the maximum heptanoate production rate was very low (0.3 g C7 l^{-1} d^{-1} (0.1 mol e eq l^{-1} d^{-1})). Toxic effects of undissociated MCFAs and/or ethanol on hydrolysis could be reasons for the low productivity. Consequently, the VFA production rate is lowered and less substrate for chain elongation is available.

The objective of this investigation was to achieve efficient high rate heptanoate production using a mixed culture chain elongation process based on propionate and ethanol. Propionate can be produced from several precursors, including glucose, lactate (Agler et al., 2011) and glycerol (Schauder and Schink, 1989). For this investigation, a synthetic medium with a high propionate concentration (7.4 g l^{-1}(100 mM)) was used to be able to compare heptanoate production with caproate production (Grootscholten *et al*., 2013b) in terms of conversion rates, concentrations and selectivity. An efficient high rate heptanoate production using chain elongation could offer an alternative for heptanoate production from castor oil.

3.2 Material and Methods

3.2.1 Reactor set-up and experiment

The reactor set-up and methods were the same as in Grootscholten et al. (2013b). In addition, pelargonate, a straight mono-carboxylate with nine carbon atoms, and propanol were measured as well. We applied a mixed culture upflow anaerobic filter with a hydraulic retention time (HRT) of 17 hours. The temperature was controlled at 30.0 ± 0.1°C and the pH was

maintained between 6.5 and 7.0. No chemical agent for methane inhibition, such as 2-bromoethanosulfonate (2-BES), was used. The substrate consisted of acetate, propionate and ethanol instead of acetate and ethanol. Table 1 shows the substrate load during the experiment. Acetate was supplemented to sustain biomass concentrations as it is a required compound for protein synthesis in *C.kluyveri* (Tomlinson and Barker, 1954).

Table 1: Experimental overview of the investigation in different phases

Phase	Days after start experiment	Acetate load ($g\ l^{-1}\ d^{-1}$)	Propionate load ($g\ l^{-1}\ d^{-1}$)	Ethanol load ($g\ l^{-1}\ d^{-1}$)
I	Day 0 to day 7	1.7	10.4	13.0
II	Day 8 to day 14	0.9	10.4	13.0
III	Day 15 to day 21	0.9	10.4	19.5

After one week, the acetate load was lowered as sufficient acetate was internally produced (Figure 1b). Two weeks after the start of the experiment, the ethanol load was increased to eliminate the ethanol limitation, because the ethanol concentration was below 1.0 $g\ l^{-1}$.

3.2.2 Calculations

3.2.2.1 Selectivity

The selectivity is defined as the concentration of electrons in the formed product divided by the net consumed electrons from the fed acetate, propionate and ethanol (1). Acetate contains 8 mol electrons per mole, propionate 14 mol electrons per mole, ethanol 12 mol electrons per mole, butyrate 20 mol electrons per mole, valerate 26 mol electrons per mole, caproate 32 mol electrons per mole, heptanoate 38 mol electrons per mole and caprylate 44 mol electrons per mole.

Selectivity = [Product] (mol e eq l^{-1}) / consumed ethanol and acetate (mol e eq l^{-1}) (1)

3.2.2.2 Volumetric production rate

The volumetric production rate of the products (butyrate, valerate, caproate, heptanoate and caprylate) is defined by its concentration divided by the HRT (2). The unit of the volumetric production rate is g l^{-1} d^{-1} and moles electron equivalents l^{-1} d^{-1} (mol e eq l^{-1} d^{-1}).

Volumetric production rate = [Product] (g l^{-1} or mol e eq l^{-1}) / HRT (d) (2)

3.3 Results and discussion

3.3.1 High concentrations of heptanoate and traces of pelargonate were produced

High rate heptanoate production was demonstrated in a steady state operating continuous flow bioreactor. A heptanoate concentration of 3.2

g l^{-1} (25 mM) was obtained at a production rate of 4.5 g l^{-1} d^{-1} (1.3 mol e eq l^{-1} d^{-1}) with a selectivity of 23%. The obtained concentration is higher than the solubility of heptanoic acid (undissociated form) in water under standard conditions, which is 2.4 g l^{-1} (18 mM). In other words, this concentration shows sufficient potential to develop separation methods for heptanoate removal from fermentation broths. Compared to the work of Smith and McCarty (1989), we doubled the maximum heptanoate concentration and improved the heptanoate production rate by more than 25 times. Moreover, the formed heptanoate was not degraded probably due to absence of hydrogenotrophic methanogens (methane percentages were below 0.1% in the headspace of the reactor). The absence of methanogens could be related to the inoculum which may not have contained active methanogens (Grootscholten *et al*., 2013b). If present, hydrogenotrophic methanogens could lower the partial hydrogen pressure and can make heptanoate oxidation thermodynamically feasible (*e.g.* Stams, 1994).

During the two last days of the experiment, low concentrations of pelargonate (< 25 mg l^{-1}) were found as well. In analogy with caproate and caprylate (Steinbusch *et al*., 2011), pelargonate could be formed from heptanoate and ethanol. Pelargonate formation from caproate and propanol might also be a possibility, as propanol can also be used by *C.kluyveri* (Table 3, reaction 3; Kenealy and Waselefsky, 1985).

Before the start of the experiment, the reactor produced mainly caproate from acetate and ethanol (Grootscholten *et al.*, 2013b). Directly after adding propionate, valerate and heptanoate were produced (Figure 1). The

absence of a lag phase indicates the biomass in the reactor did not need to adapt from acetate elongation to propionate elongation.

3.3.2 Low heptanoate selectivity due to propanol, caproate and valerate formation

The maximum heptanoate selectivity in this investigation is only 23% (Figure 2), which is much lower than the caproate selectivity (85%) in Grootscholten *et al.* (2013b). Main reasons for the low selectivity are the formations of propanol, caproate, and valerate. These products were also observed in Smith and McCarty (1989).

The exact mechanism of propanol formation in our reactor could not be determined. According to Smith and McCarty (1989), it is likely formed from propionate and ethanol (Table 3, reaction 4). However, Wu and Hickey (1996) reported that other reactions could also be responsible for propanol production during ethanol degradation as well, including propionate reduction (Table 3, reaction 5).

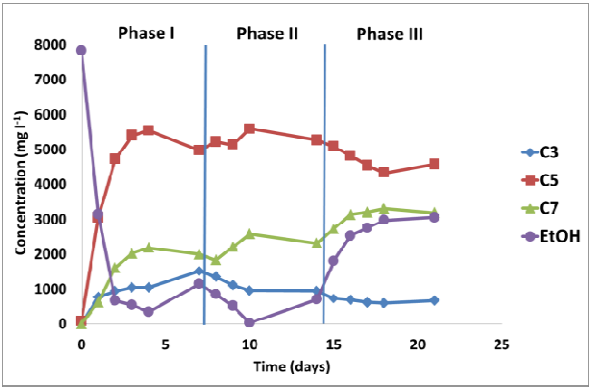
The caproate concentrations in the investigations of Smith and McCarty (1989) never exceeded 0.8 g l^{-1} (7 mM), while highest caproate concentration in this investigation reached 5.0 g l^{-1} (43 mM). Explanations for the high caproate concentrations can be found in the side reactions (Table 3, reactions 2 and 3). The first side reaction is the ethanol oxidation to acetate (Table 3, reaction 2). This internal acetate production can be further elongated to butyrate, caproate and caprylate (Table 3, reaction 1). In the second side

reaction, caproate could be produced by propionate elongation with propanol. (Table 3, reaction 3; Kenealy and Waselefsky, 1985).

Table 2: Overview of the results of the investigation. The subscript ss denotes steady state

Phase	$Heptanoate_{ss}$ ($g\ l^{-1}$)	$Caproate_{ss}$ ($g\ l^{-1}$)	$Valerate_{ss}$ ($g\ l^{-1}$)	Heptanoate production rate ($g\ C7\ l^{-1}\ d^{-1}$)	Heptanoate selectivity ($mol\ e\ eq\ l^{-1}\ mol^{-1}\ e\ eq\ l^{-1}$ * 100%)
I	2.0	3.9	5.2	2.8	17
II	2.2	3.7	5.3	3.1	19
III	3.2	4.9	4.6	4.5	23

a)



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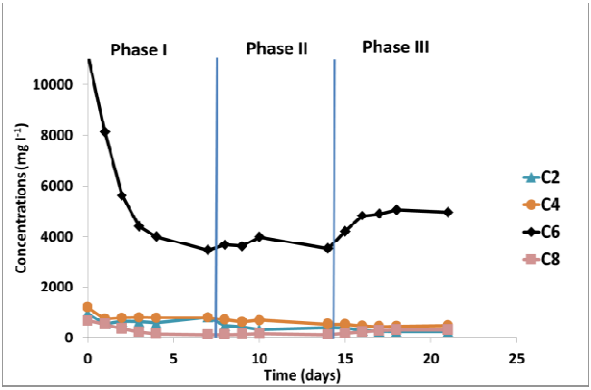

Figure 1a,b. Concentration overview of carboxylates and ethanol during experiment. Odd numbered carboxylates and ethanol (C3, C5, C7and EtOH) are presented in the upper figure (1a), while even numbered carboxylates (C2, C4, C6 and C8) are presented in lower figure (1b). Besides heptanoate (C7), mainly valerate (C5) and caproate (C6) are produced.

If acetate had not been added to the medium, the heptanoate selectivity would have been higher. On day 8, the acetate load was reduced by 50% to 0.9 g l^{-1} d^{-1} resulting in a lower caproate production and a larger heptanoate production (Table 2). Due to the large internal acetate production, there is no need to add acetate as substrate. Hence, heptanoate production could be made more selective as less ethanol would be directed to butyrate, caproate and caprylate.

Another reason for the low heptanoate selectivity is the formation of valerate, which can be produced from propionate and ethanol and/or from acetate and propanol (Bornstein and Barker, 1948; Kenealy and Waselefsky, 1985). Valerate in propionate elongation can be considered as an intermediate product, like butyrate in acetate elongation. The butyrate selectivity in acetate elongation was reduced to only 9% (Grootscholten et al., 2013b), while the valerate selectivity in this investigation was 28%. Moreover, the butyrate concentration in that experiment never exceeded 2.1 g l^{-1} (24 mM), while the valerate concentration in this investigation was (apart from the first days) never below 4.2 g l^{-1} (41 mM). These observations are in line with the statement of Bornstein and Barker (1948) that the elongation rate is lower with longer chain VFAs than with shorter chain VFAs.

Table 3: Overview of chemical reactions

Reaction		Process
1	$C_xH_{2x+1}COO^- + C_2H_5OH \rightarrow C_{(x+2)}H_{2(x+2)+1}COO^- + H_2O$	Ethanol elongation
2	$C_2H_5OH + H_2O \rightarrow CH_3COO^- + H^+ + 2\,H_2$	Ethanol oxidation
3	$C_xH_{2x+1}COO^- + C_3H_7OH \rightarrow C_{(x+3)}H_{2(x+3)+1}COO^- + H_2O$	Propanol elongation
4	$C_2H_5COO^- + C_2H_5OH \rightarrow C_3H_7OH + CH_3COO^-$	Propanol formation from propionate and ethanol
5	$C_2H_5COO^- + H^+ + 2\,H_2 \rightarrow C_3H_7OH + H_2O$	Propanol formation from propionate and hydrogen

3.3.3 Increasing the ethanol load increased heptanoate production, but also caproate and propanol production

Increasing the ethanol load in phase III, reduced the valerate selectivity and increased the heptanoate production rate and its selectivity (Figure 2). These results are in line with our previous investigation with acetate elongation (Grootscholten *et al.*, 2013b). In that investigation, caproate selectivity was increased after increasing the ethanol load. However, after increasing ethanol load in this investigation, a large part of the electrons were also directed to caproate and propanol. Therefore, the heptanoate selectivity only improved by 4%, while the valerate selectivity decreased with

11%. The impact of the increased ethanol load on the heptanoate selectivity was smaller than expected, due to the formation of caproate and propanol (Figure 2).

3.3.4 Outlook

Although the heptanoate selectivity was low (23%), propionate elongation is useful and efficient for the production of a mixture of valerate, caproate, heptanoate and caprylate (selectivity of 86%). Such a mixture can directly or after distillery fractionation be used for biodiesel, bioplastic and lubricant production. For more specialised applications, improvements of the heptanoate selectivity are required. Due to the ethanol oxidation to acetate and its elongation, the heptanoate selectivity from propionate will always be lower than the caproate selectivity from acetate. Pure cultures might be applied to improve heptanoate selectivity by reducing ethanol oxidation to acetate. This possibility is less attractive in case of waste streams due to sterilization requirements for pure cultures. Instead of improving the heptanoate selectivity during the fermentation, selective removal of MCFAs by extraction and feedback of the fermentation broth could improve the heptanoate selectivity such as demonstrated by Agler *et al*.(2012). They removed MCFAs selectively using the increased hydrophobicity of MCFAs. However, more research is required to determine if selective heptanoate removal is possible, because the extraction efficiencies of caproate, heptanoate and caprylate in the study of Agler *et al*.(2012) were similar.

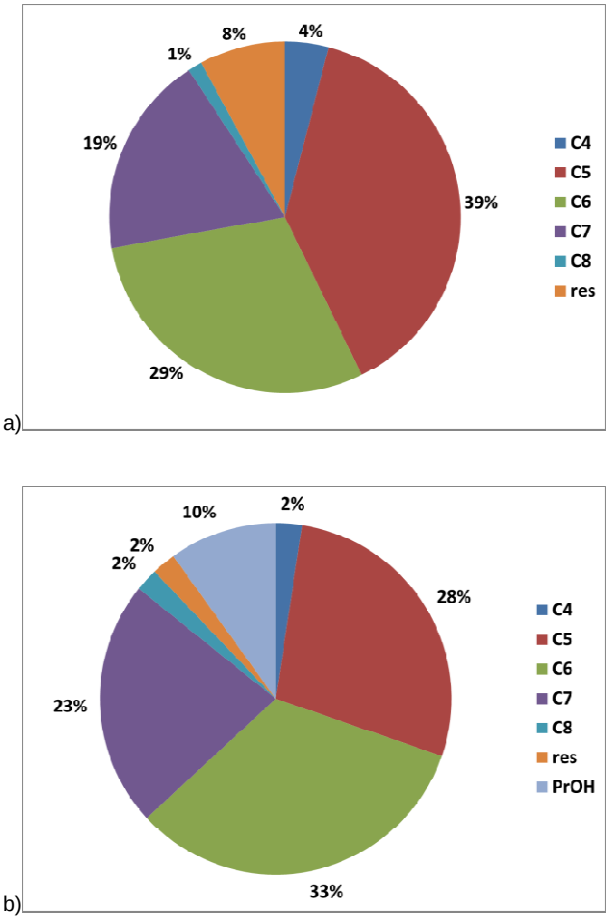

Figure 2 a,b. Increasing the ethanol load leads to higher heptanoate (C7), caproate (C6) and propanol (PrOH) selectivity at expense of mainly valerate (C5) selectivity. Figure 2a shows the product selectivity during phase II (propanol was not measured in that phase). During phase III (Figure 2b), a higher ethanol load was applied.

3.4 Conclusions

High rate heptanoate production from propionate and ethanol using a mixed culture chain elongation process was demonstrated in this investigation. We achieved a heptanoate production rate of 4.5 g l^{-1} d^{-1} (1.3 mol e eq l^{-1} d^{-1}) combined with a heptanoate concentration of 3.2 g l^{-1}. These results show sufficient potential to consider this approach as an alternative for heptanoate production from castor oil. Heptanoate selectivity improvements were demonstrated by lowering the acetate load and by increasing the ethanol load. Nevertheless, future improvements are still required to increase the heptanoate production rate, the heptanoate concentration and especially the heptanoate selectivity.

3.5 Acknowledgements

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4. Improving medium chain fatty acid productivity using chain elongation by reducing the hydraulic retention time in an upflow anaerobic filter.

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Abstract

The objective of this investigation was to further increase the medium chain fatty acid (MCFA) production rate by reducing the hydraulic retention time (HRT) in an upflow anaerobic filter. The results showed that the volumetric MCFA production rate was increased to 57.4 g MCFA l^{-1} d^{-1}, more than three times higher than previous work. Despite the lower MCFA concentrations at 4 hours HRT, the MCFA selectivity remained above 80%. Extra carbon dioxide additions and higher yeast extract concentrations were required to increase the MCFA production rate. More research related to substrates and (micro)nutrients in mixed culture continuous reactors needs to be performed to reduce yeast extract use in chain elongation.

4.1 Introduction

Chain elongation is an anaerobic fermentation in which (diluted) ethanol and volatile fatty acids (VFAs), monocarboxylic acids up to a chain length of five carbon atoms, are converted into medium chain fatty acids (MCFAs), monocarboxylic acids up to a chain length of eight carbon atoms (Steinbusch *et al.*, 2011; Agler *et al.*, 2012; Grootscholten *et al.*, 2013a; Grootscholten *et al.*, 2013b). MCFAs can be used either directly as corrosion inhibitors (*e.g.* Kuznetsov and Ibatullin , 2002) or indirectly as precursors for biodiesel production (Renz, 2005; Levy *et al*. 1981) and other biochemical processes, such as bioplastics production for medical applications (Witholt and Kessler, 1999). Due to the increased carbon chain length, which makes the molecules more apolar, MCFAs are easier to separate from water than VFAs and/or ethanol. Moreover, MCFAs can be produced from diluted ethanol, like yeast fermentation beer (Agler *et al.*, 2012). Using diluted ethanol avoids the energy intensive ethanol distillation step, which makes the chain elongation process more economical. Furthermore, chain elongation can be performed as a mixed culture fermentation and does not necessarily require a costly sterilization step.

Production of butyrate and caproate from acetate and ethanol has been known for decades with *Clostridium kluyveri* as pure culture (*e.g.* Barker, 1947), while caproate and caprylate production in mixed culture fermentations (with *C.kluyveri* as dominant species) was discovered more recently (Steinbusch *et al.*, 2011). VFAs (including acetate) can be derived from waste materials (Agler *et al.*, 2011), making chain elongation a process that can

valorise waste materials. Moreover, low concentrations of MCFAs (< 5 g l^{-1}) could also be produced in a single step reactor by additions of ethanol during acidification of the organic fraction of municipal solid waste (OFMSW) (Grootscholten *et al.*, 2013a).

Grootscholten *et al.* (2013b) made a major improvement in the volumetric MCFA production rate (up to 16.6 g l^{-1} d^{-1} (4.6 mole e l^{-1} d^{-1})) by performing chain elongation (based on acetate and ethanol) in an upflow anaerobic filter. In this system, more MCFA producers are retained than in a continuous stirred tank reactor (CSTR) resulting in a higher MCFA production rate. Competitive acetotrophic methane producers were limited by the shear stress (due to the upflow velocity) and the short hydraulic retention time (HRT) of 17 hours, which washed-out of large part of suspended acetotrophic methanogenic biomass.

Kenealy and Waselefsky (1985) stated that the maximum growth rate of *C. kluyveri* was higher than 0.1 h^{-1}. However, in their chemostat experiment *C. kluyveri* was not washed-out at a dilution rate of 0.28 h^{-1}, indicating that the maximum growth of *C. kluyveri* is at least 0.28 h^{-1}. Based on this maximum growth rate of *C. kluyveri*, the HRT of a chain elongation reactor can be reduced to 3.5 hours accordingly without wash-out of MCFA producers. Compared to our previous work at 17 hours HRT (Grootscholten *et al.*, 2013b), the volumetric MCFA production rate can increase more than four times, if similar MCFA concentrations are produced (11.1 g caproate l^{-1} and 0.6 g caprylate l^{-1}).

The objective of this investigation was to further increase the MCFA production rate using the chain elongation process based on acetate and ethanol in an upflow anaerobic filter by reducing the HRT. To ease separation, the produced MCFA concentrations should be close to the solubility of their undissociated form in water (about 10 g l^{-1} for caproic acid and 0.7 g l^{-1} for caprylic acid).

4.2 Material and Methods

4.2.1 Reactor set-up, inoculum and measurements

A reactor set-up, similar to Grootscholten *et al.* (2013b), with a 1 L reactor was used. The inoculum was taken from that reactor as well. To guarantee anaerobic conditions, nitrogen gas was bubbled from the bottom of the reactor with a flow rate of 3.6 l d^{-1}. This flow rate was controlled by a mass flow controller (Brooks instrument B.V., the Netherlands) (Steinbusch *et al.*, 2011). The same synthetic medium with acetate and ethanol was used as in Grootscholten *et al.* (2013b). pH was kept between 6.5 and 7.2 by additions of sodium hydroxide to the medium, ranging from 2.0 g l^{-1} to 3.6 g l^{-1}. Temperature was controlled at 30°C using a water bath (Julabo, Germany). VFAs, ethanol, MCFAs and biomass concentrations were measured in the same way as Grootscholten *et al.* (2013b). Headspace composition (O_2, N_2, CH_4 and CO_2) was measured occasionally by a gas chromatograph (Grootscholten *et al.*, 2013b).

Table 1: Experimental overview of the study in different phases

Phase	Days after start experiment	HRT (h)	Acetate load (g l^{-1} d^{-1})	Ethanol load (g l^{-1} d^{-1})	Yeast extract added (g l^{-1})
Start-up	Day 0 to day 4	30	4.8	7.4	1.0
	Day 5 to 29	18	8.0	18.4	1.0
	(day 8 to day 11)			(12.3)	
I	Day 32 to day 36	16	9.0	20.7	1.0
II	Day 37 to day 44	8	18.0	41.4	1.0
III	Day 45 to day 59	8	18.0	41.4	2.0
IV	Day 60 to day 69	4	36.0	82.8	2.0

4.2.2 Experiment

After the start-up period (until day 29), the experiment started by increasing the potassium carbonate (K_2CO_3) concentration from 4.0 g l^{-1} to 8.0 g l^{-1} (Figure 1). The HRT in Phase I (day 30 – 36) was 16 hours. On day 37 Phase II (day 37- 44) initiated and the HRT was reduced to from 16 hours to 8 hours. During this phase, the nitrogen gas flow was replaced with a carbon dioxide gas flow (4.8 l d^{-1}, from day 42 on). In phase III (day 45-59), the yeast extract concentration was doubled from 1.0 g l^{-1} to 2.0 g l^{-1} (Figure 1). In the final phase (phase IV (day 60-69)), the HRT was reduced from 8 hours to 4 hours. Table 1 gives an overview of the substrate loads during the experiment.

4.2.3 Calculations

4.2.3.1 Volumetric production rate

The volumetric production rate of the products (butyrate, caproate and caprylate) is defined by its concentration divided by the hydraulic retention time (1). The units of the volumetric production rate are g l^{-1} d^{-1} or moles electron equivalents l^{-1} d^{-1} (mol e eq l^{-1} d^{-1}).

Volumetric production rate = [Product] (g l^{-1} or mol e eq l^{-1}) / HRT (d) (1)

4.2.3.2 Selectivity

The selectivity is defined as the concentration of electrons in the formed product divided by the net consumed electrons from the fed acetate, ethanol and yeast extract (2). Because yeast extract ($CH_{1.7}O_{0.5}N_{0.24}$; MW = 25.1 (Duboc *et al*., 1995)) was not measured in the effluent, a complete conversion is assumed in the calculations. Acetate contains 8 mol electrons per mole, ethanol 12 mol electrons per mole, yeast extract contains 4.7 mol electrons per mole, butyrate 20 mol electrons per mole, caproate 32 mol electrons per mole and caprylate 44 mol electrons per mole. The amount of electrons per molecule can be derived by oxidizing the molecule to carbon dioxide, ammonia, protons and electrons (also known as electron donating half reaction, McCarty, 1975).

Selectivity =

[Product] (mol e eq l^{-1}) / consumed acetate, ethanol and yeast extract (mol e eq l^{-1}) (2)

4.3 Results and discussion

4.3.1 Reducing the HRT increased the volumetric MCFA production rate

By reducing the HRT to 4 hours, a maximum volumetric MCFA production rate of 57.4 g l^{-1} d^{-1} (15.9 mol e eq l^{-1} d^{-1}) was achieved. This rate is more than three times higher than the MCFA production rate in Grootscholten *et al.* (2013b). Compared to continuous ethanol production from glucose (96 g ethanol l^{-1} d^{-1} (25.0 mol e eq l^{-1} d^{-1}) Brethauer and Wyman, 2010), the achieved volumetric MCFA production rate is roughly a factor two lower, indicating that chain elongation can become a biochemical process of importance. However, at 4 hours HRT, the maximum concentrations of caproate and caprylate were respectively 9.3 g l^{-1} (80 mM) and 0.3 g l^{-1} (2 mM) (Table 2, Figure 1) and therefore below the solubility of their undissociated acid form in water. These MCFA concentrations make it harder to separate caproate and caprylate from water than concentrations above the solubility of their undissociated forms. Nevertheless, separation of these MCFAs could be achieved by extraction (*e.g.* Agler *et al.*, 2012).

At longer HRTs (8 and 16 hours), the obtained MCFA concentrations were higher and the caproate concentration at these HRTs was above the solubility of caproic acid in water. The highest concentrations of caproate and caprylate, respectively 12.0 g l^{-1} (103 mM) and 0.9 g l^{-1} (6 mM), were achieved at 16 hours HRT (Table 2; Figure 1). These concentrations are higher than the MCFA concentrations in Grootscholten *et al*. (2013b) (17 hours HRT). The maximum caproate concentration was only

slightly improved by 7%, while the maximum caprylate concentration was improved by 50%. It is the first time that caprylate concentrations, obtained with chain elongation, are reported above the solubility of caprylic acid in water.

Table 2: Overview of (considered) steady state results and additional substrate production in this study.

Phase	Ethanol ($g\ l^{-1}$)	Acetate ($g\ l^{-1}$)	Butyrate ($g\ l^{-1}$)	Caproate ($g\ l^{-1}$)	Caprylate ($g\ l^{-1}$)	MCFA production rate ($g\ l^{-1}\ d^{-1}$)	MCFA Selectivity* ($mol\ e\ eq\ l^{-1}\ mol^{-1}\ e\ eq\ l^{-1}$ * 100%)
I (day 32-36)	2.2	1.6	1.3	12.0	0.9	19.3	94
II (day37-44)	4.9	2.1	1.7	8.7	0.4	27.3	83
III (day 47-50 and 53-55)	2.9	1.4	1.3	11.3	0.6	35.7	86
IV (day 66-69)	5.1	2.5	1.7	8.7	0.3	54.0	80
Max (day 69)	4.6	2.3	1.6	9.3	0.3	57.4	81

* Assuming complete yeast extract conversion

4.3.2 Hydrogen recycling system could be reason for high MCFA selectivity in phase I

By reducing the HRT from 16 to 4 hours, the MCFA selectivity also decreased from 94% to 81% (Table 2). These values are comparable with MCFA selectivities in previous works (Agler *et al*., 2012; Grootscholten *et al*., 2013b). An explanation for the high MCFA selectivity in phase I (16 hours HRT) could be the production of additional acetate and/or ethanol from carbon dioxide. Conversion of carbon dioxide into acetate would require electrons that could have been provided by MCFA producers and/or by the conversion of yeast extract. MCFA producers need to oxidize a part of the consumed ethanol to acetate and hydrogen to obtain energy (Seedorf *et al*., 2008). Future research should be performed to determine if such a hydrogen recycling system can exist and/or can be applied.

Most of the unaccounted electrons are likely to belong to unconverted yeast extract. In contrast to our previous work (Grootscholten *et al*., 2013b), we assume a complete conversion of yeast extract in the selectivity calculations. In phases III and IV (with higher yeast extract concentrations), this assumption could result in lower selectivities. The unaccounted electrons are not likely to be converted into methane, because methane percentages in the headspace of the reactor were always below 0.1%.

The absence of methanogenic activity could be related to the inoculum, which may not have contained (active) methanogens. In case methanogenesis would occur, we expect little impact on the MCFA production

rate at 4 hours HRT. According to Agler *et al*. (2012), hydrogenotrophic methanogenesis and chain elongation are not competitive processes. However, acetotrophic methanogens compete MCFA producers for acetate as substrate. Due to their low maximum growth rate (about 0.015 h^{-1}), estimated by Pavlostathis and Giraldo-Gomez (1991), the impact of wash-out on acetotrophic methanogens in upflow anaerobic filters at 4 hours HRT is large, consequently acetate consumption by acetotrophic methanogens remains limited at this short HRT.

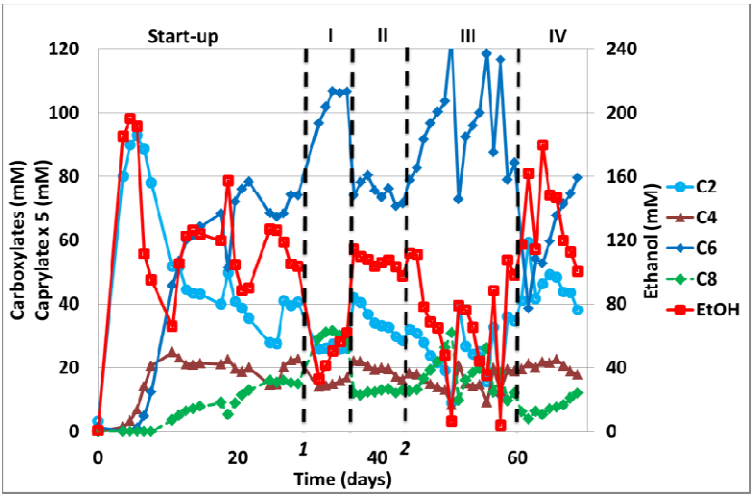


Figure 1. Concentration overview of carboxylates and ethanol during experiment. At 1 (day 32) the K_2CO_3 concentration was increased to 8.0 g l^{-1}, while at 2 (day 45) the yeast extract concentration was doubled. Acetate concentrations are given by open circles, ethanol by open squares (right y-axis), butyrate by closed triangles, caproate by closed diamonds and caprylate by closed diamonds with dashed lines.

4.3.3 Effect of carbon dioxide and yeast extract on chain elongation

4.3.3.1 MCFA production was limited by carbon dioxide and yeast extract

To improve MCFA production, larger additions of carbon dioxide and yeast extract were required. Until day 32, the MCFA production was lower than in our previous work (Grootscholten *et al*., 2013b), while substrate concentrations (acetate and ethanol; both > 2.0 g l^{-1}) did not seem to limit the MCFA production. The experimental set-up and the medium composition were the same as in the previous work; only in this work a continuous nitrogen flow was added. The nitrogen flush was likely responsible for a lower amount of carbon dioxide in the reactor. Because it is known that *C.kluyveri* requires carbon dioxide for its protein synthesis (*e.g.* Tomlinson and Barker, 1954), a lower amount of carbon dioxide in the reactor could have lowered the MCFA production. On day 32, the amount of K_2CO_3 in the medium was doubled from 4.0 g l^{-1} to 8.0 g l^{-1}, resulting in an increase of MCFA production (Figure 1). Due to the increased K_2CO_3, the pH changed from 7.1 to 6.9, possibly due to production of more acetate (discussion above). The lowered pH itself could be a reason for the higher MCFA production, but the lowered pH also increased the CO_2 percentage of the total carbon dioxide concentration from 14% (pH 7.1) to 20% (pH 6.9)[4]. If protein synthesis proceeds faster with CO_2 than with HCO_3^-, the higher CO_2 percentage could be another reason for the higher MCFA production. When the MCFA concentrations rose above the solubility of

[4] The distribution of carbon dioxide into CO_2, HCO_3^- and CO_3^{2-} is pH-dependent. For the calculations (around pH 7), only CO_2 and HCO_3^- were taken into account (pKa = 6.3).

their undissociated acid form in water (Table 2), the HRT was reduced to 8 hours.

After reducing the HRT to 8 hours, the volumetric MCFA production rate increased from 19.3 g l^{-1} d^{-1} (5.4 mol e eq l^{-1} d^{-1}) to 27.3 g l^{-1} d^{-1} (7.6 mol e eq l^{-1} d^{-1}). This rate was much lower than the volumetric MCFA production rate we expected (close to 38.6 g l^{-1} d^{-1} (10.8 mol e eq l^{-1} d^{-1})) due to lower MCFA concentrations in phase II (8 hours HRT) than in phase I (16 hours HRT) (Table 2). Because the concentrations of acetate and ethanol were still above 2.0 g l^{-1} (Table 2), chain elongation was likely not limited by low substrate concentrations. To test whether the total amount of carbon dioxide was still limiting chain elongation, additional carbon dioxide was added by replacing the nitrogen flush with gaseous carbon dioxide on day 42. Gaseous carbon dioxide was added instead of increasing the K_2CO_3 concentration in the medium to avoid possible influences of higher salt concentrations. Despite the increased amount of carbon dioxide in the reactor and the resulting decrease in pH (from 6.9 to 6.7), the MCFA production rate did not further improve and was likely not limited anymore by the total amount of carbon dioxide.

Because the substrate concentrations were still above 2.0 g l^{-1}, MCFA production seemed to be limited by another factor likely related to the growth of MCFA producers. Therefore, the yeast extract concentration was increased from 1.0 g l^{-1} to 2.0 g l^{-1} on day 45, which also resulted in a higher MCFA production rate (35.7 g l^{-1} d^{-1} (9.9 mol e eq l^{-1} d^{-1})). Moreover, the

caproate concentration rose above the solubility of caproic acid in water (Table 2; Figure 1)[5].

4.3.3.2 Implications of carbon dioxide and yeast extract use

Both carbon dioxide additions and increased yeast extract concentrations improved the volumetric MCFA production rate, probably by stimulating growth of MCFA producers. Because carbon dioxide and yeast extract are by-products of ethanol fermentations and the use of ethanol itself, chain elongation seems to be an ideal process to upgrade undistilled ethanol from ethanol fermentation plants. In case chain elongation is not connected to an ethanol fermentation plant, the implications of carbon dioxide and yeast extract can be different.

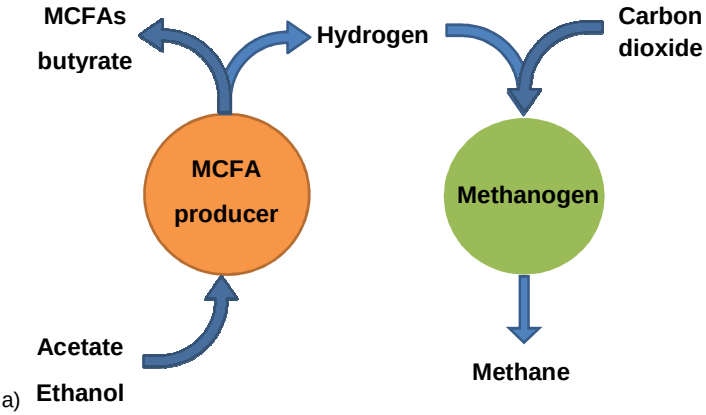
Carbon dioxide can be cost-effective, because it is a waste product of several fermentations (including ethanol fermentations). Furthermore, carbon dioxide additions can lower the partial hydrogen pressure in the reactor, which might be beneficial for chain elongation, as Schoberth and Gottschalk (1969) demonstrated that high partial hydrogen pressures (above one atm) limit growth of *C.kluyveri* in batch cultures. Finally, it could be used to produce more substrate (acetate and/or ethanol) internally for the chain elongation process when the produced hydrogen from MCFA producers is used (discussion above). If (active) hydrogenotrophic methanogens are present, they can compete for the hydrogen (*Figure 2a*) and produce methane

[5] Some scaling problems occurred during the experiment (day 51 and 58). A power cut caused the disturbance on day 56.

instead of substrate for chain elongation (*Figure 2b*) (*e.g.* Cord-Ruwisch *et al*., 1988). To avoid this competition, hydrogenotrophic methanogens are preferably avoided in the chain elongation process. Heat shocks could be applied to inhibit methanogens, while spore-forming MCFA and acetate/ethanol producers can survive the heat shock and repopulate the reactor afterwards (*e.g.* Steinbusch *et al*., 2009).

In contrast to carbon dioxide, yeast extract could be costly, because it is commonly applied in several fermentations and therefore it has a commercial value. In case chain elongation is based on waste materials that contain (dead) microbial biomass, the use of yeast extract could be reduced and the chain elongation process could become more cost-effective. According to Barker (1947), the growth factors biotin and para-aminobenzoic acid can replace yeast extract completely for cultivation of *C.kluyveri*.

Apart from their presence in the added yeast extract, both growth factors were also added to our reactor via a B-vitamin solution (2 ml l^{-1} medium), containing a biotin concentration of 1 g l^{-1} and a para-aminobenzoic acid concentration of 0.1 g l^{-1}. The findings of Barker (1947) were made in batch bottles with a pure culture of *C.kluyveri*. Therefore, it is not known whether these components could completely replace yeast extract in continuous reactors with mixed cultures or whether other components are required as well. More research related to substrates and (micro)nutrients in mixed culture continuous reactors needs to be performed to reduce yeast extract use in chain elongation.



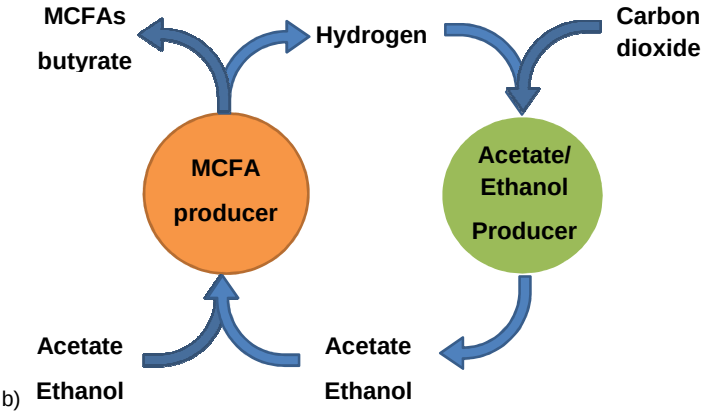


Figure 2a,b. Possible interactions between carbon dioxide based hydrogen scavengers and MCFA producers. If hydrogen is consumed by acetate/ethanol producers (b), additional substrate for chain elongation could be produced from carbon dioxide.

4.4 Conclusions

By reducing the HRT to 4 hours, a maximum volumetric MCFA production rate of 57.4 g l^{-1} d^{-1} (15.9 mole e l^{-1} d^{-1}) was achieved, more than three times higher than the MCFA production rate in our previous work. Despite the lower concentrations at 4 hours HRT, the MCFA selectivity was still above 80%, indicating that chain elongation at these short HRTs remains efficient. Extra carbon dioxide additions and higher yeast extract concentrations were required to increase the MCFA production rate. Future research should reduce yeast extract use to make chain elongation more cost-effective.

Intermezzo: In-situ inhibition of methanogenesis during dry anaerobic digestion of OFMSW for VFA production.

Abstract

During the anaerobic digestion of OFMSW other valuable products than methane, like VFAs and alcohols, are produced as intermediates. These intermediate products could be used as alternative source for chemical and fuel production instead of fossil sources. To obtain high VFA and alcohol production from anaerobic digestion of OFMSW, methanogenesis should be prevented, preferably without a reduction of hydrolysis and acidogenesis rates. Methanogenesis should be inhibited for a period of at least 21 days, the time required to hydrolyse OFMSW. In this investigation, the effect of pH on net VFA production was tested in lab-scale batch reactors. Reactors operated at pH 6.0 and pH 5.5 were externally controlled, besides which reactors were operated without external pH control. The reactors without external pH control showed the highest net production of VFA for a period of 35 days. The pH in these reactors was naturally buffered to a value around 5.0. Although acetate consumption could be prevented, hydrolysis rate constants of OFMSW in these experiments were low (0.004 – 0.005 d^{-1}).

Introduction

Due to the threat of climate change and concerns on oil availability and prices, alternative sources for chemicals and fuels production are investigated. The organic fraction of municipal solid waste (OFMSW) is an in part easily biodegradable and inexpensive worldwide available feedstock (Bolzonella *et al.*, 2005). Several technologies have been developed to digest OFMSW anaerobically into biogas, a gas mixture mainly consisting of methane and carbon dioxide (Ten Brummeler, 1993; De Baere *et al.*, 1986; Begouën *et al*., 1988). Anaerobic digestion of OFMSW can be understood as a four step sequential process, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Pavlostathis and Giraldo-Gomez, 1991). Hydrolysis is the process in which complex insoluble organic polymers in OFMSW are converted into soluble monomers, acidogenesis is the formation of volatile fatty acids (VFAs), alcohols, carbon dioxide and hydrogen from the hydrolyzed monomers, acetogenesis is the formation of acetate from VFAs, alcohols, carbon dioxide and hydrogen and finally methanogenesis is the formation of methane from acetate or carbon dioxide and hydrogen. The consumption of hydrogen by the methanogens leads to a lowering of the hydrogen partial pressure and stimulates the conversion of alcohols into VFA (Bolzonella *et al.*, 2005).

VFA and alcohols produced from OFMSW, could replace fossil fuels as chemical building blocks for the production of chemicals and fuels (Levy *et al.*, 1981). Although biogas can replace fossils fuels as well, by burning the biogas for heat or electricity, it cannot be used to make chemical additives.

Furthermore, the energy density of pure methane gas is 8.0 GJ m^{-3} (298 K, compressed at 200 atm) is low compared to the energy density of liquid biofuels e.g. the energy density of ethanol is 23.4 GJ m^{-3} (298K, 1 atm). The production of VFA and alcohols from OFMSW as an alternative for methane is thus an interesting option.

To obtain high VFA and alcohol production during anaerobic digestion of OFMSW, methanogenesis should be prevented, preferably without a reduction of hydrolysis and acidogenesis rate. Here we study the inhibition of methanogens by a low pH. This is an attractive option, as VFA production lowers the pH naturally. Moreover, the undissociated form of VFA is more toxic to methanogens than the dissociated form. At high undissociated VFA concentrations (larger than 30 mg l^{-1}) inhibition of the methanogenic population has been observed (Anderson *et al.*, 1982). According to equations (1-3), the undissociated concentration of VFAs increases when the pH decreases.

Several other methods have been applied to prevent a methanogenic population of growing, including specific chemical inhibiters, such as 2-bromoethane sulfonic acid (2-BES) (Valdez-Vazquez *et al*., 2005), and heat shocks (Steinbusch *et al.*, 2009). However, these methods can be questioned to be environmental sustainable or cost effective methods for methanogenic inhibition.

$$\frac{[VFA^-][H^+]}{[VFA]} = K_a = 10^{-4.8} \ (pK_a = 4.8) \quad (1)$$

$$Total\,VFA = [VFA] + [VFA^-] \quad (2)$$

$$[VFA] = \frac{[H^+]}{K_a + [H^+]} Total\ VFA \quad (3)$$

where

[*VFA*] is the undissociated concentration of VFA (mol l^{-1})

[VFA^-] is the dissociated concentration of VFA (mol l^{-1})

[H^+] is the proton concentration (mol l^{-1})

K_a is the equilibrium constant[6] (mol l^{-1})

According to Taconi *et al.* (2008) methanogenic bacteria can adapt to acidic pH conditions, even at a pH as low as 4.0 in the presence of 2 grams acetate per litre. However, these conditions increase the lag time of methanogenic activity up to a maximum of 38 days. When using a batch wise operated dry anaerobic digester it is thus important that the inhibition time for methanogenic bacteria should be longer than the sludge retention time of OFMSW, which is about 15 to 21 days at mesophilic conditions in dry anaerobic reactors (at least a total solids content of 20 % w/w) (De Baere, 2000; ten Brummeler, 2000). This period is required to hydrolyse the OFMSW

[6] The selected pK_a value represents an approximation of the exact value. Individual VFA species differ slightly from this value (*e.g.* pK_a of acetic acid is 4.75)

for production of VFAs. Dry anaerobic digestion is an attractive technique for the anaerobic digestion of OFMSW. It requires a lower amount of added water, less energy of intensive mixing and a lower amount of energy for heating up the reactor (ten Brummeler, 1993).

This investigation should lead to inhibiting methanogenic bacteria in dry anaerobic reactors, while maintaining hydrolysis from OFMSW. We expect that dry anaerobic reactors will yield higher concentrations of VFA compared to wet systems and consequently will contribute to the methanogenic inhibition. The inhibition period of methanogenesis should be long enough to develop a VFA production process that requires minimal use of additional chemicals and energy. Veeken *et al.* (2000) showed that the hydrolysis rate of OFMSW at pH 7 was more than two times higher than at pH 5. So we expect that a more acidic pH will improve methanogenic inhibition, but at cost of a lower hydrolysis rate. Therefore we investigate in this intermezzo both the effect of pH on the methanogenic lag phase and the effect of pH on the hydrolysis rate.

Material and methods

OFMSW and inoculum

A representative sample of OFMSW was collected at the composting plant of Essent Milieu (de Wijster, The Netherlands). The OFMSW consisted about 90 % v/v garden waste, mainly fallen leafs and hardwood (due to the season it was collected), and about 10 % v/v kitchen waste. The OFMSW was cut with a 10 litres stainless-steel commercial kitchen blender

(Eduard Müller & Söhne, Germany) and afterwards it was stored in a cellar at 12°C for one month in case of the first experiment and for five months in case of the second experiment. Because OFMSW already contains acidogenic bacteria, no inoculum was added. The Total Solids (TS) content of the OFMSW was 0.343 g per kg OFMSW and Volatile Solids (VS) content of the OFMSW was 0.203 g per kg OFMSW.

Reactor set-up

The reactor set-up was based on the dry anaerobic digestion process BIOCEL (ten Brummeler, 1993), i.e. the total solids (TS) in the reactor should be higher than 200 g per kg OFMSW. In *Figure 1*, a schematic overview of the reactor set-up is presented. The experiments were performed in stainless steel cylindrical reactors with an effective volume of five-litres.

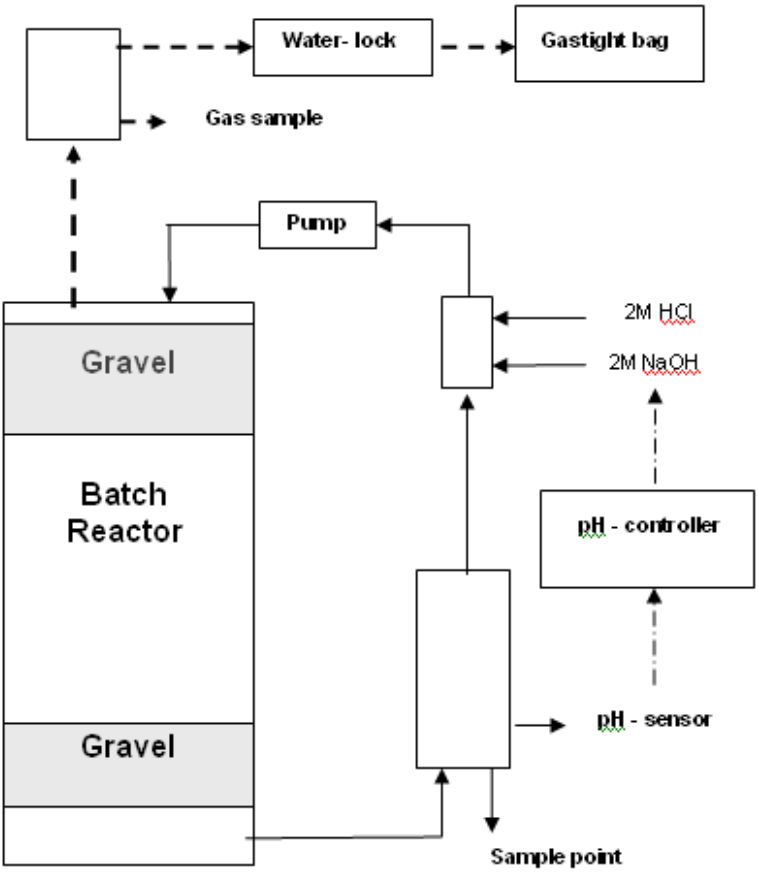


Figure 1. Experimental set-up of the reactors. Solid lines represent liquid streams, while the dashed lines stand for gas streams. The dash-dotted lines are signals to and from the pH-controller.

Peristaltic pumps (Marlon-Watson 323U, U.K.) were used for percolation and recirculation of the leachate through the OFMSW. To enhance hydrolysis of the OFMSW, a leachate recirculation of 10.8 l/h was applied (ten Brummeler and Koster, 1990). To prevent clogging in the reactor set-up, the OFMSW was packed in between two gravel beds with washed 4-6mm diameter gravel. The leachate was pumped up from the bottom to a sampling vessel, where liquid samples were taken and the pH was measured. In case of pH-control, the leachates were controlled with a proportional controller (Endress + Hauser, Switserland) by adding 2M HCl or 2M NaOH to the leachate in another small vessel downstream. The gas phase of the reactors consisted of gas sample cell, a water lock and a ten-litre gastight bag. The reactor was placed in a water bath with a recirculation heater (Julabo MD, Germany) controlled at 30 ± 0.5 °C.

Experiments

The investigation consists of two experiments. The first experiment consisted of three reactors; one reactor was controlled at pH 6.0, one reactor was controlled at pH 5.5 and one reactor did not have pH-control. The second experiment was executed to validate the results of the best performing reactor from the first experiment. The second experiment was conducted in quadruplet, i.e. in four reactors. On average, each reactor contained 2.2 kg of fresh OFMSW, representing a Chemical Oxygen Demand (COD) of 446 g, one gram of VS is considered to have a COD-value of one gram COD (ten Brummeler, 1993). In the first experiment, one litre of demineralised water was added, while in the second experiment 1.4-litre was added to obtain a

better liquid recirculation. Before the start of each experiment, the reactors were flushed with nitrogen to remove residual oxygen.

Measurements and Analytical methods

Liquid samples were taken three times a week to measure VFA and alcohol concentrations. Both VFA (C2-C6) and alcohols (C1-C2) were measured using a gas chromatograph (HP 5890 series II GC, Germany), with a glass column packed with 10% Fluorad 431 on Supelco-port 100-120 mesh, using different column temperatures (130°C for VFA and 80°C of alcohols). The produced gas was collected in a ten-litre gastight bag. Gas samples were taken to measure the methane, nitrogen, carbon dioxide and oxygen percentages in the produced biogas by analysing the sample with a gas chromatograph (Steinbusch *et al.*, 2008). The total solids (TS) content and solids (VS) contents were determined according to standard methods (APHA, 1985).

Calculations

To convert the amount of methane in the biogas into COD equation (3) and (4) were used in combination with the volume of the produced biogas. Table 1 shows the meaning of the symbols in equations (4) and (5) and the values used in this research.

$$CH_4\ (gCOD) = \frac{p \cdot V_{methane}}{R \cdot T} \cdot M_{W,\,methane} \cdot c_{methane} \qquad (4)$$

with $V_{methane} = V_{gas} \cdot \%\, CH_4$ (5)

The hydrolysis rate of the OFMSW was calculated based on the COD balance and assuming that hydrolysis follows first order kinetics. Rearrangements of the COD balance results into equation (6).

$$k = \frac{\ln\left(\frac{bCOD - COD_r}{bCOD}\right)}{-t} \quad (6)$$

where

k is the hydrolysis rate of OFMSW (d^{-1})

bCOD is the biodegradable COD,

assumed to be 0.8 of total COD (356g) (g COD)

COD_r is the amount COD produced in the form of VFA and/or alcohol (g COD)

t is time of the calculation of the hydrolysis rate (d)

Table 1 : Values and signification of symbols in equations (2) and (3).

Symbol	Signification	Value [Unit]
p	pressure	$1.01 * 10^5$ [Pa]
V_{gas}	total gas volume	$12 * 10^{-3}$ [m^3]
$V_{methane}$	total methane volume	$5.28 * 10^{-3}$ [m^3]
% CH_4	percentage methane in gas phase	44 [%]
R	gas constant	8.31 [$J\ K^{-1}\ mol^{-1}$]
T	Temperature	303 [K]
$M_{w,\ methane}$	Molecular Weight methane	16 [$g\ mol^{-1}$]
$c_{methane}$	COD conversion factor methane	4 [$g\ O_2\ (g\ CH_4)^{-1}$]

Results

Production and consumption of VFAs and alcohols at different pH

Figure 2 shows the results of the first experiment where three different pH conditions were tested, respectively uncontrolled pH, controlled at pH 5.5 and controlled at pH 6.0. During the first days, until day 8, the amount of VFAs and alcohols increased in all three reactors as result of hydrolysis and acidification. After day 8 different trends were observed for the acidification at different pH values. At pH 6, from day 8 on, net acetate consumption was observed, together with gas production. In the reactor controlled at pH 5.5, net

acetate consumption and biogas production started at 15 days. At uncontrolled pH conditions, the pH dropped to 4.8 (close to the pKa-values of VFAs) at day 12. At this pH, no net acetate consumption or significant gas production (more than one litre biogas) was observed until the end of the experiment (day 36).

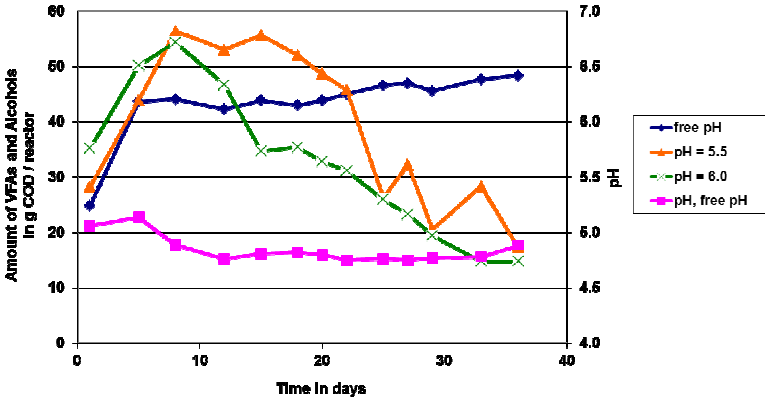


Figure 2. Comparison of total intermediate production in leachate for three pH conditions.

Methane percentages in the produced gas were at day 15 respectively 48% (pH 6.0) and 26% (pH 5.5). This confirmed that the acetate consumption and gas production were caused by methanogenic bacteria.

The reactor controlled at pH 5.5 was only pH controlled until day 22. At that time a large amount of methane, representing more than 13 g COD, had already been produced. This value was calculated using equations (4), (5) and the values in table 1. Additionally, the cumulative sodium hydroxide

consumption at that time was 536 mmol, while the hydrochloric acid consumption was 309 mmol. After switching off the pH control (and with a methanogenic microbial population present), the pH increased from 5.5 to a value of 7.3 at day 36 due to the consumption of VFAs.

Validation of the methanogenic inhibition without external pH-control

The first experiment showed that methanogenic inhibition for at least 21 days was only obtained in the reactor without pH control (with a pH below 5.0). To validate the methanogenic inhibition of this reactor, a second experiment consisting of four reactors without pH-control was performed. In *Figure 3*, the average production of the VFAs and alcohols and the average pH of all the reactors in the second experiment are presented. The second experiment confirmed the previous results of the reactor without pH-control. Some acetate consumption (± 5% of COD) by methanogenic bacteria was seen until day 35. The pH was naturally buffered at a pH of 4.8. In Table 2, the values of total VFAs in the leachate are presented, recorded at the time methanogenic bacteria became active in the reactor or at the end of the experiment. In that case little net acetate consumption (< 5% of produced COD) was observed.

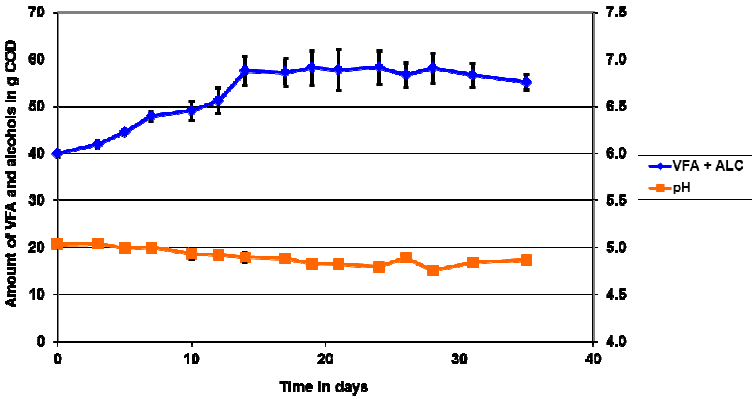


Figure 3. The second experiment confirmed that large acetate consumption could be prevented in a dry anaerobic reactor without pH control.

Discussion

Controlling pH of leachate at 5.5 is not sufficient to prevent large acetate consumption

The reactors with the controlled pH conditions (pH 5.5 and 6.0) in the first experiment demonstrated that more than ten per cent of the produced acetate was consumed by methanogenic bacteria in these reactors. At a total VFA concentration of approximately 14 g l^{-1} at pH 5.5, the undissociated VFA concentration, calculated with equations (1-3), equalled 2.3 g l^{-1}. This concentration is 75 times higher than the indicated toxicity level for methanogenic bacteria by Anderson *et al.* (1982). Yet, it was not sufficient to prevent significant acetate consumption (more than ten per cent COD of the produced VFAs and alcohols). However, our result is in line with the findings of Taconi *et al.* (2008). The undissociated VFA concentrations in their research were approximately 1.7 g l^{-1} at a pH of 4 (calculated with equations (1-3)).

Table 2: VFA concentration at time of initial acetate consumption or at the end of the experiment

	1st experiment pH 6.0	1st experiment pH 5.5	1st experiment free pH	2nd experiment free pH
day	8	15	36	35
Acetate (g l^{-1})	8.3	7.7	9.6	9.7 ± 0.3
Propionate (g l^{-1})	2.7	2.3	2.6	3.1 ± 0.2
Butyrate (g l^{-1})	2.2	2.4	2.5	1.3 ± 0.1
Valerate (g l^{-1})	0.6	0.8	0.5	0.3 ± 0.0
Caproate (g l^{-1})	0.7	0.5	0.2	0.0 ± 0.0
Total VFA (g l^{-1})	14.5	13.7	15.4	14.5 ± 0.5

Hydrolysis rate at high VFA concentrations in reactors without pH control

Equation (5) was used to calculate the hydrolysis rate. In the first experiment, the OFMSW in the reactor without pH control a hydrolysis rate of 0.004 d^{-1} was calculated, while the average hydrolysis rate of the OFMSW in the second experiment was calculated at 0.005 d^{-1}. These calculated hydrolysis rates of the reactors without pH control were low compared to values of different biowastes (0.076– 0.264 d^{-1}), described by Veeken and Hamelers (1999). It is known in anaerobic waste treatment that high VFA

concentrations in combination with low pH inhibit hydrolysis (Llabrés-Luengo and Mata-Alvarez, 1988). This can also be seen in *Figures 2 and 3* where after 15 days, no further increase of VFAs and/or alcohols takes place. The VFA and alcohol yield from the OFMSW is about 15 % in terms of COD, these results are in line with previous results at pH 5.0 (ten Brummeler, 1993; De Baere *et al.*, 1986).

Conclusions and Recommendations

For inhibition of methanogenic bacteria in OFMSW in a period of 35 days, reactor systems without pH control showed the highest VFA production and the lowest methane production. In reactors controlled at pH 6.0 and pH 5.5, acetate consumption of more than ten per cent COD of the produced VFAs and alcohols could not be prevented. The lag phase of methanogenic activity was seven days longer in the reactor controlled at pH 5.5 (15 days) than in the reactor controlled at pH 6.0 (8 days). Nevertheless, a lag phase of methanogenic activity of 15 days is not long enough to prevent VFA consumption during hydrolysis of OFMSW. Additionally, the consumption of salts in the reactor controlled at pH 5.5 was large (536 mmol NaOH and 309 mmol HCl) compared to the total VFA production of 532 mmol (Calculated from table 2.). In case of a perfect pH controller, the minimal amount of NaOH addition could be reduced to 227 mmol (subtracting the amount of HCl from the total amount of NaOH). Per mol VFA produced, a minimal amount of 427 mmol NaOH would still be required. Due to expenses of NaOH additions, it would more difficult to make a VFA production process cost-effective with pH control than without one.

The reactor system without pH-control has two additional benefits, beside the lack of addition of chemicals. First, the pH is naturally buffered to a pH value at 4.8 which limits methanogenesis. Second, the in-situ methanogenic inhibition is enhanced by VFA production, more VFA production yields less favourable conditions for methanogenic bacteria (lower pH and higher VFA concentrations). In the experiments, we demonstrated that large acetate consumption by methanogenic bacteria could be prevented for a period of at least 35 days (*Figures 2 and 3*). Without the inhibition of these methanogenic bacteria, a feasible VFA production process from OFMSW cannot exist.

Nevertheless, improvements in the hydrolysis rates and in the yields of VFAs are essential to develop an economically attractive VFA production process. To improve hydrolysis rates the removal of VFA is required, since high VFA concentrations inhibit hydrolysis (Llabrés-Luengo and Mata-Alvarez, 1988). This would also result into a higher yield of VFAs and alcohols. However, to prevent methanogenesis the pH, mainly determined by VFA concentrations, should remain below 5.5. Hence, an optimal VFA removal strategy has to be developed to improve hydrolysis of the OFMSW. Additionally, the process temperature could be increased to thermophilic conditions to improve the reaction rates of hydrolysis and acidification and to reduce the duration of the batch experiments.

5. Promoting chain elongation in mixed culture acidification reactors by addition of ethanol

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Abstract

In this research we investigate a microbial production process to produce medium chain fatty acids (MCFAs) based on the organic fraction of municipal solid waste (OFMSW). In this microbial production process, called chain elongation, bacteria produce medium chain fatty acids (MCFAs) from ethanol and volatile fatty acids (VFAs). MCFAs could be used as new biomass based building blocks for the chemical and fuel industry. The objective of this article is to investigate whether chain elongation can be promoted during acidification of OFMSW by addition of ethanol. The results show that chain elongation can be promoted during acidification of OFMSW by addition of ethanol. However, the hydrolysis rate and the carboxylic acid yield of the OFMSW in reactors with ethanol additions were lower than the hydrolysis rate and the carboxylic acid yield than in reactors without ethanol additions. Further research is required to determine whether a combined chain elongation and acidification reactor or a separated reactor system is more advantageous for MCFA production from OFMSW.

5.1 Introduction

To reduce the dependence on fossil carbon sources for chemical and fuel production, biomass based chemical and fuel production processes are being developed. To avoid competition with food resources that some biomass based chemical and fuel production processes face, we investigate a microbial production process to produce chemicals based on the organic fraction of municipal solid waste (OFMSW). OFMSW is a waste stream and its generation does not require land use, which could be used for the production of food. In the microbial production process, called chain elongation, bacteria produce medium chain fatty acids (MCFAs) from ethanol and volatile fatty acids (VFAs). MCFAs are straight carboxylic acids with a chain length of six to eight carbon atoms, while VFAs are defined as carboxylic acids with a chain length of two to five carbon atoms.

Formation of caproate (C6) and heptanoate (C7) from VFAs and ethanol has been known for decades (Barker, 1947; Smith and McCarty, 1989). The production of caprylate (C8) from acetate, ethanol and hydrogen was recently described by Steinbusch *et al.* (2011). Smith and McCarthy (1989) temporarily produced caproate and heptanoate after applying perturbations of ethanol and propionate concentrations using mixed undefined bacterial populations in propionate and ethanol fed continuous reactors. Kenealy *et al.* (1995) demonstrated the stable production of 4.4 g caproate from 6 g cellulose and 4 g ethanol in co-culture one litre batch reactors at a pH of 7. In these defined co-cultures, a saccharolytic enzyme producing bacteria converted the cellulose to intermediate products (acetate or succinate), while

Clostridium kluyeri consumed these intermediate products together with ethanol and produced another intermediate product, butyrate (max 2.6 g), and caproate (max 4.4 g). Steinbusch *et al.* (2011) produced (using a mixed bacterial culture) 8.2 g caproate l^{-1} and 0.3 g caprylate l^{-1} from acetate, ethanol and hydrogen in a batch reactor. From their microbial analysis, it became clear that *Clostridium kluyeri* was one of the dominant bacterial species. To suppress methanogenesis in their reactor, they applied 10 g 2-bromoethanosulfonic acid per litre.

Suppression of methanogenesis is important, because both methanogenic bacteria and MCFA producers compete for acetate as a substrate. In this research we choose not to control the pH during acidification of OFMSW to suppress methanogenic activity. Previous experiments with OFMSW demonstrated that the final pH in dry anaerobic batch operations without pH control was in the range of 4.9 – 5.5, slightly above the pKa-value of VFAs (around 4.8) (D'Addario *et al.*, 1993). Due to this low pH-value, large part of VFA (up to 44 per cent of the total VFA concentration) is present in the undissociated form. A high concentration of undissociated VFA, above 5 mM (or 30 mg l^{-1} acetic acid), is sufficient to inhibit methanogenic bacteria (Anderson *et al.*, 1982). If sufficient undissociated VFA are produced from the OFMSW during the acidification, methanogenic activity will be suppressed and there is no need for additional chemicals.

MCFA production not only requires methanogenic suppression, but also production of intermediary products, such as butyrate and valerate. These products could also be produced from the shorter VFAs (acetate and

propionate) and ethanol. Equations (1-5) show the chain elongation reactions. Equation 1 shows the formation of butyrate from acetate and ethanol and equation 5 shows with the formation of caprylate from caproate and ethanol.

$$CH_3COO^- + C_2H_5OH \rightarrow C_3H_7COO^- + H_2O \quad \text{(equation 1)}$$

$$C_2H_5COO^- + C_2H_5OH \rightarrow C_4H_9COO^- + H_2O \quad \text{(equation 2)}$$

$$C_3H_7COO^- + C_2H_5OH \rightarrow C_5H_{11}COO^- + H_2O \quad \text{(equation 3)}$$

$$C_4H_9COO^- + C_2H_5OH \rightarrow C_6H_{13}COO^- + H_2O \quad \text{(equation 4)}$$

$$C_5H_{11}COO^- + C_2H_5OH \rightarrow C_7H_{15}COO^- + H_2O \quad \text{(equation 5)}$$

The possibility to produce petroleum-like chemicals from MCFAs was already acknowledged by Levy *et al.* (1981). Their research mainly focussed on the processing step of MCFAs to fuel chemicals by means of the Kolbe-electrolysis. A more common applied industrial process to use MCFAs is ketonization (Renz, 2005). In ketonization, two MCFA molecules are coupled to produce a long chain ketone and a carbon dioxide molecule. The produced ketones could be blended into kerosene or diesel.

MCFAs could also be used as pre-cursors for polyalkanoate (PHA) production. PHAs can be directly be used as bioplastics. They can be formed inside aerobic bacteria when these bacteria experience nutrient limitations (*e.g.* Johnson *et al.*, 2010; Hong *et al.*, 2009 for more information about PHA production). By harvesting the PHA containing biomass and disrupt their cell

walls, PHAs can be extracted and purified. When applying MCFAs as a substrate of PHA producing bacteria, the MCFA based PHAs have a larger degree of flexibility and could have different applications than the shorter chain PHAs produced with VFAs (Witholt and Kessler, 1999).

As production of MCFAs from OFMSW requires VFAs, at least an acidification reactor is required in the chain elongation process. The objective of this article is to investigate whether chain elongation can be promoted during acidification of OFMSW by addition of ethanol. If the results are successful, chain elongation could be performed in a single reactor. An additional chain elongation reactor after the acidification reactor would increase the investment costs for the chain elongation process significantly compared to a single reactor combining chain elongation and acidification.

5.2 Material and methods

5.2.1 OFMSW and inoculum

A representative sample of OFMSW was collected at the composting plant of Essent Milieu (de Wijster, The Netherlands). The OFMSW consisted of about 90 % v/v garden waste, mainly weed (due to the season it was collected in), and about 10 % v/v kitchen waste. The OFMSW was cut with a 10 litres stainless-steel commercial kitchen blender (Eduard Müller & Söhne, Germany). For the first experiment, fresh waste was used, while in the second experiment frozen waste was used. The frozen waste had been stored in a commercial freezer (Bosch, Germany) at a temperature of -30°C. Because OFMSW already contains acidogenic bacteria, no inoculum was

added. The Total Solids (TS) content of the OFMSW was 453 ± 18 g per kg OFMSW and Volatile Solids (VS) content of the OFMSW was 224 ± 23 g per kg OFMSW.

2.2 Reactor set-up

The reactor set-up was based on the dry anaerobic digestion process BIOCEL (ten Brummeler, 1993), i.e. the total solids (TS) in the reactor should be higher than 200 g per kg OFMSW. Peristaltic pumps (Marlon-Watson 323U, U.K.) were used for percolation and recirculation of the leachate through the OFMSW. To enhance hydrolysis of the OFMSW, a leachate recirculation of 7.2 l per h was applied (Veeken and Hamelers, 2000). To prevent clogging in the reactor set-up, the OFMSW was packed between two gravel beds with washed 4-6mm diameter gravel. The leachate was pumped up from the bottom to a sampling vessel, where liquid samples were taken and the pH of the leachate was measured. The gas phase of the reactors consisted of gas sample cell, a water lock and gas metre (Ritter, Germany). The reactor was placed in a water bath with a recirculation heater (Julabo MD, Germany) controlled at 30 ± 0.5 °C.

5.2.3 Experiments

Three experiments were performed in stainless steel cylindrical reactors with an effective volume of 5-L. Each experiment consisted of two batch reactors, which were all operated as duplicates. The first experiment was performed with fresh waste and ran for 35 days. It was used to observe ethanol and caproate formation during acidification and to determine the ethanol addition strategy in the second experiment. The second experiment

was performed with frozen waste and ethanol additions and ran for 28 days. It was used to investigate the possibility of chain elongation during acidification with ethanol additions. The third experiment was performed with frozen waste without ethanol additions and ran for 28 days. It was performed at the same time as the second experiment and was used to compare the results of the second experiment with ethanol additions. Table 1 shows an overview of the experiments.

On average, each reactor contained 2.0 kg of OFMSW (fresh or frozen), representing a Chemical Oxygen Demand (COD) value of 407 ± 58 g; one gram of VS is considered to have a COD-value of one gram COD (ten Brummeler, 1993). To maintain a continuous recirculation, 2 L of demineralised water was added. Before the start of each experiment, the reactors were flushed with nitrogen to remove residual oxygen.

Table 1: Overview experiments

Experiment	Number of reactors	Condition of OFMSW	Addition of ethanol
1 (observation)	2 (duplicate)	Fresh	No
2 (ethanol addition)	2 (duplicate)	Frozen	Yes
3 (control, no additions)	2 (duplicate)	Frozen	No

To test adsorption of caproate and caprylate to OFMSW, either a 250 ml caproate solution (50 mM) or a 250 ml caprylate solution (10mM) was added to 55 g of OFMSW in a 500 ml glass bottle. These adsorption tests were performed as triplicates. Afterwards, the pH was adjusted with NaOH to a pH value of 5.5. Finally, the gas phase of the glass bottles were flushed with nitrogen gas.

5.2.4 Measurements and Analytical methods

Liquid samples were taken three times a week to measure carboxylic acids and alcohol concentrations. Around ethanol additions, additional liquid samples were taken to observe the effect of the additions. For the adsorption tests, caproate or caprylate were measured two hours after addition to the OFMSW. Both carboxylic acids (C2-C8) and alcohols (C1-C2) were measured using a gas chromatograph (HP 5890 series II GC, Germany), with a glass column packed with 10% Fluorad 431 on Supelco-port 100-120 mesh. For the carboxylic acid measurements, the column temperature was 130°C for four minutes, increased linearly with a ramp of 12°C per minute to 160°C and kept on 160°C for 6.5 minutes. For alcohol measurements, the column temperature was constant at 70°C. Soluble COD was measured by means of Dr. Lange tubes (HACH, Germany). Gas samples were taken to measure the methane, nitrogen, hydrogen and oxygen percentages in the produced biogas by analysing the sample with a gas chromatograph (Steinbusch *et al.*, 2008). The total solids (TS) content was determined after drying the sample for 24 hours in a 105°C stove, while the volatile solids (VS)

contents was determined after heating the residual of the TS measurement in a 550°C stove for 8 hours.

5.2.5 Calculation of the hydrolysis rates

The hydrolysis rates (k_h) were calculated based on the amount of soluble COD concentration produced from the OFMSW after 28 days. After rearrangements, the exact calculation of the hydrolysis rates is shown in Equation (6).

$$k_h = \frac{\ln\left(\frac{bCOD - (COD_r + COD_{hydrogen})}{bCOD}\right)}{-t} \qquad \text{(equation 6)}$$

where

k_h is the hydrolysis rate of OFMSW (d^{-1})

bCOD is the biodegradable COD of the OFMSW,

assumed to be 0.8 of total COD (326g) (g COD)

COD_r is the amount soluble COD present in the reactor after

subtraction of the added COD in the form of ethanol (if added) (g COD)

$COD_{hydrogen}$ is the amount of formed hydrogen during acidification (g COD)

t is time of the calculation of the hydrolysis rate (d)

5.3 Results and Discussion

5.3.1 Caproate and heptanoate formation from fresh waste without ethanol addition

During experiment 1 (with fresh waste), we observed the formation of caproate and heptanoate without ethanol additions to determine the ethanol addition strategy. Due to presence of easily bioavailable carbohydrates (mainly sugars), a rapid acidification increases the partial hydrogen pressure within the first three days, according to equation 7. Consequently, ethanol formation from acetate is stimulated (equation 8) More detailed information about this reaction can be found in Steinbusch *et al.* (2008)

$$C_6H_{12}O_6 + 4\ H_2O \rightarrow 2\ CH_3COO^- + 2\ HCO_3^- + 4\ H_2 + 4\ H^+ \quad \text{(equation 7)}$$

$$CH_3COO^- + H^+ + 2H_2 \rightarrow C_2H_5OH + H_2O \quad \text{(equation 8)}$$

Figure 1 shows an increase of ethanol to 1.3 g l^{-1} (28 mM) at day four. However, from day four on, the ethanol concentration drops to a concentration below 0.1 g l^{-1} (2 mM) on day nine and remains low until the end of the experiment. After a lag phase of at least two days, caproate production initiates. During the ethanol concentration drop, the caproate concentration increases from 0.2 mg l^{-1} (2 mM) on day four to 1.5 g l^{-1} (13 mM) on day nine (*Figure 1*). In the same period, the heptanoate concentration increases from not detectable concentrations to 0.4 g l^{-1} (3 mM). Smith and McCarty (1989) also observed a lag phase before caproate and heptanoate production started after their ethanol perturbations.

The formation of caproate from ethanol requires butyrate as intermediate, while the formation of heptanoate requires valerate. Hence, butyrate and valerate formation are essential for MCFA formation. Directly from the start of the experiment until day four, there is a rapid increase of the butyrate concentration to 3.0 g l^{-1} (34 mM). The butyrate could be either formed directly from glucose or indirectly from acetate and ethanol. During the ethanol concentration drop, the butyrate concentration slightly decreases to 2.8 g l^{-1} (32 mM) confirming consumption of butyrate for caproate formation. As butyrate is also formed in the same period, its concentration drop is small. In case of valerate formation, we see a similar mechanism to butyrate formation. There is a rapid increase until day four to 1.0 g l^{-1} (9 mM) and during the ethanol concentration drop, the valerate concentration remain about the same concentration. Hence, the formation rate of valerate from propionate and ethanol is more or less equal to the consumption rate of valerate to heptanoate.

5.3.2 MCFA formation during acidification of frozen waste with ethanol additions

The experiment with fresh waste demonstrated that MCFA production took place after a delay of some days. Hence, in the experiment with ethanol additions (experiment 2), we supplied additional ethanol after we observed an ethanol concentration drop. We observed the hydrogen partial pressure increased, ethanol was formed and at day 6 the ethanol concentration dropped, as depicted in *Figures 2 and 3*. Consequently, we added 0.1 mol (± 33 mM) to the reactors at day six and we kept on monitoring

the ethanol concentrations in the following days. Two days later (day eight), we saw a new ethanol concentration drop and added another 0.1 mol. Similar actions were taken at day ten and day thirteen. From day thirteen the ethanol concentration did not drop anymore below 1.0 g l^{-1}.

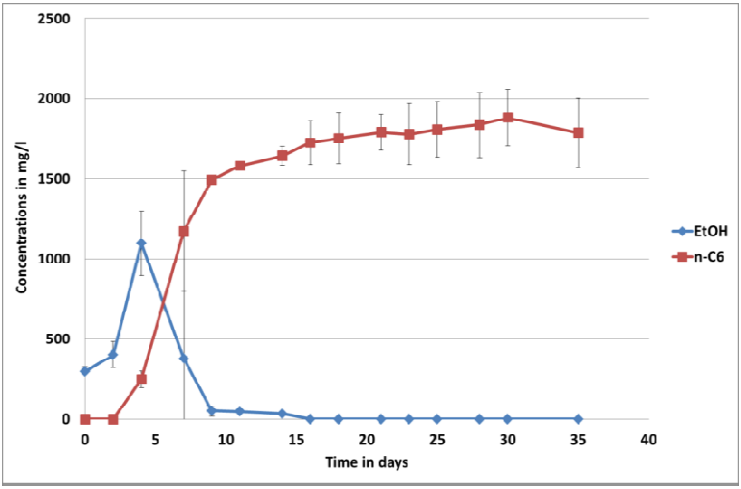

Figure 1. During acidification of fresh waste at 30°C, caproate formation (squares) and ethanol formation (diamonds) were observed. Most of the caproate formation coincided with ethanol degradation from day four until day nine, indicating that caproate is formed from ethanol.

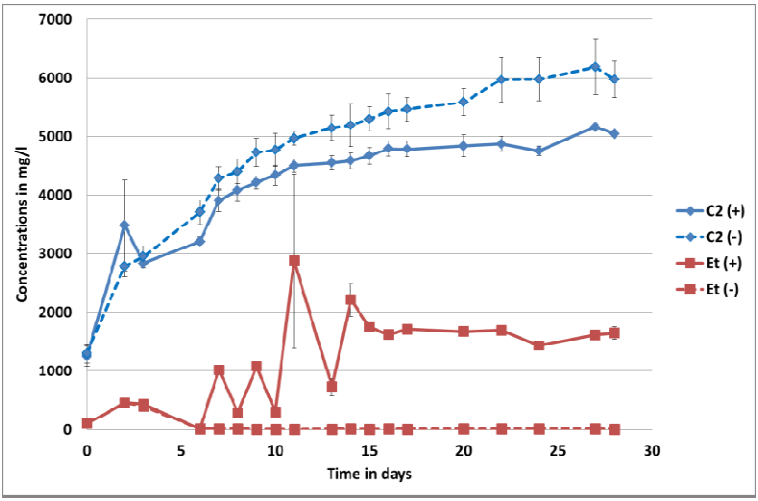

Figure 2. Acetate and ethanol concentrations during experiment with ethanol additions (solid lines) and during control experiment (dashed lines).

A possible reason for the ceased ethanol consumption could be the high unionized carboxylic acid concentrations, more than 28 mM, at a pH of 5.4. Kenealy and Waselefsky (1985) stated that *C.kluyveri* can grow in an pH range from 5.2 until above 8.0, but they did not report anything about toxicity levels of undissociated carboxylic acid concentrations for this organism. The MCFA production in the reactors with ethanol additions is obvious larger than in the reactors without ethanol additions (Table 2). In *Figure 4*, the concentrations of MCFA in this experiment are presented. After ethanol additions, we obtained a caproate concentration of 2.8 g l^{-1} (24 mM), a heptanoate concentration of 1.6 g l^{-1} (12 mM) and a caprylate concentration of 0.6 g l^{-1} (4 mM). In the reactors without ethanol additions, the MCFA concentrations of caproate, heptanoate and caprylate were respectively 1.6 g

l^{-1} (14 mM), 0.4 g l^{-1} (3 mM) and 0.1 g l^{-1} (< 1 mM). The highest caproate production rate was obtained after the first ethanol addition (day six to eight) and had a value 0.7 g caproate l^{-1} d^{-1} (6 mM C6 l^{-1} d^{-1}). This rate is in line with the results of Steinbusch *et al.* (2011), who produced caproate from acetate, hydrogen and ethanol at a maximum rate of 0.5 g caproate l^{-1} d^{-1}.

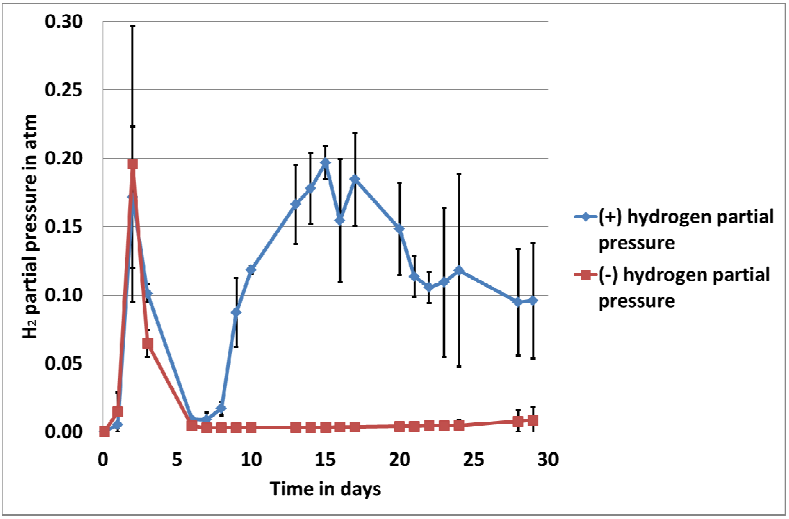

Figure 3. Hydrogen partial pressures during experiments with frozen waste.

5.3.3 Effect of ethanol additions on intermediate products

The first ethanol addition did not change significantly the concentrations of the intermediate products (propionate, butyrate and valerate). However, at the same time in reactors without ethanol additions the concentrations of the intermediate products were still increasing. That indicates that there was consumption of the intermediate products after the

first ethanol addition. After the second ethanol addition, the consumption became obvious and the concentrations of propionate, butyrate and valerate decreased, as shown in *Figure 5*.

Table 2: final carboxylic acid concentrations in the leachate

Experiment	n-C2 ($g\ l^{-1}$)	n-C3 ($g\ l^{-1}$)	n-C4 ($g\ l^{-1}$)	n-C5 ($g\ l^{-1}$)	n-C6 ($g\ l^{-1}$)	n-C7 ($g\ l^{-1}$)	n-C8 ($g\ l^{-1}$)
1 (fresh)	6.1 ± 0.1	1.8 ± 0.0	3.3 ± 0.1	1.1 ± 0.1	1.8 ± 0.2	0 .4 ± 0.1	-
2 (frozen, ethanol)	5.0 ± 0.1	0.5 ± 0.0	1.0 ± 0.1	0.6 ± 0.0	2.8 ± 0.0	1 .6 ± 0.1	0.6 ± 0.1
3 (frozen, control)	6.0 ± 0.3	2.0 ± 0.0	4.0 ± 0.3	1.3 ± 0.1	1.7 ± 0.2	0 .4 ± 0.0	0.0 ± 0.0

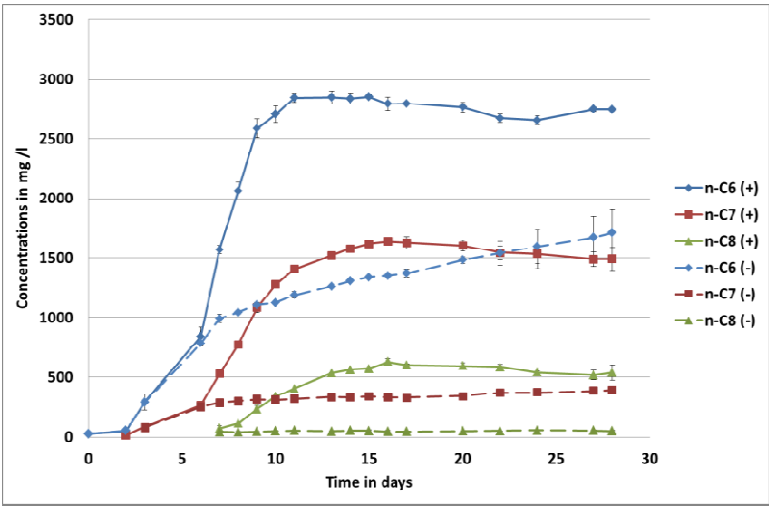

Figure 4. After ethanol additions, caproate (solid diamonds), heptanoate (solid squares) and caprylate (solid triangles) concentrations increased clearly during acidification of frozen waste at 30°C . The dashed lines indicate the MCFA concentrations in the control experiment without ethanol additions.

Remarkably, the difference in the concentration of acetate between reactor with ethanol additions and without additions is relatively much smaller (-20%) than the difference of the intermediate product concentrations (from –62% up to -75%) (*Figure 2*). An explanation for the higher acetate concentration can be found in the metabolism of *C.kluyveri* as a significant part of the consumed ethanol is oxidized to acetate and hydrogen to provide energy for the chain elongation reaction (Seedorf *et al.*, 2008). Additionally, the released hydrogen (or other hydrogen present in the reactor) could be converted together with carbon dioxide into more acetate via homoacetogenesis.

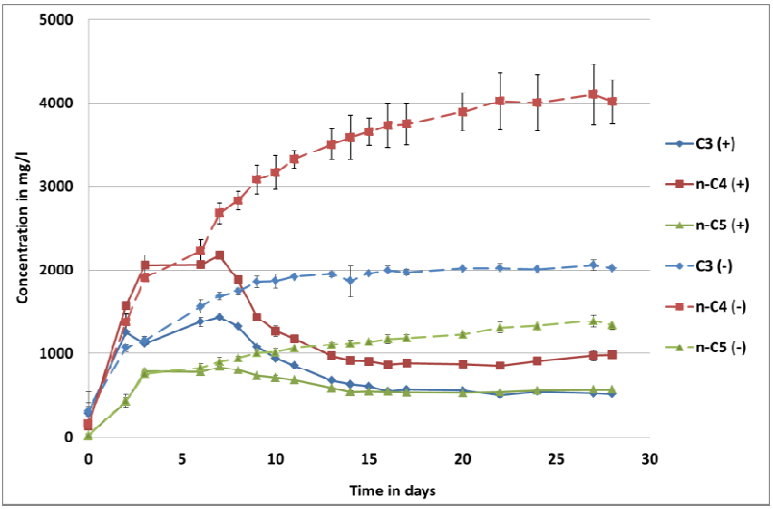

Figure 5. Intermediate product concentrations (propionate (solid diamonds), butyrate (solid squares) and valerate (solid triangles)) clearly decreased after ethanol additions during acidification of frozen waste at 30°C. The dashed lines indicate the intermediate product concentrations in the control experiment without ethanol additions.

5.3.4 Effect of ethanol additions on hydrolysis of frozen waste

The total amount of carboxylic acids (Σ C2 - C8) produced in reactors with ethanol additions is 28 % smaller than in reactors without ethanol additions (*Figure 6*). This is not in line with our expectations as we expected that the added ethanol would be converted to additional carboxylic acids (either to MCFAs or acetate). Moreover, the soluble COD concentration in the reactors with ethanol additions is only marginally higher, 33.5 ± 0.2 g l^{-1}, than the soluble COD concentration in the other reactors, 31.3 ± 1.8 g l^{-1}. When we subtract the COD value of ethanol additions (12.8 g COD l^{-1}) from

the soluble COD concentration at day 28, we find a hydrolysis constant of 0.008 d^{-1} for the reactors with ethanol additions and a hydrolysis rate of 0.012 d^{-1} in the reactors without ethanol additions. COD loss to methane production can be excluded as an explanation, for the reason that the methane percentage in the gas phase of the reactors was below one per cent.

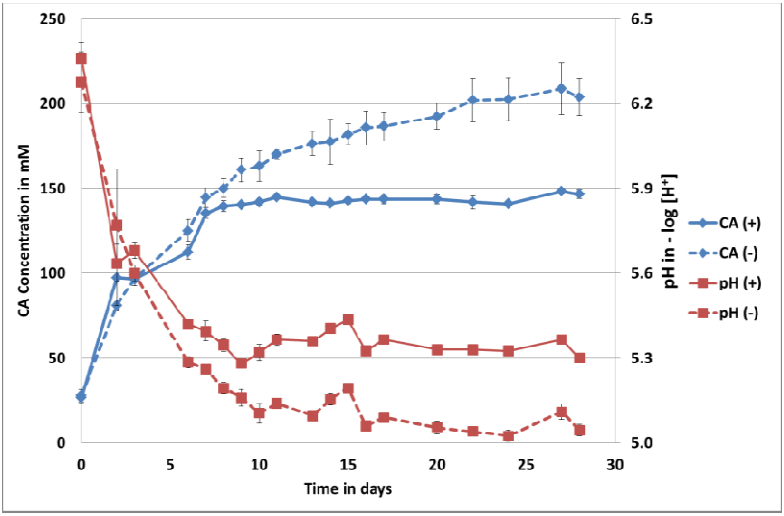

Figure 6. The total carboxylic acid (CA = Σ C2-C8) production during acidification with ethanol addition (solid diamonds) is significantly lower than the total carboxylic acid (CA) production without ethanol additions (dashed diamonds). As result, the pH is higher in reactors with ethanol additions (solid squares) than in the reactors without ethanol additions (dashed squares).

According to Skrivanova *et al.* (2006) and Liu *et al.* (2008) caprylic acid, the undissociated form of caprylate, can inhibit bacteria and fungi. Besides caprylic acid, high ethanol concentrations (> 40 mM) can inhibit these

micro-organisms as well (*e.g.* Chen and Jin, 2006). An inhibitory effect of caprylic acid (perhaps combined with other MCFAs) or ethanol on acidogenic bacteria could be an explanation for the lower amount of carboxylic acids and soluble COD as these bacteria are required to hydrolyse the OFMSW and convert the hydrolysed OFMSW to VFAs.

To reduce an inhibitory effect of undissociated MCFAs and to improve hydrolysis at low pH values, in-line extraction could be performed. Recently, Agler *et al.* (2012) demonstrated improved caproate production from diluted beer after in-line extraction of caproate. Another method to reduce an inhibitory effect of undissociated MCFA is to raise the pH during acidification. Consequently, a larger percentage of the MCFAs is present in the dissociated form instead of the more toxic undissociated form. Additionally, an inhibitory effect of the pH itself on hydrolysis could also be reduced (Veeken *et al.*, 2000). However, by raising the pH an alternative method to suppress methanogenic activity would be required.

Instead of raising the pH during acidification or in-line extraction, a second reactor could be added to reduce an inhibitory effect of undissociated MCFAs by adding ethanol in the second reactor that performs chain elongation. In such a way, an inhibitory effect of produced undissociated MCFAs on hydrolysis in the acidification step is eliminated. Moreover, a separated reactor system also avoids a toxicity effect of high ethanol concentrations on hydrolysis in the acidification step. This is an advantage over an in-line extraction system that only can reduce toxicity of undissociated MCFA. If the pH is raised in the chain elongation reactor, it would also require

an alternative method to suppress methanogenic activity in this second reactor.

Another plausible reason for the lower measured COD in the reactors with ethanol additions could be the adsorption of MCFAs to the OFMSW, due to their hydrophobicity. Additional batch tests (Table 3) show that there is partial adsorption of caprylic acid to the OFMSW. For caproic acid, however, no significant adsorption to the OFMSW was found. As the batch tests were performed in wet anaerobic conditions (TS of ± 90 g kg^{-1} OFMSW), while the reactor experiments were performed in dry anaerobic conditions (TS of ± 290 g kg^{-1}), it is possible that during the reactors experiments a larger percentage of adsorption of caprylic acid to OFMSW occurred. To quantify the adsorption of caprylic acid to OFMSW further research is required.

Table 3 : Caprylate has a clearly stronger adsorption to OFMSW than caproate

	pH	Concentration (mM) at t = 0 h	Concentration (mM) at t = 2 h	Adsorption percentage
Caproate (C6)	5.5 ± 0.1	45 ± 1	43 ± 2	4 %
Caprylate (C8)	5.5 ± 0.1	9.0 ± 0.2	7.3 ± 0.9	19 %

5.4 Conclusions

In this paper, we demonstrated that chain elongation can be promoted during acidification of OFMSW by ethanol additions. Caproate concentrations rose to 2.7 g l^{-1}, while heptanoate and caprylate increased to respectively 1.5 g l^{-1} and 0.5 g l^{-1} after ethanol additions in reactors with frozen OFMSW. MCFA production ceased after day ten, probably due to low pH (5.4) and/or high unionized carboxylic acid concentrations. Despite the increased MCFA production, the hydrolysis rate and the carboxylic acid yield in reactors with ethanol additions were lower than the hydrolysis rate and carboxylic acid yield in the reactors without ethanol additions. Additionally, partial adsorption of produced caprylic acid to OFMSW occurs. Further research is required to determine whether a combined chain elongation and acidification reactor or a separated reactor system is more advantageous for MCFA production from OFMSW.

5.5 Acknowledgement

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6. Two-stage medium chain fatty acid (MCFA) production from municipal solid waste and ethanol

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T.I.M. Grootscholten, D.P.B.T.B. Strik, K.J.J. Steinbusch, C.J.N. Buisman and H.V.M. Hamelers. Two-stage medium chain fatty acid (MCFA) production from municipal solid waste and ethanol.

Abstract

Chain elongation is an anaerobic fermentation that produces medium chain fatty acids (MCFAs) from volatile fatty acids and ethanol. These MCFAs can be used as biochemical building blocks for fuel production and other chemical processes. Producing MCFAs from the organic fraction of municipal solid waste (OFMSW) is attractive because it combines waste treatment and biochemical production. We investigated whether higher MCFA production rates can be achieved from OFMSW by separating acidification and chain elongation instead of using a single-stage system. We obtained higher MCFA production rates with a two-stage system than with a single-stage system. Also, the obtained caproate concentrations were above the solubility of caproic acid in water, which favours separation. Additionally, it is easier to control competitive processes in a two-stage system. However, more research is required to prevent too much production of butyrate instead of MCFAs, which occurred several times during the experiments.

6.1 Introduction

Alternative processes based on biomass are being developed to generate electricity and reduce the world's dependence on oil. One of these biobased processes involves a mixed-culture fermentation leading to chain elongation. In chain elongation, bacteria produce medium chain fatty acids (MCFAs), which are straight monocarboxylic acids with six to eight carbon atoms, from diluted ethanol and volatile fatty acids (VFAs), which are straight monocarboxylates with up to five carbon atoms (Steinbusch *et al.*, 2011; Agler *et al.*, 2012; Grootscholten *et al.*, 2013a; Grootscholten *et al*., 2013b; Grootscholten *et al.*, 2013c; Grootscholten *et al.*, 2013d). MCFAs can be used as antimicrobials (Woolford, 1975) and corrosion inhibitors (Kuznetsov and Ibatullin, 2002) or as precursors in biodiesel production (Renz, 2005), bioplastics production (*e.g.* Witholt and Kessler, 1999) and other biobased chemical production processes (*e.g.* Levy *et al.*, 1981). As VFAs and ethanol can be produced from lignocellulosic materials (Agler *et al.*, 2011; Sarkar *et al.*, 2012), which cannot be digested by humans, the food-fuel debate could be avoided for the chain elongation process.

Chain elongation can be performed with a mixed culture. Consequently, a sterilization step is not necessary. High production rates of MCFAs (up to 57.4 g MCFA l^{-1} d^{-1}) have been achieved with high MCFA concentrations (to ease separation) and high selectivity (> 90%) with an improved chain elongation reactor design (Grootscholten *et al.*, 2013b; Grootscholten *et al.*, 2013c, Grootscholten *et al.*, 2013d). Chain elongation could valorise the organic fraction of municipal solid waste (OFMSW), if it is

used as source for VFA production. In that way, it provides an alternative for methane production via anaerobic digestion.

VFAs are produced during acidification of the organic fraction of municipal solid waste (OFMSW), especially if methanogens are inhibited. Often, the pH is lowered (also by VFA production itself) during this process to avoid acetate consumption by methanogens (Agler *et al.*, 2011); other methods are also available to reduce the impact of methanogens (*e.g.* heat shocks). Besides VFAs, also MCFAs are produced in low concentrations (< 5 g l^{-1}) during acidification of OFMSW without ethanol addition (Holtzapple *et al.*, 1999; Grootscholten *et al.*, 2013a).

Kenealy *et al.* (1995) demonstrated that caproate (MCFA with six carbon atoms) could be produced from chemical-grade cellulose (a large constituent of OFMSW) and ethanol with a defined co-culture (without methanogens). Grootscholten *et al.* (2013a) were able to use mixed cultures to produce MCFAs from OFMSW and ethanol in a single reactor. However, the hydrolysis rate of the OFMSW with ethanol addition was lower than the hydrolysis rate of the OFMSW without ethanol addition. High concentrations of undissociated MCFA and/or high ethanol concentrations may have been the reason for the lower hydrolysis rate. To decrease the effect of undissociated MCFAs, these components can be removed from the reactor, *e.g.* by in-line extraction (Agler *et al.*, 2012). However, even if in-line extraction would be successful in eliminating the toxicity of undissociated MCFAs, ethanol toxicity could still affect the hydrolysis rate.

By using a two-stage system, which separates acidification and chain elongation, toxicity effects of both MCFAs and ethanol on hydrolysis could be avoided. Other advantages of a two-stage system over a single-stage system would be (i) the possibility to optimize acidification and chain elongation separately and (ii) the avoidance of MCFA adsorption to solid OFMSW particles (Grootscholten *et al.*, 2013a). Disadvantages of a two-stage system over a single-stage system are its increased complexity and higher capital costs due to need of two bioreactors instead of one. The objective of the research described in this paper was to investigate whether the MCFA production rate from OFMSW and ethanol would be higher in a two-stage system than in a single-reactor system.

6.2 Material and methods

In the first stage, VFAs were produced from OFMSW by mixed-culture acidification. The leachate of the acidification reactor was fed together with ethanol to the second stage, in which chain elongation took place. The chain elongation reactor was operated with a short hydraulic retention time (HRT) of 11 h at near-neutral pH; the impact of acetate consumption by acetotrophic methanogens was expected to be limited at this HRT because of their low maximum growth rate ($\mu_{max} < 0.02\ h^{-1}$). Moreover, MCFA producers like *Clostridium kluyveri* produce more MCFAs at near-neutral pH values than at slightly acidic pH values ($5 < pH < 6$) (Kenealy and Waselefsky, 1985; Kenealy *et al.*, 1995; Steinbusch *et al.*, 2011).

6.2.1 OFMSW and acidification reactor setup

A new representative sample of OFMSW was collected at a waste treatment plant of Attero in Venlo, the Netherlands every two weeks. During the experiment, there were seasonal variations in the composition of the OFMSW. Part of the OFMSW was used directly for acidification, while the remainder was stored in a controlled cold-storage room at a temperature of 4°C. Each week, approximately 12 kilograms of wet OFMSW was used together with 21 litres of tap water. No additional pre-treatment step was applied. No inoculum was added because OFMSW already contains acidogenic bacteria.

Acidification was performed in a 40-L dry anaerobic batch reactor with leachate percolated from the top, and based on the BIOCEL process (ten Brummeler *et al.*, 1991). A separate liquid vessel (about 12 L) was connected to the leachate recycle loop to keep the anaerobic batch reactor in dry conditions (total solids > 200 g kg^{-1} OFMSW), while obtaining sufficient leachate to perform chain elongation at a HRT of 11 h (about 18 L per week). The temperature in the acidification reactor was controlled at 35°C with a water bath (Julabo, Germany), which pumped the water through the water jacket of the reactor. We decided not to control the pH to reduce the impact of methanogens. After filling the acidification reactor with OFMSW, we flushed the head space with nitrogen gas to remove residual oxygen. Figure 1 depicts the complete experiment setup. After 7 days, the leachate (containing VFAs) was collected in a hard plastic container (about 25 L), flushed with nitrogen to

retain anaerobic conditions, and stored in the same cold storage room as the OFMSW (4°C).

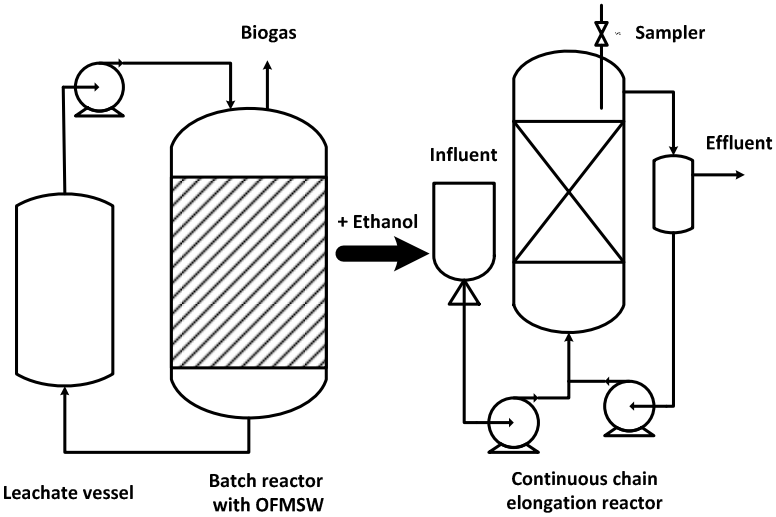


Figure 1. Overview of experimental setup, which consists of an acidification batch reactor to produce VFAs from OFMSW and a continuous chain elongation reactor to produce MCFAs from VFAs and ethanol.

6.2.2 Chain elongation reactor setup and experiment

For the chain elongation step, a similar 1-L anaerobic upflow filter was used as described by Grootscholten *et al.* (2013b), except that the K_2CO_3 was replaced by gaseous CO_2, which was bubbled from the bottom of the reactor. The temperature in the chain elongation reactor was controlled at 30°C with a water bath (Julabo, Germany). Before feeding the chain elongation reactor with OFMSW, the reactor was running on acetate and ethanol with active MCFA producers. Together with addition of chemical-grade

ethanol (varying between 9 and 22 g l^{-1} but >18.4 g l^{-1} from day 123), the collected leachate (containing VFAs) was fed to the chain elongation reactor from a 9-L anaerobic medium bag (MSR, USA), which was stored in a refrigerator at 4°C (Bosch, Germany). Until day 102, the pH was regulated by addition of NaOH to the medium bag. As large pH variations occurred, we installed a pH controller (Endress & Hauser, Switzerland) combined with a 5M NaOH solution on day 102 to maintain the pH between 6.5 and 7.0.

6.2.3 Measurements

Five times a week, we took liquid samples from the acidified leachate, the medium bag and the chain elongation reactor. Carboxylates (VFAs and MCFAs) and ethanol were measured with gas chromatographs (Hewlett Packard 5890-II, USA), using the same procedure as Steinbusch *et al.* (2011). Soluble chemical oxygen demand (COD) was measured with commercial test kits (Hach, Germany). The headspace of the reactor was monitored for CH_4, N_2, O_2 and CO_2 by taking gas samples and measuring them with another gas chromatograph (Shimadzu GC-2010, Japan). A gas sample of 2 ml was injected into a loop, from which a 50-µl sample was taken automatically and injected into the Porabond Q column (50m x 0.53mm, 10µm, Varian CP7355) at 120°C. The column temperature and column pressure were 75°C and 1 atm, respectively. The components were detected with a thermal conductivity detector at 150°C.

6.2.4 Calculations

6.2.4.1 Selectivity in the chain elongation step

The selectivity in the chain elongation step is defined as the concentration of electrons in the formed product divided by the net consumed electrons from the fed acetate, propionate and ethanol (Equation 1). Acetate contains 8 moles of electrons per mole, propionate 14 moles of electrons per mole, butyrate 20 moles of electrons per mole, valerate 26 moles of electrons per mole, caproate 32 moles of electrons per mole, heptanoate 38 moles of electrons per mole, caprylate 44 moles of electrons per mole and ethanol 12 moles of electrons per mole.

Selectivity = [Product] (mol e eq l^{-1}) / consumed ethanol and VFAs (mol e eq l^{-1}) (1)

6.2.4.2 Volumetric production rate

The volumetric production rate of each product (butyrate, valerate, caproate, heptanoate and caprylate) is defined by its concentration divided by the hydraulic retention time (Equation 2). The unit of the volumetric production rate is g l^{-1} d^{-1} or moles of electron equivalents l^{-1} d^{-1} (mol e eq l^{-1} d^{-1}). For the total volumetric production rate, the residence time of the entire system was taken, which was 7 days and 11 hours (or 7.46 days).

Volumetric production rate = [Product] (g l^{-1} or mol e eq l^{-1}) / HRT (d) (2)

6.3 Results and discussion

6.3.1 MCFA production rate and caproate concentration

Based on the results, the experiment can be divided in three periods (Table 1, Figure 2). In period I, excessive oxidation of ethanol to acetate occurred. In period II, excessive ethanol oxidation was prevented, but MCFA production was unstable because of large pH variations (from pH 5 to pH 12). Installation of a pH controller on day 102 marked the beginning of period III with substantial production of MCFA and butyrate.

Table 1: Periods during experiment in chain elongation step

Period	Days	Characteristic	Action at end of period
Period I	0 - 70	Substantial oxidation of ethanol to acetate	Carbon dioxide addition reduced
Period II	71 - 102	Variable MCFA production due to large pH variations	pH controller installed
Period III	103 - 171	Substantial MCFA and butyrate production	Experiment ended

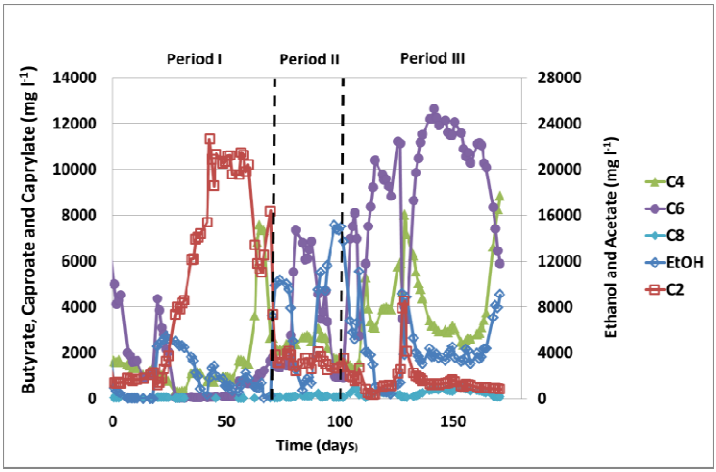
The entire two-stage system achieved a maximum volumetric MCFA production rate of 1.9 g MCFA l^{-1} d^{-1} (0.5 mol e eq l^{-1} d^{-1}) on day 142 (including hydrolysis and acidification), which is approximately two times higher than the maximum MCFA production rate in a previously tested single-stage system (Grootscholten *et al.*, 2013a). However, the acidification in this

study was performed at 35°C, but at 30°C in the previous study. After correction for the temperature (using the Arrhenius equation), the MCFA production rate in the overall two-stage system was still 30% higher than in the single-stage system.

The maximum volumetric MCFA production rate in the chain elongation reactor was 28.1 g MCFA l^{-1} d^{-1} (7.8 mol e eq l^{-1} d^{-1}) on day 142 (see Table 2), almost 70% higher than in the experiments of Grootscholten *et al.* (2013b), but roughly two times lower than in the experiments of Grootscholten *et al.* (2013d). The maximum MCFA selectivity based on the consumed VFAs and ethanol in the chain elongation reactor was 78% (Figure 3). This selectivity is lower than the selectivity achieved in previous chain elongation reactors (Agler *et al.*, 2012; Grootscholten *et al.*, 2013b; Grootscholten *et al.*, 2013d), which is mainly caused by butyrate production (7%) and valerate production (5%). Based on the results of the soluble COD measurements, we think that most of the converted electrons (10%) unaccounted for were probably converted by methanogenesis.

In period III, the caproate concentration was higher than the solubility of the caproic acid in water for 30 days (day 135 – day 165), with a maximum concentration of 12.6 g l^{-1} (109 mM) on day 142 (Figure 2b). These results demonstrate that caproate production from OFMSW and ethanol in a two-stage system is feasible.

a)



b)

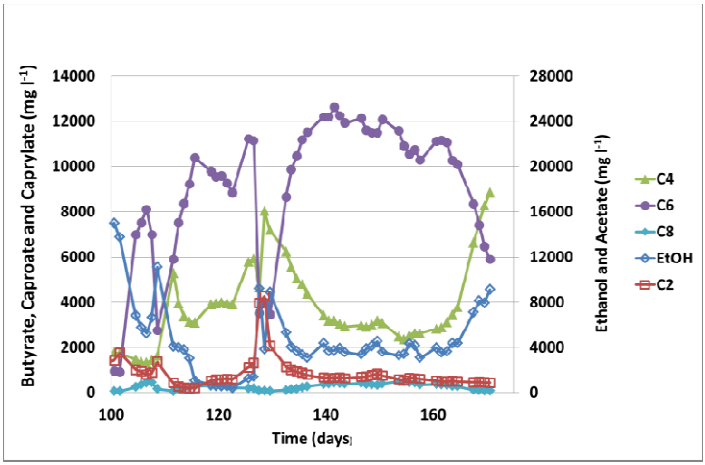


Figure 2. Overview of experimental results (chain elongation reactor) in the complete experiment (a) and the experimental results of period III (after installing the pH controller on day 102) (b).

Despite the higher production rates of heptanoate and caprylate, their concentrations were lower in this two-stage system than in our previous single-stage system (Grootscholten *et al.*, 2013a). The maximum heptanoate concentration of 1.2 g l^{-1} (9 mM) occurred on day 154, while the maximum caprylate concentration of 0.4 g l^{-1} (3 mM) coincided with the maximum caproate concentration on day 142. Further improvements are required to increase the heptanoate and caprylate concentrations to the solubility of their undissociated forms in water.

6.3.2 Effect of hydrolysis of OFMSW on MCFA production

By separating hydrolysis and chain elongation, possible negative effects of MCFA and ethanol on hydrolysis were eliminated, resulting in a higher total MCFA production rate. Table 3 lists the average VFA production from the OFMSW in the first step. The total VFA production was lower in the present study than in our previous work (Grootscholten *et al.*, 2013a), but that is caused by the shorter period of operation (7 d *vs*. 28 d). Despite the separation of the processes, hydrolysis is likely to be the limiting step for MCFA production from OFMSW because the MCFA production rate in the entire two-stage system (1.9 g MCFA l^{-1} d^{-1}) was much lower than the MCFA production rate in the chain elongation reactor (28.1 g MCFA l^{-1} d^{-1}).

Table 2: Carboxylates and ethanol concentrations in chain elongation reactor at maximal MCFA production and on final day

Stream (day)	pH	EtOH ($g\ l^{-1}$)	n-C2 ($g\ l^{-1}$)	n-C3 ($g\ l^{-1}$)	n-C4 ($g\ l^{-1}$)	n-C5 ($g\ l^{-1}$)	n-C6 ($g\ l^{-1}$)	n-C7 ($g\ l^{-1}$)	n-C8 ($g\ l^{-1}$)	COD* ($g\ l^{-1}$)
Effluent (142)	7.4	3.7	1.3	0.5	3.2	2.0	12.6	0.9	0.4	54.8
Influent (140)	5.3	19.3	3.3	1.8	1.9	1.1	0.7	0.4	0.0	56.8
Consumption or production		15.6	2.0	1.3	1.3	0.9	11.9	0.5	0.4	2.0
Effluent (171)	6.8	9.1	0.8	0.8	8.9	2.0	5.9	0.3	0.1	59.5
Influent (168)	5.6	22.0	2.0	1.8	2.8	1.6	0.7	0.3	0.0	60.6
Consumption or production		12.9	1.2	1.0	6.1	0.4	5.2	0.0	0.1	1.1

* Measured COD, not calculated COD

Hydrolysis could still be affected by low pH and/or high undissociated VFA concentrations (Veeken *et al.*, 2000; Agler *et al.*, 2011). By increasing the pH to a value of 6, a faster hydrolysis can be achieved than at pH 5.5 (Veeken *et al.*, 2000). Moreover, the effect of undissociated VFAs would also be lower at the same total VFA concentrations, as most of the VFAs are present in the dissociated form at pH 6. However, inhibition of acetotrophic methanogens in the acidification step is less severe at this pH value, so other methods to reduce their impact could be required (*e.g.* heat shocks).

Another method to improve the hydrolysis of OFMSW could be the addition of a pre-treatment step, in which complex polymeric structures are converted into simple monomeric structures that might be easier consumed by bacteria. However, it is not certain that one specific pre-treatment is suited for OFMSW owing to its complex and varying composition (Hendriks and Zeeman, 2009). Moreover, the costs of such a step might not be economically feasible for MCFA production, depending on the future value of MCFAs.

Table 3: Carboxylic acid concentrations in leachate of acidification reactor during experiment

n-C2 ($g\ l^{-1}$)	n-C3 ($g\ l^{-1}$)	n-C4 ($g\ l^{-1}$)	n-C5 ($g\ l^{-1}$)	n-C6 ($g\ l^{-1}$)	n-C7 ($g\ l^{-1}$)	n-C8 ($g\ l^{-1}$)
3.0 ± 0.9	1.5 ± 0.6	1.6 ± 0.7	0.8 ± 0.4	0.6 ± 0.4	0 .3 ± 0.2	0.0 ± 0.0

6.3.3 Impact of competitive processes in the chain elongation step

6.3.3.1 Definition of competitive processes

As this chain elongation process was based on OFMSW effluent, it was important to reduce the impact of competitive processes in the chain elongation reactor. We recognize the following competitive processes:

(i) Excessive oxidation of ethanol to acetate;

(ii) Acetotrophic methanogenesis;

(iii) MCFA oxidation to acetate.

Excessive oxidation of ethanol to acetate (reaction 2, Table 4) is considered a competitive process, when other organisms consume the ethanol instead of MCFA producers. As MCFA producers also oxidize part the consumed ethanol to acetate to obtain energy (Seedorf *et al.*, 2008), we use the term 'excessive' to indicate the ethanol oxidation that must be avoided. Acetotrophic methanogenesis (reaction 3, Table 4) converts acetate into methane by reducing the substrate for chain elongation (reaction 1, Table 4). Finally, fatty acid oxidizers can degrade MCFAs to acetate (reaction 4, Table 4), resulting in loss of desired products. Fatty acid oxidation is also known as β-oxidation. Hydrogenotrophic methanogenesis (reaction 5, Table 4) is not considered a competitive process because the process itself does not consume ethanol, acetate or MCFAs. Nevertheless, hydrogenotrophic methanogenesis can have an indirect effect on chain elongation, as discussed hereafter.

Table 4: Overview of biochemical processes related to this study

Reaction		Process
1	$C_xH_{2x+1}COO^- + C_2H_5OH \rightarrow C_{(x+2)}H_{2(x+2)+1}COO^- + H_2O$	Chain elongation
2	$C_2H_5OH + H_2O \rightarrow CH_3COO^- + H^+ + 2\ H_2$	Ethanol oxidation
3	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	Acetotrophic methanogenesis
4	$C_xH_{2x+1}COO^- + 2\ H_2O \rightarrow C_{(x-2)}H_{2(x-2)+1}COO^- + H^+ + 2\ H_2$	Anaerobic MCFA oxidation
5	$HCO_3^- + H^+ + 4\ H_2 \rightarrow CH_4 + 3\ H_2O$	Hydrogenotrophic methanogenesis
6	$C_6H_{12}O_6 + 4\ H_2O \rightarrow 2\ CH_3COO^- + 2\ HCO_3^- + 4\ H^+ + 4\ H_2$	Oxidation of glucose to acetate

6.3.3.2 Effect of carbon dioxide addition on excessive oxidation of ethanol to acetate

In period I, high acetate concentrations were observed of up to 22 g l^{-1}, while ethanol was consumed (Figure 2a). These observations indicate that ethanol was converted into acetate instead of MCFAs, probably by ethanol-oxidizing acetogens. For this reaction to be thermodynamically feasible, the hydrogen partial pressure had to be low (< 10^{-1} atm) (*e.g.* Stams, 1994). Another process therefore had to be removing hydrogen to cause this low hydrogen partial pressure. This phenomenon is known as interspecies hydrogen transfer (*e.g.* Stams, 1994). By measuring the gas composition in the headspace, high methane partial pressures (> 0.9 atm) were detected.

After examining the mass balances, we concluded that the methane was most likely produced by hydrogenotrophic methanogens, which we considered responsible for the hydrogen consumption.

To reduce the hydrogen consumption by hydrogenotrophic methanogens, we reduced the carbon dioxide flow 1.0 l h^{-1} to 0.1 l h^{-1} on day 70. Subsequently, acetate concentrations decreased and ethanol concentrations increased, indicating that excessive ethanol oxidation can be avoided by maintaining an elevated hydrogen partial pressure. No excessive ethanol oxidation was observed, whereas the hydrogen partial pressure in the headspace was as low as about $3 * 10^{-2}$ atm. An explanation for the absence of excessive ethanol oxidation could be reduced growth of ethanol-oxidizing organisms. When the substrate and product concentrations approach the thermodynamical limit of the reaction, the growth rate of the involved organism(s) is reduced (Hoh and Cord-Ruwisch, 1996). MCFA producers can oxidize ethanol at higher hydrogen partial pressures than other ethanol-oxidizing organisms, probably because they can reuse the released electrons from ethanol oxidation in their elongation mechanism (Li *et al.*, 2008; Seedorf *et al.*, 2008).

We also investigated the effect of carbon dioxide addition. As MCFA producers require carbon dioxide for protein synthesis (*e.g.* Tomlinson and Barker, 1954), complete elimination of carbon dioxide addition may not be possible. On day 116 (period III), we tried to improve protein synthesis of MCFA producers by raising the carbon dioxide flow to 0.2 l h^{-1}. This action resulted in greater acetate concentrations on day 128 and 129. On day 129,

the carbon dioxide was reduced again to 0.1 l h^{-1} and acetate concentrations decreased again. As only limited carbon dioxide can be supplied, MCFA producers are more depending on other growth factors than carbon dioxide for their protein synthesis.

Our results confirm the statement of Agler *et al.* (2012) that hydrogenotrophic methanogenesis and chain elongation can proceed simultaneously in mixed cultures, although an important condition should be added, which is the prevention of excessive ethanol oxidation to acetate. This prevention could be achieved by (i) performing chain elongation without other ethanol-oxidizing organisms than MCFA producers, (ii) increasing the hydrogen partial pressure to levels that make excessive oxidization of ethanol to acetate thermodynamically unfavourable or (iii) inhibiting other ethanol-oxidizing organisms than MCFA producers in another way.

6.3.3.3 Implications for other competitive processes in the chain elongation step

We did not observe large impacts of acetotrophic methanogenesis and MCFA oxidation to acetate in the chain elongation step during this study. However, these processes may occur in future research and therefore we discuss them here.

Acetotrophic methanogens were likely present in our experiments because of their presence in OFMSW and consequently in the OFMSW effluent. However, the short HRT (11 h) in the upflow anaerobic filter and the low maximum growth rate of acetotrophic methanogens (about 0.015 h^{-1}; see

Pavlostathis and Giraldo-Gomez, 1991) limited their direct impact in the chain elongation step. As acetotrophic methanogens convert acetate into methane and carbon dioxide, they also can have an indirect effect on chain elongation by increasing the amount of carbon dioxide in the reactor. This additional amount of carbon dioxide can result in a lower hydrogen partial pressure, which may cause excessive oxidation of ethanol to acetate (see discussion above). If the indirect impact of acetotrophic methanogens is too large, additional actions are required. These actions could include further reducing the HRT and/or lowering the pH to reduce the indirect impact on chain elongation, although these actions might lower the chain elongation rate as well. Ultimately, heat shocks might have to be applied to limit methanogens. As *C.kluyveri* is a spore-forming bacterium, it can survive such heat shocks and recolonise the reactor afterwards.

It may not be possible to suppress fatty acid oxidizers (0.005 h^{-1}-0.05 h^{-1}; see Pavlostathis and Giraldo-Gomez, 1991) with only a shorter HRT in an upflow anaerobic filter. However, to make fatty acid oxidation thermodynamically feasible, the hydrogen partial pressure needs to be below 10^{-4} atm (Stams, 1994). Fatty acid oxidation is not likely to occur in a chain elongation reactor because the hydrogen partial pressure in the chain elongation step has to be kept at least above $3 * 10^{-2}$ atm to avoid excessive ethanol oxidation.

6.3.3.4 Controlling competitive processes

The hydrogen partial pressure is a key parameter in limiting the impact of competitive processes. In this study, we were able to elevate the

hydrogen partial pressure by limiting the hydrogen consumption after reducing the carbon dioxide flow. That made excessive oxidation of ethanol to acetate thermodynamically unfeasible. As we added this carbon dioxide externally, we were able to control its addition almost completely and heavily influence the hydrogen partial pressure. The hydrogen partial pressure can also be lowered by sulphate reduction to hydrogen sulphide by fast-growing organisms (*e.g.* Lens *et al.*, 1998). However, the amount of sulphate in OFMSW is limited. Consequently, sulphate reduction is likely not a relevant process for chain elongation based on OFMSW.

The ability to control large amounts of carbon dioxide is an important advantage of a two-stage system over a single-stage system for MCFA production from OFMSW with mixed cultures. In a single-stage system, a large part of the sugars is converted into acetate, carbon dioxide, protons and hydrogen (reaction 6, Table 4). Initially, this leads to elevated hydrogen partial pressures and sometimes even to ethanol formation (*e.g.* Grootscholten *et al.*, 2013a). However, when hydrogenotrophic methanogens become active, sufficient carbon dioxide is present to reduce the hydrogen partial pressure and allow significant ethanol oxidation.

In a single-stage system, additional actions could be performed to maintain the hydrogen partial pressure. These actions include removal of carbon dioxide (*e.g.* by using NaOH pellets in the headspace) and addition of hydrogen to the headspace. Arslan *et al.* (2012) added hydrogen to the headspace and did not observe a negative effect on hydrolysis of starch. Yet,

the effect of hydrogen addition on the hydrolysis of OFMSW requires more research.

In a two-stage system, large amounts of carbon dioxide are separated from the OFMSW effluent in the acidification step, especially if this step is performed under mild acidic conditions (5.0 < pH < 6.0) (as most of the carbon dioxide is present as gaseous carbon dioxide in this case). Therefore, it is probably easier to prevent excessive oxidation of ethanol to acetate in the chain elongation step by controlling the carbon dioxide addition than by removing carbon dioxide and/or adding hydrogen in a single-stage system.

6.3.4 Large production of butyrate instead of MCFAs

Occasionally (day 64 – 67, day 129 -133 and day 168 – 171), high butyrate concentrations (> 6 g l^{-1}) were produced instead of MCFAs. In all these periods, the estimated hydrogen partial pressure was between 5 * 10^{-3} and 5 * 10^{-2} atm indicating that a hydrogen partial pressure below 5 * 10^{-2} atm might be the reason for high butyrate concentrations instead of high MCFA concentrations. MCFA producers (like *C.kluyveri*) also produce butyrate besides MCFAs (*e.g.* Seedorf *et al.*, 2008). Therefore, it is not clear to us whether MCFA producers or other butyrate producers were responsible for the high butyrate concentrations.

Apart from estimated hydrogen partial pressures below 5 * 10^{-2} atm, MCFA production might also have been limited by (micro)nutrients in this period. If other organisms than MCFA producers were responsible for the observed butyrate production, they might have outcompeted the MCFA

producers for nutrients. In contrast to in our previous work (*e.g.* Grootscholten *et al.*, 2013d), we did not add yeast extract this time because we expected OFMSW to contain sufficient (micro)nutrients and/or dead bacterial biomass for MCFA producers. A lack of (micro)nutrients may be an alternative explanation for the higher butyrate concentrations. Further research is required to determine the cause of high butyrate concentrations in the chain elongation step.

6.4. Conclusions

In this study, we demonstrated that MCFA production from OFMSW in a two-stage system results in higher MCFA production rates and higher caproate concentrations than in a previously tested single-stage system. An additional advantage of separating chain elongation and hydrolysis is the easier control of competitive processes by controlling the carbon dioxide addition and by manipulating the hydrogen partial pressure. Hydrolysis of the OFMSW was the limiting process in this two-stage MCFA production system and requires further improvements. Occasionally, MCFA production was lowered by substantial butyrate production and this requires more research.

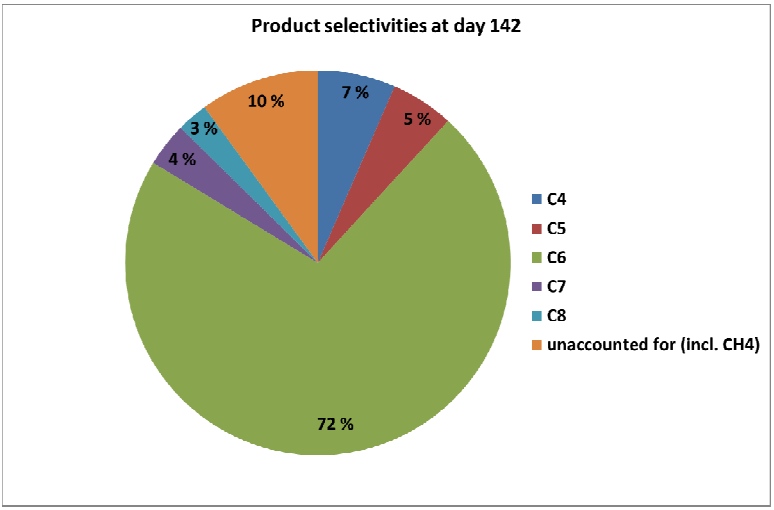


Figure 3. Product selectivities (based on electrons) on day of maximum MCFA production rate (day 142).

6.5 Acknowledgements

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7. General discussion

7.1 Chain elongation, a serious alternative for anaerobic digestion

In this thesis, we have made major improvements in the chain elongation process. We were able to improve the volumetric MCFA productivity more than 120 times compared to Steinbusch (2010). The volumetric productivity is now such that it shows potential for industrial applications of chain elongation. In this way, chain elongation will become a serious alternative for methane production via anaerobic digestion. The high productivity was achieved while MCFA concentrations were above the solubility of their unionized forms in water. Such high concentrations make it possible to develop cost-effective downstream processes. Additionally, the MCFA selectivity was higher than 70%, indicating that competitive processes did not have a large impact. A new reactor design enabled retention of MCFA producing biomass without retaining large biomass concentrations of acetotrophic methanogens. In this way, toxic chemical agents for inhibition of methanogens were no longer required. Finally, chain elongation based on municipal solid waste and ethanol was demonstrated, creating new possibilities to valorise municipal solid waste. In this thesis, a two-phase system (chapter 6) showed a higher MCFA productivity than a single phase system (chapter 5).

In this chapter, we would like to discuss the theoretical potential of the chain elongation process based on dissolved substrates. This discussion

is needed to investigate whether the currently observed volumetric productivity is reaching a limit or that major breakthroughs in volumetric productivity can be expected. Based on this analysis, it can be decided to perform research towards higher volumetric productivities or towards improvements in other aspects of chain elongation, such as steering in product formation. Moreover, we would like to discuss shortly alternative sources for expensive substrates ((diluted) ethanol and yeast extract) to make the chain elongation process more cost-effective. Finally, an application for selective production of heptanoate from municipal solid waste and ethanol is discussed. In chapter 3, the selectivity of heptanoate was only 23 percent. By improving this selectivity, heptanoate production could become more economical attractive.

7.2 Kinetic considerations of chain elongation to estimate its potential

7.2.1 Specific substrate utilization rate

To estimate the maximal volumetric productivity of the chain elongation process, we used microbial process kinetics. The first important observation is that the free biomass concentrations, observed during the complete research, described in this thesis never exceeded 0.6 gVSS l^{-1}, indicating that the chain elongation process does not require high biomass concentrations to obtain high volumetric MCFA production rates. In other words, the chain elongation process has a high specific substrate utilization rate. Based on the relation between attached and suspended (methanogenic) biomass in a anaerobic upflow filter (Show and Tay, 1999), we assume the

total biomass concentration in our reactor is twice the free biomass concentration. Using this assumption, we estimate a total biomass concentration of 0.3 g VSS l^{-1} and find a maximum specific substrate utilization rate of 58 mole e g^{-1}VSS d^{-1} (or 461 gCOD g^{-1}VSS d^{-1}) at 69 days of operation of the continuous reactor as reported in chapter 4. After recalculating the data in Table 1 of Kenealy and Waselefsky (1985)[7], we find a maximum specific substrate utilization rate of 42 mole e g^{-1}VSS d^{-1} (or 337 gCOD g^{-1}VSS d^{-1}) for *C.kluyveri*, which is in same order of magnitude as our work.

Acetotrophic methanogens have a lower specific substrate utilization rate of approximately 1 mole e g^{-1}VSS d^{-1} (8 gCOD g^{-1}VSS d^{-1}) (Pavlostathis and Giraldo-Gomez,1991). Hence, (acetate based) high rate methane producing reactors need larger concentrations of biomass. Consequently, systems with large biomass retention, such as UASBs, have been developed. Due to the high specific substrate utilization rate of MCFA producers, chain elongation does not necessarily require such reactors to obtain high MCFA production rates. Yet, better biomass retention can lead to higher volumetric MCFA production rates and better ethanol removal efficiencies. On the other hand, it could also lower the selectivity of the chain elongation process, because suppression of competitive processes is likely to be less effective in these high biomass retention systems, especially at relative longer HRTs. Therefore, it depends on the requirements of the user

[7] First, the volumetric production rate was calculated from the formed products (butyrate and caproate). Then, this value was divided by the biomass concentration, which was derived from the OD 660 measurements.

whether higher volumetric MCFA production rates and/or higher ethanol removal efficiencies or higher MCFA selectivities are required.

7.2.2 Estimated maximum growth rate, maintenance coefficient and yield of *C.kluyveri*

To make an estimation of maximum specific substrate utilization rate of chain elongation bacteria, estimations of the maximum growth rate, maintenance coefficient and yield of *C.kluyveri* have been made in this paragraph.

Using a Lineweaver-Burk plot of the chemostat results reported by Kenealy and Waselefsky (1985) (Figure 1), we derived a maximum growth rate for *C.kluyveri* of approximately 1.0 h^{-1} (24 d^{-1}), which is comparable to the maximum growth rate of *Clostridium acetobutylicum* (Dabrock *et al.*,1992; Napoli *et al.*, 2011). Kenealy and Waselefsky (1985) already stated that a higher growth rate of *C.kluyveri* can be achieved in continuous reactors than in batch cultures. According to them, at high growth rates (or low HRTs) some micronutrient seems to be limiting growth. CO_2 could also have been limiting the growth of *C.kluyveri* in their experiment as well, because they did not increase the gas flow rate (with 95% N_2 /5% CO_2) at higher dilution rates (or shorter HRTs).

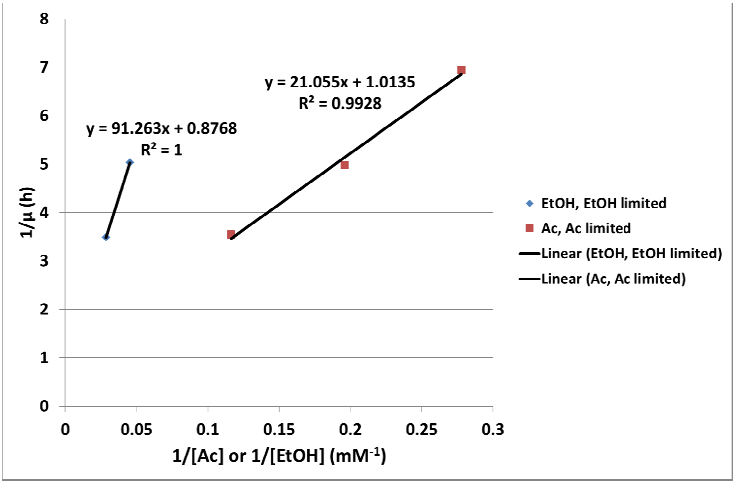

Figure 1. Lineweaver-Burk plots of C.kluyveri based on the data of Kenealy and Waselefsky (1985).

An estimated maintenance coefficient can be found by making a Tempest plot of the specific substrate utilization rate versus the dilution rate of their work (Figure 2). From the graph, we can extract a maintenance coefficient of approximately between 1.1 and 2.5 mole e g^{-1}VSS d^{-1} (or 9-20 gCOD g^{-1}VSS d^{-1}). This maintenance value is about 40 to 400 times higher compared the maintenance value of acetotrophic methanogens (0.05-0.25 gCOD l^{-1} d^{-1}) (Guiot *et al*., 1989) and indicates that at low dilution rates (or long HRTs) a larger percentage of the converted substrate is converted for biomass maintenance than at higher dilution rates (or shorter HRTs). Because of this high maintenance value of *C.kluyveri*, batch systems do not seem useful to study and improve the mixed culture chain elongation process.

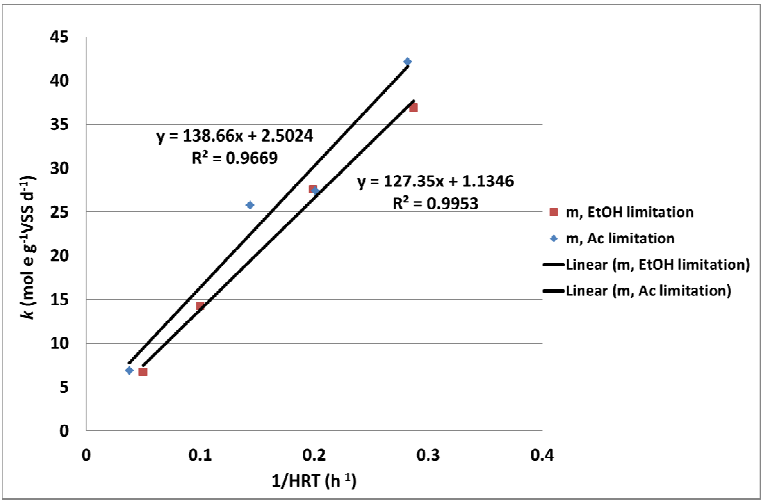

Figure 2. Tempest plots of C.kluyveri (based on data of Kenealy and Waselefsky (1985)) provide information to estimate the maintenance coefficient.

The final kinetic parameter we need to know to estimate the potential of the chain elongation process is the maximal yield. In the work of Kenealy and Waselefsky (1985), the yield was in the range of 0.14-0.26 gVSS per mole consumed electrons from ethanol. For our further estimations, we assume their average yield of 0.2 gVSS per mole consumed electrons from ethanol (0.05 gVSS g^{-1}EtOH).

7.2.3 Estimation of maximum specific substrate utilization rate

By combining the maximum growth, the maintenance coefficient and the yield of *C.kluyveri,* we can estimate a maximum specific substrate

utilization rate of 122 mol e g^{-1}VSS d^{-1} (or 976 gCOD g^{-1}VSS d^{-1}) for chain elongation by means of the Pirt equation (equation 3) (Guiot *et al.*, 1989).

$$k = \mu/Y + m \qquad (3)$$

where:

k is specific substrate utilization rate	(mole e g^{-1}VSS d^{-1})
μ is the maximum growth rate	(24 d^{-1})
Y is the yield of biomass from ethanol	(0.2 gVSS $mole^{-1}$e)
m is the maintenance coefficient	(2 mole e g^{-1}VSS d^{-1})

Because limited information about kinetic parameters is currently present, the used parameters and therefore also the estimation of the maximum specific substrate utilization rate could deviate from the given value here and should not be treated as a fact. Especially, a small deviation in the biomass yield (*Y*) has a large impact on the estimation of the specific utilization rate. Nevertheless, the estimated value can be a valuable tool to determine the potential of a gram MCFA producing biomass.

7.3 Estimation of maximum MCFA concentrations in the fermentation broth

Apart from specific substrate utilization rate, an estimate of the maximum MCFA concentrations in the fermentation broth is an important parameter to determine the potential of chain elongation. An important parameter in the maximum MCFA concentrations is the pH, due to product

toxicity. At acidic pH values (< pH 6.0), a considerable fraction of MCFAs is present in the unionized form (Intermezzo, chapter 5). These unionized MCFAs could be toxic for MCFA producers, which is probably one of the reason for the low MCFA productivity found in chapter 5 and in the work of Steinbusch (2010) at pH 5.5. Based on a maximum unionized carboxylic acid (Σ C2-C8) concentration of 30 mM (similar to chapter 5), a pKa of 4.8 and a pH in the fermentation broth of 5.5, the maximum total carboxylic acid (unionized and ionized) concentration in the fermentation broth could be approximately 180 mM. In case of an efficient fermentation, an assumption can be made that 75% of the total carboxylic acids is present as MCFAs. Hence, the maximum MCFA concentration could be approximately 135 mM.

By increasing the pH towards pH 7, the amount of unionized acids is reduced at the same total carboxylic acid concentration. Consequently, MCFA productivity is likely not limited anymore by unionized acids. Moreover, the growth rate of *C.kluyveri* is higher at near-neutral pH vales than at acidic pH values (Kenealy and Waselefsky, 1985; Weimer and Stevenson, 2012). However, at near-neutral pH values other factors could start limiting the maximum MCFA concentrations. In chapter 4, it was demonstrated that higher yeast extract concentrations were required to obtain high MCFA concentrations at short HRTs. Another factors that have not been addressed as consequence of high MCFA concentrations at near-neutral pH values are cation toxicity, product toxicity and /or salinity.

To keep the pH at near neutral values and maintain electron neutrality, the anions (mainly ionized carboxylic acids) needs to compensated

with cations, like Na^+ and K^+. Because the ionized carboxylic acids and cations are together in the same solution, determination whether reduced productivity is caused by high acid concentrations or by high cation concentrations can be difficult. Zoetemeyer *et al*., 1982 demonstrated that the maximum dilution rate (related to growth rate) of mixed culture acidification reactor decreased with increasing acid concentrations (while maintaining pH at 6.0). In Herrero *et al*., 1985, the growth rate of *Clostridium thermocellum* was reduced by more than 50% at a sodiumbutyrate concentrations of 200 mM. Kugelman and McCarty (1964) investigated the effect of cation toxicity on acetotrophic methanogens. They demonstrated the reaction rate was reduced by 50%, when 300 mM sodium or 150 mM potassium was present.

Although the mentioned reference do not provide direct evidence that chain elongation is also affected by high cation concentrations, high carboxylic acid concentrations and/or salinity, it is likely that these aspects will limit the MCFA productivity. Another indication for this argument is that no report has ever mentioned the presence of *C.kluyveri* is marine sediment (± 500 mM NaCl) or saline sediments (> 500 mM NaCl). However, Kenealy and Waselefsky (1985) were able to obtain a *C.kluyveri* strain from brackish sediments. The maximum caproate concentration that Weimer and Stevenson (2012) achieved in batch bottles was 110 mM with a *C.kluyveri* strain derived from a cow rumen, while the total acid concentration was 170 mM. Based on the presence of *C.kluyveri* in brackish sediments, an estimated maximum total carboxylic acid concentration of 300 mM might be feasible. In case also the assumption is made that 75% of the total carboxylic acids is present as

MCFAs, the estimated total MCFA concentration is 225 mM. Because these maximum concentrations have not been reported (yet), this analysis is highly speculative. Future research is required to determine the real maximum MCFA concentration.

In this discussion, the effect of in-situ product removal is not incorporated, although several investigations have demonstrated that in-situ product removal improves MCFA productivity (*e.g.* Roddick and Britz, 1997; Agler *et al*., 2012). Main reason for excluding these in-situ product removal techniques in this discussion is the current unknown application to use the produced MCFAs. For some applications, such as PHA production, there is no need to apply an expensive and complicated in-situ product removal techniques.

7.4 Alternative substrates for ethanol and yeast extract

To make the chain elongation process more cost-effective, alternatives for expensive substrates should be investigated. In our research, we consider (diluted) ethanol and yeast extract as expensive substrates, which should be reduced and/or replaced to improve cost-effectiveness of the process.

Although the chain elongation process can use diluted ethanol, ethanol is more expensive than VFAs and by replacing ethanol with a cheaper substrate the chain elongation process becomes more cost-effective. One candidate to replace ethanol is methanol. Caproate formation from methanol has been demonstrated with the anaerobic methylotrophic bacterium

Eubacterium limosum (Genthner *et al.*, 1981). However, it is not clear if high rate caproate formation from methanol in a mixed culture is possible (> 4 g C6 l^{-1} d^{-1}). Methanol is cheaper than ethanol and is produced from various sources, including natural gas, syngas and biogas. Alternatively, methanol can be produced from carbon dioxide and hydrogen. In case hydrogen is produced from renewable electricity, methanol based chain elongation could have a low environmental impact.

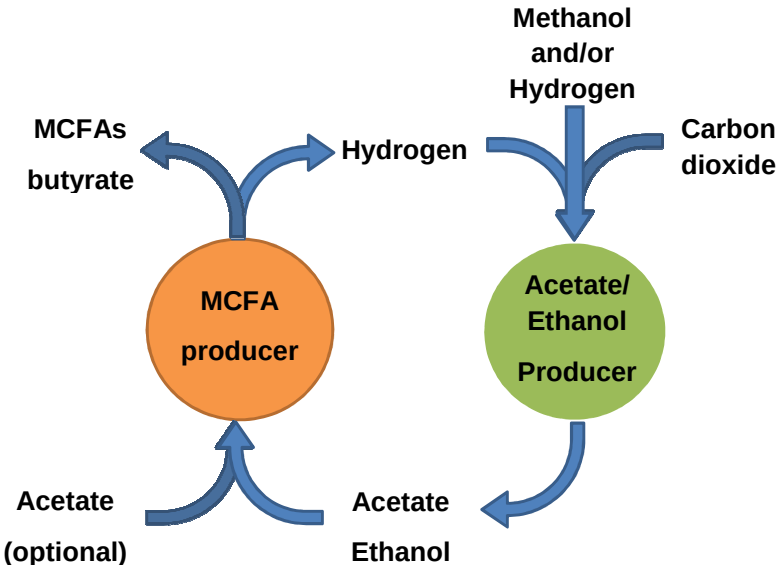

Figure 3. Possible consortium to replace ethanol with methanol and/or hydrogen/carbon dioxide

Instead of using methanol to replace ethanol, ethanol could also be replaced with a H_2/CO_2 mixture. In such a process, carbon dioxide and

hydrogen are converted by a first group of anaerobic bacteria into acetate and/or ethanol, while chain elongation is performed by a second group of anaerobic bacteria (*Figure 3*). In chapter 4, it was indicated that such a consortium could exist in a chain elongation reactor. Conversion of a H_2/CO_2 mixture into ethanol has been demonstrated with several anaerobic bacteria, including *Clostridium Ljungdahlii* (Ljungdahl, 1986; Drake and Daniel, 2004; Köpke *et al*., 2010). The use of a H_2/CO_2 mixture to replace ethanol requires two additional major challenges, being (i) the mass transfer of hydrogen to the water phase and (ii) the competition for hydrogen in a mixed culture with other groups of bacteria, especially hydrogenotrophic methanogens and sulphate reducers (*e.g.* Cord-Ruwisch *et al*., 1988). The mass transfer of hydrogen to the water phase can solved by using a gas lift reactor system (*e.g.* van Houten *et al*., 1997), which is a more complex reactor system than the upflow anaerobic filter used in this thesis. Competitive processes for hydrogen could be avoided by heat shocks (*e.g.* Steinbusch *et al*., 2009) and controlling the sulphate addition. Heat shocks can eliminate hydrogenotrophic methanogens, while a controlled sulphate addition limits hydrogen consumption by sulphate reducers. However, heat shocks could be costly on industrial scale, so alternative methods to reduce competitive processes need to be developed.

To reduce or even replace yeast extract, the other expensive substrate, more research is required to determine the nutrient requirements of the MCFA producers or the complete consortium in the chain elongation reactor. Especially at short HRTs (Chapter 4), when the highest yeast extract concentration was used, the protein formation rate for synthesis of MCFA

producing biomass could have been limiting the MCFA producing biomass concentration and possibly also the volumetric MCFA production rate. By performing chain elongation with an influent that contains (dead) microbial biomass (*e.g.* Chapter 6; Agler *et al*., 2012), yeast extract addition did not seems to be necessary, possibly due to lysis of the added (dead) microbial biomass. However, long term research is required to proof if yeast extract can be completely replaced by (dead) microbial biomass, especially at short HRTs.

7.5 Heptanoate production from OFMSW in combination with methanogens

A mixture of VFAs elongated with ethanol (Chapters 3, 5 and 6) leads to a mixture of MCFAs and consequently to lower specific product selectivities (either caproate, heptanoate or caprylate). This mixture of MCFAs is likely to result in lower MCFA concentrations, which could make MCFA separation from the fermentation broth less effective. Because only limited MCFA concentrations can be achieved due to cation toxicity, product inhibition and/or salinity, a combined production with high concentrations of caproate, heptanoate and caprylate might be difficult to obtain. Depending on the user requirements, selectivity heptanoate production may need to be developed. Here, we discuss two-stage heptanoate production from OFMSW and ethanol in combination with (acetotrophic and hydrogenotrophic) methanogens. *Figure 4* gives an schematic overview of the two-stage heptanoate production process.

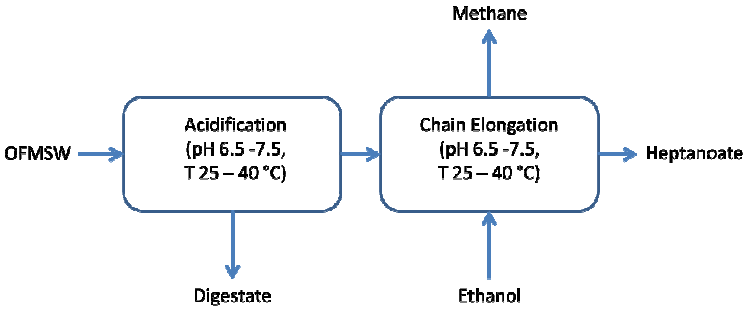

Figure 4. Schematic overview of the two-stage heptanoate production process.

Although heptanoate production from propionate and ethanol proceeds slower than caproate production from acetate and ethanol (4.5 g C7 l^{-1} d^{-1} vs 15.7 g C6 l^{-1} d^{-1} (chapters 2 and 3)), heptanoic acid has a lower solubility in water than caproic acid (2.4 g C7 l^{-1} vs. 10 g C6 l^{-1}) and therefore it is easier to separate from the fermentation broth than caproic acid. In case of caproate/caprylate production, acetotrophic methanogenesis is a competitive process that consumes a substrate for chain elongation (acetate). However, methanogenesis is not a competitive process in heptanoate production, because acetate is not a substrate for heptanoate production. Moreover, acetate consumption by methanogens can be beneficial in heptanoate production, because the acetate consumption reduces ethanol consumption for the production of butyrate and caproate.

Due to the avoidance of ethanol consumption towards butyrate and caproate, ethanol is directed more selectively towards valerate and

heptanoate, resulting in a higher heptanoate selectivity than 23% (chapter 3). Keep in mind that it remains important to monitor the partial hydrogen pressure to avoid (i) excessive ethanol oxidation to acetate, (ii) heptanoate oxidation to propionate, (iii) propionate oxidation to acetate and (iv) reduce propanol formation from propionate reduction, which could occur at elevated partial hydrogen pressures (pH_2 > 0.1 atm). Regulation of carbon dioxide addition could be used to heavily manipulate hydrogen consumption by hydrogenotrophic methanogens.

To produce heptanoate, selective propionate production from OFMSW is required during the acidification phase. To produce propionate selectively, it is important to reduce butyrate formation from OFMSW. A method to achieve selective propionate production is by maintaining the pH between pH 6.5 and 7.5 and keep the temperature in the mesophilic range (25 - 40°C). Nagao *et al*. (2012) was able to produce a mixture of VFAs from food waste of which 70% consisted of propionate. The dominant remaining VFA of the mixture was acetate with 20%. By applying acetotrophic methanogens to consume the acetate, a selective propionate stream can be obtained.

This high propionate stream can be fed to the second phase in which ethanol is added to elongate the propionate to heptanoate selectively. At this stage, it is unknown how selectivity this heptanoate production can be, although it will be less than 85% (maximum caproate selectivity) due to the combination of ethanol oxidation to acetate and acetate conversion into methane. In this second phase, a similar pH is maintained as in the first phase. Besides acetate consumption, methanogens could provide another

benefit to the reactor system. Due to lysis, methanogens can provide growth factors and/or other nutrient, which could reduce yeast extract additions to the chain elongation reactor.

Although heptanoate production from OFMSW and ethanol in combination with methanogens uses a smaller part of the OFMSW than combined MCFA production (chapter 6), heptanoate production in combination with methanogens offers the potential to use the ethanol more efficiently than combined MCFA production without methanogens. Whether it is more attractive to produce heptanoate than a mixture of MCFAs is unknown at this moment. Nevertheless, heptanoate could be used for more specialized applications, such as lubricant production for low-temperature applications. Additionally, less alternative processes are available for heptanoate production via chain elongation than for caproate production via chain elongation, because caproate can also be produced from glucose.

Summary

To reduce dependence on oil, alternative fuel and chemical production processes are investigates. In this thesis, we investigated the production of medium chain fatty acids (MCFAs) using an anaerobic chain elongation process from the organic fraction of municipal solid waste (OFMSW) and (diluted) ethanol. MCFAs, monocarboxylic acids up to a chain length of eight carbon atoms, can be used directly as antimicrobial and corrosion inhibitor or indirectly as biobased building blocks for the production of biodiesel, bioplastic and other biochemicals. By using OFMSW for the production of MCFAs, OFMSW can be valorised. Moreover, the food-fuel discussion can be avoided, as long as the ethanol is not produced from food resources.

To produce MCFAs from OFMSW and ethanol, volatile fatty acid (VFA) production from OFMSW is required, because the MCFA producing bacteria elongate the VFAs together with ethanol into MCFAs. MCFAs are less soluble in water than VFAs and ethanol and therefore separation of MCFAs from fermentation broth is likely easier than separation of VFAs and/or ethanol from fermentation broths.

In this thesis, a mixed culture chain elongation process was investigated to avoid a costly sterilization step. As a consequence, the mixed culture chain elongation process requires methods to reduce the impact of competitive bacteria. Acetotrophic methanogenesis was considered to be the most important competitive process, because acetate (VFA with two carbon

atoms) is a substrate for both acetotrophic methanogenesis and chain elongation.

In **chapter 2**, selective acetate elongation was investigated in an upflow anaerobic filter. To limit the impact of acetotrophic methanogenesis, the reactor was operated at a short hydraulic retention time (HRT, 17 h HRT) and a large upflow velocity (> 1 m h^{-1}). In the upflow anaerobic filter biomass retention is biomass retention is lower than in an upflow anaerobic sludge blanket (UASB). Because chain elongating bacteria, such as *Clostridium kluyveri*, are fast growing bacteria (μ_{max} > 0.1 h^{-1}) and acetotrophic methanogens are slow growing bacteria (μ_{max} < 0.02 h^{-1}), non-attached acetotrophic methanogens can be washed-out by a short HRT. To reduce the impact of attached competitive bacteria, a large upflow velocity was used to create a shear stress that limits the size of the methanogenic biofilm. Additionally, chapter 2 demonstrated that an aqueous stream with an ethanol percentage of less than 2.5% (v/v) is sufficient for the chain elongation process. By reducing the HRT, the caproate (C6) productivity was improved by more than 7 times. The caprylate (C8) productivity was even improved by more than 16 times.

In **chapter 3**, the majority of the acetate was replaced by propionate to test whether selective heptanoate (C7) production was feasible in an upflow anaerobic filter. The results demonstrated that heptanoate production from propionate and ethanol has potential to compete with heptanoate production from castor oil, although future research is required to improve the heptanoate production using chain elongation. The heptanoate productivity was improved

by more than 25 times compared to previous continuous propionate elongation work. Moreover, the heptanoate concentration was higher than the solubibity of heptanoic acid in water (standard conditions). However, the heptanoate selectivity was rather low (23%) and needs improvements.

In **chapter 4**, it was demonstrated that chain elongation can achieve industrial acceptable production rates (> 2 g MCFA l^{-1} h^{-1}) by reducing the HRT to 4 hours. To obtain these high production rates, extra carbon dioxide additions and higher yeast extract concentrations were necessary. At the longest HRT (16h HRT), the MCFA selectivity was 94 per cent, probably due to a hydrogen recycling system in which the produced hydrogen (by MCFA producers) is converted together with carbon dioxide into acetate and/or ethanol. Because high yeast extract concentrations were required at shorter HRTs, more research in continuous reactors is required to determine the essential components of yeast extract for MCFA producers.

The **intermezzo** is the introduction for the production of MCFAs from OFMSW instead of synthetic media (Chapters 2,3 and 4). In this intermezzo, the effect of pH on hydrolysis and acetotrophic methanogens during dry acidification of OFMSW was investigated. The results demonstrated that a low pH (< pH 5.5) in combination with undissociated VFAs can prevent acetate consumption for a period longer than 21 days. At a pH of 5.5, the lag phase of the acetotrophic methanogens was extended compared to the lag phase of the acetotrophic methanogens at a pH of 6.0, but acetate consumption was not prevented. Although a low pH (< pH 5.5)

combined with the undissociated VFAs prevented acetate consumption, the hydrolysis rate and consequently the VFA production were also affected.

In **chapter 5**, ethanol was added during the acidification of OFMSW to produce MCFAs in a single stage system. The ethanol additions promoted the MCFA production, but also affected the hydrolysis rate either by undissociated MCFAs and/or high ethanol concentrations. Another interesting aspect was the preference of the MCFA producers to elongate propionate, butyrate and valerate rather than acetate.

In **chapter 6**, a two stage system was investigated for the production of MCFAs from OFMSW and ethanol. In the two stage system, a higher MCFA productivity was achieved than in the single phase system of chapter 5, probably due to avoidance of undissociated MCFAs and/or ethanol toxicity effects on hydrolysis. Hydrolysis was still the rate limiting factor in the two stage system and requires further improvements. An additional advantage of the two stage system over the single phase system is the easier control of competitive processes, such as excessive ethanol oxidation and acetotrophic methanogenesis.

In the **discussion**, kinetic considerations on chain elongation are presented as well as an estimation of the maximum MCFA production rate without in-situ product removal. Additionally, recommendations are given to reduce ethanol and yeast extract, which are (currently) the most expensive substrates in chain elongation. Finally, a case is presented to produce heptanoate selectively from OFMSW and ethanol.

Samenvatting

Om de afhankelijkheid van olie te verminderen worden alternatieve brandstof en chemicaliën productieprocessen onderzocht. In dit proefschrift is de productie van middellange vetzuurketens (MCFAs) uit groente, fruit en tuinafval (OFMSW) en ethanol onderzocht door middel van een anaeroob ketenverlengingsproces. MCFAs, vetzuren met één zuur groep en een ketenlengte tot acht koolstof atomen, kunnen direct gebruikt worden als conserveringsmiddel of corrosie werende chemicaliën, daarnaast kunnen ze ook indirect gebruikt worden als bouwsteen voor biodiesel, bioplastic en biochemicaliën. Door het gebruikt van OFMSW in de productie van MCFAs kan OFMSW gebruikt worden voor meer waardevollere toepassingen. Bovendien kan ook de voedsel-brandstof discussie vermeden worden zolang ethanol niet uit voedsel geproduceerd wordt.

Om MCFA te produceren vanuit OFMSW en ethanol is de productie van korte vetzuurketens (VFAs) uit OFMSW noodzakelijk, omdat de MCFA producerende bacteriën VFAs verlengen met ethanol naar MCFAs. Omdat MCFAs zijn minder goed oplosbaar in water dan VFAs en ethanol, is het waarschijnlijk makkelijker om MCFAs te scheiden van fermentatiemengsels dan de scheiding van VFAs en ethanol uit fermentatiemengsels.

In dit proefschrift is een gemengd bacterie cultuur ketenverlengingsproces onderzocht om een dure sterilisatiestap te vermijden. Als gevolg daarvan is het noodzakelijk om methodes te ontwikkelen om de invloed van concurrerende processen te verminderen. Methaan productie uit

acetaat werd beschouwd als belangrijkste concurrerend proces, omdat acetaat (VFA met twee koolstof atomen) een substraat is voor zowel methaan productie uit acetaat als voor ketenverlenging.

In **hoofdstuk 2**, selectieve acetaat verlenging was onderzocht in een omhoog stromende anaerobe filter (reactor). Om de invloed van methaan productie uit acetaat te beperken, de reactor werd geopereerd met een korte hydraulische verblijft tijd (HRT, 17uur HRT) en met een hoge opstroomsnelheid (> 1 m uur^{-1}). In de omhoog stromende anaerobe filter is de biomassa retentie lager dan in een omhoog stromende anaeroob slib deken (UASB). Omdat ketenverlengende bacteriën, zoals *Clostridium kluyveri*, snel groeiende bacteriën zijn (μ_{max} > 0.1 uur^{-1}) en acetaat consumerende methaan producerende bacteriën langzaam groeiende bacteriën zijn (μ_{max} < 0.02 uur^{-1}), kunnen de niet-vastzittende (of vrije) acetaat consumerende methaan producerende bacteriën uitgespoeld worden doormiddel van een korte HRT. Om de invloed van de vastzittende concurrerende bacterien te verminderen, een hoge opstroomsnelheid werd gebruikt om een wrijvingskracht uit te oefenen, die het grootte van het biofilm met methaan producerende bacterien beperkt. Daarnaast toonde hoofdstuk 2 aan dat een water stroom met een alcoholpercentage lager dan 2.5 % (vol.%) voldoende is het voor ketenverlenging. Door de HRT te verminderen werd de capronaat(C6) productiviteit verbeterd met meer dan 7 keer. De caprylaat (C8) productiviteit werd zelfs verbeterd met meer dan 16 keer.

In **hoofdstuk 3** werd de meerderheid van de acetaat vervangen door propionaat om te testen of selectieve heptanoaat (C7) productie haalbaar

was in de omhoog stromende anaerobe filter. De resultaten lieten zien dat heptanoaat productie uit propionaat en ethanol voldoende potentie heeft om te concurreren met heptanoaat productie uit castor olie, hoewel toekomstig onderzoek vereist is om de heptanoaat productie met behulp van ketenverlenging te verbeteren. Vergeleken met vorig propionaat verlengings onderzoek in continue reactoren was de heptanoaat productiviteit verbeterd met meer dan 25 keer. Bovendien was de heptanoaat concentratie hoger dan de oplosbaarheid van heptaanzuur in water (onder standaard condities). Echter, the heptanoaat selectiviteit was nogal laag (23%) en vereist verbeteringen.

In **hoofdstuk 4** werd aangetoond dat ketenverlenging industrieel aanvaardbare productie snelheden (> 2 g MCFA l^{-1} uur^{-1}) kan bereiken door de HRT te verlagen naar 4 uur. Om deze hoge productie snelheden te behalen waren extra koolstofdioxide en hogere gist extractie concentraties nodig. Tijdens de langste HRT (16h HRT), MCFA selectiviteit was 94 procent waarschijnlijk door een waterstof hergebruik mechanisme waarin het geproduceerde waterstof (door MCFA producerende bacterien) wordt omgezet met koolstofdioxide in acetaat en/of ethanol. Omdat hoge gist extractie concentraties vereist waren op korte HRTs is er meer onderzoek nodig naar de essentiele componenten uit het gist extract voor MCFA producerende bacterien te bepalen.

Het **intermezzo** is een introductie voor de productie van MCFAs uit OFMSW in plaats van synthetisch media (Hoofdstukken 2,3 en 4). In dit intermezzo is het effect van pH op zowel de hydrolyse als op de acetaat

consumerende methaan producerende bacterien tijdens droge verzuring van OFMSW onderzocht. De resultaten laten zien dat een zure pH (< pH 5.5) in combinatie met ongedissocieerde VFAs kan de acetaat consumptie voor een periode langer dan 21 dagen voorkomen. Bij een pH van 5.5 was de vertragende periode van acetaat consumerende methaan producerende bacterien langer dan bij een pH van 6.0, maar acetaat consumptie werd niet voorkomen. Hoewel een lage pH (< pH 5.5) in combinatie met ongedissocieerde VFAs acetaat consumptie voorkomt, werden de hydrolyse en daarmee samenhangend de VFA productie ook negatief beïnvloed.

In **hoofdstuk 5** ethanol werd toegevoegd tijdens de verzuring van het OFMSW om MCFAs te produceren in enkel reactor systeem. De ethanol toevoegingen stimuleerde de MCFA productie, maar zorgde ook voor een negatieve invloed op de hydrolyse door de giftigheid van ongedissocieerde MCFAs en/of hoge ethanol concentraties. Een ander interessant aspect was de voorkeur van de MCFA producerende bacterien om propionaat, butyraat en valeraat te verlengen in plaats van acetaat.

In **hoofdstuk** 6 werd een twee reactor system onderzocht voor de productie van MCFAs uit OFMSW en ethanol. In het twee reactor system werden hogere MCFA productie snelheden bereikt dan in het enkele reactor system uit hoofdstuk 5, waarschijnlijk door de toxiciteits effecten van ongedissocieerde MCFAs en/of ethanol op de hydrolyse te vermijden. Desalniettemin was hydrolyse nog steeds snelheid limiterende factor in het twee reactor systeem en vereist toekomstige verbeteringen. Een bijkomend voordeel van het twee reactor systeem ten opzichte van het enkel reactor

systeem is de simpelere controle van concurrerende processen, zoals overmatige ethanol oxidatie en acetaat consumerende methaan productie.

In de **discussie** worden kinetische beschouwingen op ketenverlenging en een schatting van de maximale MCFA productie snelheid zonder in-situ verwijdering technieken gepresenteerd. Daarnaast worden aanbevelingen gegeven om de ethanol en de gist extract concentraties te verminderen; dit zijn de duurste substraten in ketenverlenging. Tenslotte is er een voorstel gepresenteerd om selectief heptanoaat te produceren uit OFMSW en ethanol. Dit zou heptanoaat productie economisch meer aantrekkelijk kunnen maken.

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List of publications

Grootscholten, T.I.M., Steinbusch, K.J.J., Hamelers, H.V.M., Buisman, C.J.N. 2013. Improving medium chain fatty acid productivity using chain elongation by reducing the hydraulic retention time in an upflow anaerobic filter. Bioresource Technology, 136, 735-738.

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- Wetsus Rabobank Water Business Challenge
- Advanced Course Downstream Processing
- Scientific writing course

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- *Medium Chain Fatty Acid production from the organic fraction of municipal solid waste.* SENSE "Microbes for Sustainability" symposium, Wageningen University, 5-4-2012, Wageningen, The Netherlands
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