Liquid biofuel production from volatile fatty acids

Kirsten J.J. Steinbusch

Thesis committee

Thesis Supervisor
Prof. dr. ir. C.J.N. Buisman
Professor of Biological Recycling Technology

Thesis (Co) Supervisors
Dr. ir. H.V.M. Hamelers
Assistant Professor, sub-department of Environmental Technology

Other members
Prof. dr. ir. A.J.M. Stams, Wageningen University
Prof. dr. J.P.M. Sanders, Wageningen University
Prof. dr. L. Diels, University of Antwerp/ VITO, Belgium
Dr. ir. R.J. Zoetemeyer, PURAC

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Thesis

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Kirsten J.J. Steinbusch
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Index

1 General introduction

1.1 Societal demand for sustainable replacement for oil

Environmental and economic drivers

Due to increased human economic activity since the Industrial Revolution, we momentarily face two major global constraints to further worldwide economic development, both of which are related to burning fossil fuel. The first global constraint is warming of the Earth's surface, an indirect effect of burning fossil fuels. By burning captured carbon from deeper earth layers, carbon dioxide is released into the atmosphere and accumulates there. Accrual of carbon dioxide, a greenhouse gas, is one of the major causes of global warming, which is evident by the observed increases in global average air and ocean temperatures, widespread melting of snow and ice and rising global average sea level (IPCC, 2007). The effects of climate change on the natural and human systems are difficult to determine, but can result in, for example, changes in temperature and precipitation patterns that affect both water supply and food security, and decrease in biodiversity due to extinction of species that cannot adapt fast enough to the change in weather patterns. To limit or diminish the effects of global warming, carbon dioxide emissions should be reduced by employing alternative energy resources that replace fossil fuels such as oil, gas or coal.

The second economical global constraint is the limited amount of accessible fossil oil for use as an energy resource. Fossil oil was responsible for 42.6% of the world energy consumption of 347 EJ in 2007 (IEA, 2009), indicating its key position within international energy budgets. According to the latest prognosis of the International Energy Agency, it is expected that, with growing world population and increase of welfare, the energy consumption in 2030 will increase 20 to 40% depending on economic growth and political

interventions (IEA, 2009). Oil production is expected, though, to have reached its peak before 2030, because the global supply of 'conventional oil' will be constrained by physical depletion (Sorrell and Speirs, 2009). Large resources of conventional oil may still be available, but these are unlikely to be accessed quickly and may make little difference to the timing of the global oil peak. Thus, based on present oil consumption patterns it can be concluded that there will be a shortage in crude oil supply somewhere in the near future.

The limited supply of oil will drastically affect two sectors that almost completely rely on fossil oil as a primary resource: the transport sector and the chemical industry. Transportation is a significant energy consuming economic activity, which accounted for 26% of the world energy consumption in 2007, equal to 90.4 EJ. This accounts to 61.2% of total world oil consumption (IEA, 2009). At 16.8%, the chemical industry uses less oil, but it is expected that this percentage will grow (IEA, 2009). Since the demand for energy will increase, other resources such as natural gas, coal, nuclear, geothermal heat and renewables will become progressively more important to the energy supply. Demand for mobility will worldwide increase, leading to an expected 2.1% annual growth in the transport sector. Thus, the need for alternative resources to sustain the current demand and to support growth within the transport sector and chemical industry is evident.

As a result of environmental and economic concerns, legislation has been formulated to stimulate the production of renewable fuels from biomass as an alternative to oil. An EU directive states a minimum share of renewable fuel of 10% in 2020 for all member states (2003). The US stipulate expanded biofuel production in the Energy Independence and Security Act (2007), which requires national annual biofuel production of 79 billion liters in 2022. Brazil has set by far the highest goals concerning biofuel use in the transportation industry. Since July 2007, it is mandatory for light vehicles to use a blend of 25% anhydrous ethanol with 75% gasoline per volume (Stephanes, 2007).

First generation biofuels

Amongst the renewable fuels, bio-ethanol and bio-diesel are produced on a large scale. Primarily driven by government policies, world ethanol production

for transport fuel tripled between 2000 and 2007 from 17 billion to more than 52 billion liters, while biodiesel expanded eleven-fold from less than 1 billion to almost 11 billion liters (Bringezu et al., 2009). These sources together fueled 1.5% of the world's transport in terms of energy units (1.42 EJ)(IEA, 2009). Ethanol can be blended with gasoline as an oxygenate (MTBE) or a fuel extender for use in gasoline vehicles; or it can be used alone in "flexible-fuel vehicles" that run on any blend of ethanol and gasoline. Bio-ethanol is mainly produced from sugar containing biomass, most commonly sugar cane, corn, grain or other sugar crops. In large scale fermentation processes, sugar is converted by yeasts to ethanol. Bio-diesel consists of fatty acid methyl esters FAMEs or fatty acid ethyl esters FAEEs and can be blended with normal diesel. Bio-diesel is produced by the trans-esterification of oil or fats from biological origin with methanol, yielding FAMEs and glycerol as a by-product. Currently, bio diesel is mainly produced from vegetable oil from plant seeds.

Sustainability

Sustainability and environmental issues have been raised in response to the large scale production and use of conventional biofuels (Williams et al., 2009). First generation biofuel production from sugar-containing crops or seed oils competes for arable land with food production, have low energy efficiency, requires high rates of chemical and energy input (e.g. fuel, fertilizers and pesticides) and reduces biodiversity (Dias De Oliveira et al., 2005; Engelhaupt, 2007; Groom et al., 2008). Sugar and vegetable oil can be used for fuel production, but are also sources of food for humans and livestock. This is an important drawback, given the fact that food scarcity is a serious problem in certain geographical areas and that the prices of the feedstocks will rise due to increasing demand for biofuel production (OECD-FAO, 2008). The land required to produce enough starting material to supply worldwide biofuel demand would be so large that this will compete locally with land required for food production and nature resources (Durrett et al., 2008). Finally, high energy input in the large scale biomass production and production processes of biofuels, for example the ethanol distillation, are considered to limit the realized benefit of emissions reductions (Dias De Oliveira et al., 2005).

1.2 Feedstock choice for sustainable biofuels

The sustainability of biofuels or chemicals is substantially determined by the type of biomass feedstock used (Dias De Oliveira et al., 2005; Tilman et al., 2009). Consequently, the EU directive added to the biofuel directive that the biomass used for biofuel production should be produced sustainably. According to the Dutch government, sustainable biomass should fulfill criteria that are defined for "greenhouse gas balance; competition with food, local energy supply, medicine and building materials; biodiversity; economic prosperity; social well-being and environment" (Cramer, 2006).

Tilman et al. (2009) distinguished five biomass feedstocks that comply with the sustainability criteria and thus can be used to produce substantial quantities of biofuels. The five biomass feedstocks are:

1. Perennial plants grown on degraded lands abandoned by agriculture
2. Crop residues
3. Sustainably harvested wood and forest
4. Double crops and mixed cropping systems
5. Municipal and industrial organic waste

The aforementioned biomass feedstocks have a high energy content and are abundant in society (Perlack et al., 2005). In this thesis, we focus on the employment of the latter biomass feedstock for chemical and fuel production. This type of waste can generally not be used for any food of feed applications.

In summary, biomass is also a limited resource; and it is a challenge to convert biomass in an efficient manner. Efficiency can be defined in terms of carbon or energy recovery of biomass in the final product. The chemical industry aims at recovering as much as carbon from biomass to replace oil as resource, whereas the transport sector focuses on a high energy recovery. Without oil, the chemical industry fully relies on biomass as a carbon resource. In contrast, the transportation industry can diversify and use other sustainable resources than biomass such as solar or wind energy. A careful analysis should reveal for which purpose biomass should be used, either for biofuel or chemical production (Brehmer, 2008). This thesis studies biomass conversions that recover both carbon and energy content of biomass as much as possible to serve both sectors. Since biofuels have a larger and emerging market than chemicals, the conversion process in this thesis is focused on the

production of biofuels. The low value of biofuel compared to some chemicals requires a cheap and simple production process. Eventually, the final fuel product can still will be used for chemical application depending on the market.

1.3 Technological challenges to convert waste

Using biomass waste for fuel production requires a large change in chemical structure. Biomass waste varies in composition but is mostly solid, polar and often has a high water content. Fuels, conversely, are non-polar, fluid and have hydrocarbon chains with 5-20 carbon atoms depending on the type of fuel (Petrus and Noordermeer, 2006) (Table 1.1). Whereas fuels contain no oxygen in their structure, biomass, such as carbohydrates, contains one oxygen atom for each carbon atom. Converting biomass like hemicellulose $[C_5(H_2O)_4]_n$ into hydrocarbons requires removal of oxygen and addition of hydrogen.

Table 1.1 Properties of carbohydrates, and hydrocarbon fuels as gasoline, diesel and gasoil.

	Carbohydrate	*Gasoline*	*Diesel-Gasoil*
Structure	Linear/cyclic	branched/aromatic cyclic/unsaturated	linear/ saturated
C	$(5\text{-}6)_n$	5-10	12-20
O/C molar ratio	1	0	0
Phase behaviour	Solid	liquid	liquid
Polarity	Polar	non-polar	non-polar

To complicate matters, waste is comprised not only of carbohydrates, but of a mixture of solid and dissolved organic polymers including proteins, lipids, carbohydrates and organic acids. The high water content makes thermal conversion economically unfeasible. Similarly challenging is the biological conversion of structurally diverse components into a single precursor for biofuel with minimum loss of carbon or energy value of the original waste. The conversion requires most likely multiple conversion and/or separation steps, but the process should be cheap, energy efficient, robust and require low chemical input.

Anaerobic mixed culture fermentation can deal with the varied composition of wet waste and convert this mixture of components in an energy efficient manner into one product. The advantage of mixed culture fermentation is that it requires no energy input for sterilization and, owing to its ability to deal with a wide variety of biomass, it is robust, stable and inexpensive. The most widely applied anaerobic mixed culture fermentation is anaerobic digestion. During digestion, organic material is converted to biogas, a mixture of methane and carbon dioxide. The biogas produced contains up to 85-90% of the input energy and is easily separated from the liquid slurry, making anaerobic digestion a very energy efficient process (Metcalf & Eddy, 2003). Current developments on the energy market are a drive to innovate anaerobic digestion of waste streams and to invent new fuel or chemical production processes to provide an alternative to fossil fuels (Kleerebezem and van Loosdrecht, 2007).

In anaerobic digestion, four conversion steps can be distinguished; hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1.1). After each step, the biomass is converted to a smaller molecule by different types of microorganisms. To produce liquid fuels from wet waste, part of these anaerobic digestion steps can be used.

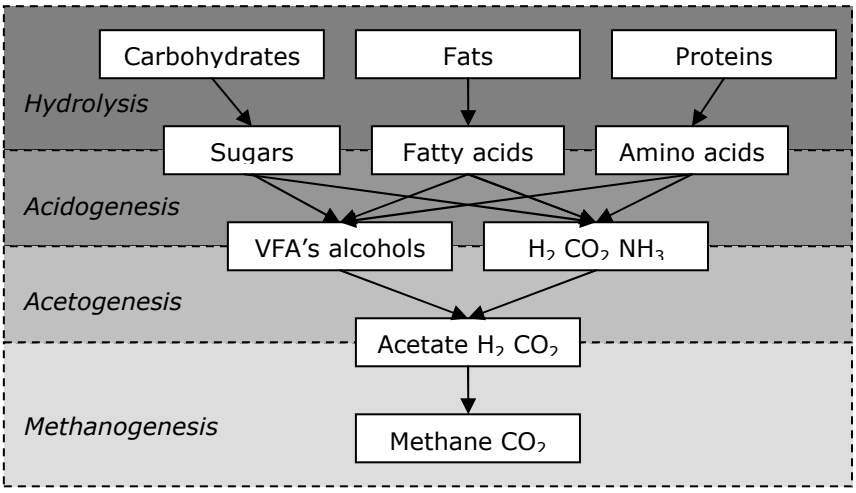

Figure 1.1 Process steps during anaerobic conversion of biopolymers to biogas.

During hydrolysis, polymers are enzymatically converted into smaller polymers by enzymes that are excreted by fermentative bacteria. Acidogenesis is the process by which the smaller dissolved polymers are assimilated by the fermentative bacteria and converted to monomers like alcohols, hydrogen, CO_2 and volatile fatty acids such as acetate, propionate, *n*-butyrate and valerate. During acetogenesis, the acidogenesis products are converted to acetate, hydrogen and carbon dioxide. The last step of digestion, methanogenesis, converts acetate, hydrogen and carbon dioxide to biogas. For liquid fuel production, these volatile fatty acids (VFA) are suitable molecules to use for further fuel conversion. VFA are key molecules within anaerobic digestion: each polymer, a lipid, a protein or a carbohydrate, is converted via VFA to biogas. VFA are involved in many anaerobic conversions. Concluding from this, VFA would be good building blocks for fuel and chemical production, as long as methanogenesis can be prevented.

1.4 VFA as building block: a sustainable alternative

1.4.1 Introduction

Volatile fatty acids (VFA) are introduced to serve as a platform molecule for liquid fuel or chemicals production. Using VFA as a building block has three major advantages for the production of fuel and chemicals. First, VFA offers flexibility of feedstock choice, because a variety of cheap and abundantly present biomass waste and residues can be used as a sustainable resource. Second, the technology used for VFA production is robust, inexpensive and most importantly requires less energy and chemical input. Finally, by converting VFA to liquid fuels such as alcohols, more carbon is recovered than converting VFA to methane.

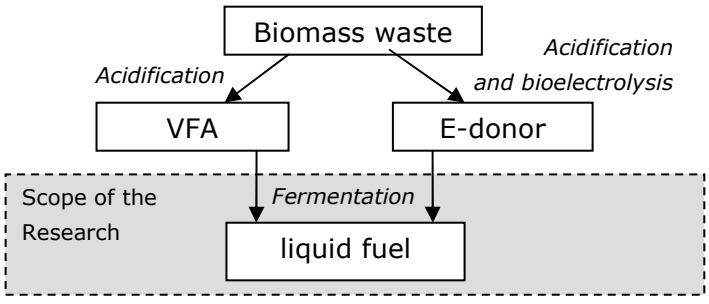

Figure 1.2 Overview of liquid biofuel production from biomass waste via VFA as a building block and hydrogen from electricity as the electron donor.

The following sections describe the production process from waste to VFA in more detail and the further conversion of VFA to fuels with the use of electron donors. The production of required electron donors such as hydrogen and electrons from the same waste as VFA will be mentioned. Finally each VFA conversion will be compared based on its energy and carbon recovery.

1.4.2 Production process of VFA

VFA are produced during acidification of biomass. Acidification comprises the first three steps of anaerobic digestion: hydrolysis, acidogenesis and acetogenesis as described in Figure 1.1. Acidification products can be alcohols, hydrogen, CO_2 and VFA such as acetate, propionate, *n*/*i*-butyrate and *n*/*i*-valerate. The exact product formation is related to the type of bacterium that dominates during the fermentation. For example, acidogenesis is a category of reactions that can be accomplished by a large, highly diverse group of fermentative bacteria (Rittmann and McCarty, 2002). The type of bacteria that will dominate and determine the final product compilation, depends on the initial type of substrate and operational parameters such as pH (Dinopoulou et al., 1988). Within acidogenesis, there are two main fermentations: propionic acid and acetate-butyrate fermentation. Each fermentation is performed by different types of microorganisms. In general, it can be said that at low pH (<6) more *n*-butyrate is produced, at pH 6<pH<8 more acetate, and at a high pH (>8) more propionate. Other parameters that influence the product formation are hydraulic retention time (HRT), substrate, temperature, influent concentration, organic loading rate and reactor type (Hawkes et al., 2002).

1.4.3 VFA conversion to fuel or chemicals

VFA themselves are unsuitable for fuel application due to the small carbon chain and the high oxygen/carbon ratio. VFA should be further converted, which can be done either thermally or biologically. Examples of thermal conversion have been described by Levy (1981) and in a MixAlco process (Holtzapple et al., 1999). In this process, biomass is first pretreated with lime at 100ºC and then converted by mixed cultures to VFA with mainly acetic acid (40% wt of total VFA). VFA, that directly precipitate as calcium carboxylates, were separated and further converted to alcohols in a thermal hydrogenation process. The separation of short carboxylates and conversion into fuel requires large input of chemicals and energy.

Biological conversion process with mixed cultures requires normally low chemical and energy input. There are two enzymatic reactions that increase energy density of VFA without carbon loss:

- biohydrogenation to an alcohol
- chain elongation to a longer chain fatty acid

Hydrogenation or elongation of acetate increases the energy density in the final products to ethanol or *n*-butyrate or even longer to caproate or caprylate with 6 and 8 carbon atoms, respectively (Figure 1.3). After these conversions, products have a higher energy density than VFA or the starting biomass material as sugar, but have still a lower energy density than gasoline or diesel. Ethanol and butanol can directly be blend in with gasoline, whereas caproic and caprylic acids need to be further processed to diesel or kerosene-like components by for example ketonization (Gaertner et al., 2009).

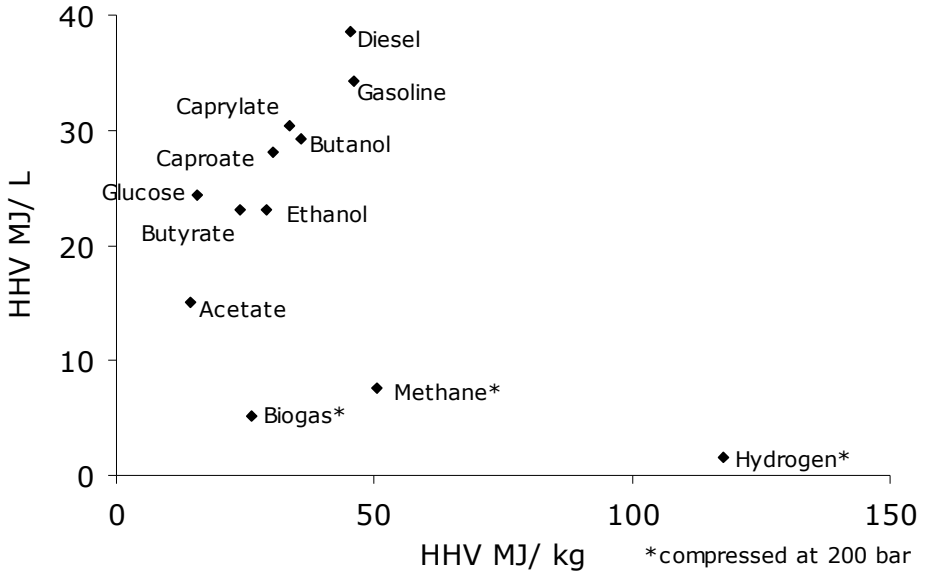

Figure 1.3 Volumetric and mass energy density of biomass, liquid fuels and gas.

Both biological reactions, biohydrogenation and chain elongation are catalyzed by enzymes present in anaerobic bacteria (Schlegel, 1986). However, until now, no experimental data have been published for mixed-culture fermentations to produce these desired components for biotechnological application.

Biohydrogenation

Biohydrogenation of the carboxylic group of aliphatic and aromatic compounds has been described for several pure cultures for the synthesis of specialty chemicals, including aromatic as well as aliphatic alcohols. The reduction of carboxylic acids has been described for a limited number of mesophilic microorganisms *Nocardia* (Chen and Rosazza, 1994; Li and Rosazza, 2000), *Clostridium formicoaceticum* (Fraisse and Simon, 1988), *Clostridium thermoacticum* (Simon et al., 1987) and fungi (Arfmann and Abraham, 1993) as well as for hyperthermophilic archaeus *Pyrococcus furiosus* (Ban et al., 1999). The biological reduction of acids to alcohols, as described previously, is catalyzed by at least two enzymes in presence of formate, carbon monoxide or hydrogen as electron donors. Reduction of a VFA by mixed cultures has been mentioned only once in the literature by Smith and McCarty (1989). They observed propionate reduction in a reaction coupled to ethanol oxidation. According to the authors, this reaction was mediated by ethanol oxidizing organisms during high rates of ethanol utilization. No literature was found that described an attempt to produce alcohols from VFA by mixed cultures with solely hydrogen as an electron donor.

Chain elongation

Chain elongation of short chain fatty acids to long fatty acids is called reversed β-oxidation. Reversed β-oxidation has been described for the strictly anaerobic bacteria *Megasphera elsdenii*, *Eubacterium pyruvatiorans* (Wallace et al., 2003) and different species of Clostridia. Anaerobic bacteria use chain elongation as a mechanism to release reducing equivalents in conjunction with growth or as a mechanism to detoxify the medium (Wiesenborn et al., 1989). Bacteria that use chain elongation as a growth mechanism *are Clostridium Kluyveri* (Barker et al., 1945) or *Eubacterium pyruvatiorans* (Wallace et al., 2003). They convert acetate and ethanol to butyric acid or even longer medium chain fatty acids such as caproic acid. It was even reported that caproate could be produced by co-cultures of *Clostridium kluyveri* with ruminal cellulolytic bacteria from ethanol and cellulose(Kenealy et al., 1995). Levy et al. discussed the possibility to produce caproate and caprylate with mixed cultures, but no experiments or data were described (Levy et al., 1981).

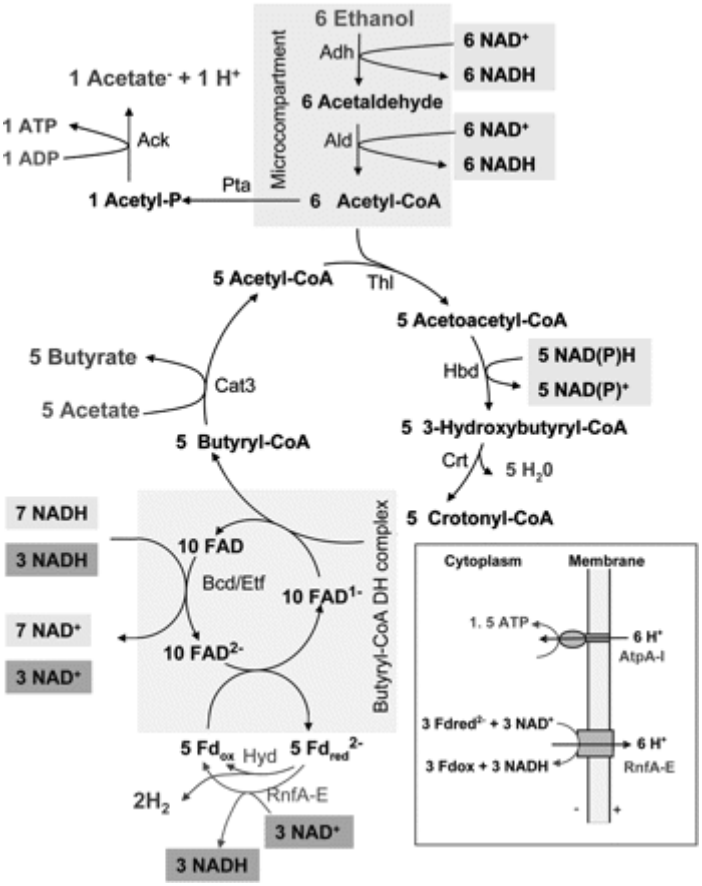

Figure 1.4 Fermentation pathway of *Clostridium kluyveri* (Seedorf et al., 2008).

Chain elongation is a cyclic pathway of four enzymatic steps using acetyl-CoA, NADH and $FADH_2$ as energy carriers (Figure 1.4). In one cycle, acetyl-CoA is coupled to another CoA derivative to form a CoA derivative with an additional 2 carbon atoms (Lynen and Ochoa, 1953). The key issue in elongation is the activation of the fatty acid to a CoA derivative. The activation of the fatty acid is performed by either addition of CoA to a fatty acid with ATP or via transfer of the CoA group of one CoA derivative to another CoA derivative. Acetate, as key molecule in the fermentation, can be activated by the addition of an electron donor. This can occur either with hydrogen to convert VFA into ethanol via biohydrogenation or by the adding ethanol to acetate itself (this mechanism is seen in the *C. kluyveri* as shown in figure 1.4).

1.4.4 Electron donor

Hydrogenation and chain elongation are both energy efficient mechanisms performed by anaerobic organisms to convert VFA into products with a higher energy density or into biomass. Electron donors and protons are needed in this conversion to increase energy density of the product and to eliminate oxygen in the form of water. Possible donors that are commonly used in biological reactions are mentioned below (Fraisse and Simon, 1988). All potentials are given at standard conditions at pH=7 and are expressed versus Normal Hydrogen Electrode (NHE):

1. Hydrogen E_0 = - 414 mV vs NHE
2. Formate E_0 = - 432 mV
3. Carbon monoxide E_0 = - 520 mV
4. Ethanol (to CO_2) E_0 = - 315 mV
5. Electricity and protons E_0 = variable mV

In this thesis only hydrogen, ethanol and electricity were studied as electron donor, as only those can be biologically produced from residual biomass.

Hydrogen

Hydrogen can be generated from organic waste by acidification (described in 1.4.1), and by bioelectrolysis. During acidification of carbohydrate, a theoretical maximum of 4 mol hydrogen per mol glucose can be obtained with acetate as acidification product (Equation 1.6).

$$C_6H_{12}O_6^- + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2C_2H_4O_2 \quad (1.6)$$

$$C_6H_{12}O_6^- \rightarrow 2H_2 + 2CO_2 + C_4H_8O_2 \quad (1.7)$$

In practice, hydrogen yields are lower, because a mixture of acetate (Equation 1.6) and *n*-butyrate (Equation 1.7) are formed as fermentation products. The hydrogen yield can be very low when propionate or other reduced products such as alcohols and lactic acid are formed during acidification. As described in 1.4.1, the type of fermentation reaction, and therefore the hydrogen yield, depends on the reactor operation, the substrate, and the biodegradability of the substrate. A general prediction of hydrogen production from organic waste, though, is unreliable and systematic studies of the effect of key process parameters, are needed (Li and Fang, 2007). One of the first researchers that yielded a significant

amount of hydrogen from an organic fraction of municipal solid waste, obtained a hydrogen headspace of 60% (Lay et al., 1999). Afterwards many researchers followed and produced hydrogen with many other substrates (Li and Fang, 2007). Bioelectrolysis is a recently developed method for directly converting biodegradable material into hydrogen using bioelectrochemical systems (Rozendal et al., 2008). In a bioelectrochemical system, hydrogen can be produced at the cathode by using the energy of acetate oxidation at the anode. Although electricity is still required as energy input in the endothermic reaction, in this way less energy is needed to generate hydrogen than by electrolysis of water.

Electrons

Microorganisms can also directly and indirectly use electrons from an electrode in a bioelectrochemical system as an electron donor. Using an electrode directly as an electron source has been demonstrated for several inorganic conversions, including the reduction of nitrate to nitrite (Gregory et al., 2004), complete denitrification (Peter Clauwert, 2007), reductive dechlorination of TCE with a MV modified electrode (Aulenta et al., 2007), chromium(VI) reduction (Tandukar et al., 2009) and proton reduction (Rozendal et al., 2008). The indirect use of an electrode as an electron donor has also been demonstrated in mixed culture environments where iron reduction at the cathode was coupled with microbial oxidation of iron(II) to iron(Ye et al.) (Ter Heijne et al., 2006). Theoretically, organic compounds such as acetate can also be reduced at the cathode by mixed cultures. Direct consumption of electrons and protons at the cathode minimizes the amount of hydrogen that is needed for biohydrogenation or chain elongation. The use of electrodes in a large biofuel producing bioreactor, though, can cause electron transport limitations. The current density of an electrode, and therefore the productivity, can be enhanced by using a mediator.

Competition for electrons

Methane formation is the last step in anaerobic digestion. Without intervention, acetate would be utilized by acetoclastic methanogens and hydrogen by hydrogenotrophic methanogens. Parameters that reduce hydrogen consumption by methanogenesis have been extensively studied in research of dark fermentation by mixed cultures (Li and Fang, 2007). One of the most effective ways to inhibit methanogenesis is to apply a low pH (Chen

et al., 2002; Kim et al., 2004; Oh et al., 2003). Moreover, pretreatment of the inoculum with a simple heat shock removed any hydrogen consuming non-spore forming bacteria (Oh et al., 2003).

1.4.5 Energy and carbon conservation in fermentation product

Biomass conversion to liquid fuels with hydrogenation or coupling, increases the energy density of biomass and recovers the carbon of the biomass. Subsequently, the conversion reactions should be energy efficient. Energy conversion efficiency can be calculated with the Gibbs free energy change. It is the energy of the reactants that is recovered into products without losing energy in for example heat. The Gibbs free energy change for the reaction can be written as

$$\Delta G_r = \Delta G_r^0 + RT \ln Q \tag{1.8}$$

with T as temperature, R as the gas constant, and Q the reaction quotient. The quotient is the mathematical product of the concentrations (or partial pressures) of the products of a reaction divided by the mathematical product of the concentrations (or partial pressures) reactants of a reaction. The Gibbs free energy should be lower than zero to have a spontaneous reaction. The closer the Gibbs free energy is to zero, the more energy is recovered in products and the more efficient the reaction is. For example, biohydrogenation of acetate with hydrogen, VFA and protons as the reactants and alcohol and water as products (Equation 1.10) yields at standard conditions -9.1 kJ.

$$\Delta G_r = \Delta G_r^0 + RT \ln \frac{[Alcohol]}{[VFA_t]pH_2{}^2} \tag{1.9}$$

In this reaction, 99.3% of the combustible energy in acetate and hydrogen is converted into ethanol. The energy efficiencies of acetate to *n*-butyrate chain elongation (Equation 1.11) and acetoclastic methanogenesis (Equation 1.12) are a bit lower but still above 96%.

Table 1.2 Gibbs free energy of VFA conversions and high heating value of reactants.

VFA conversions		ΔG_r^0 kJ mol^{-1}	HHV reactants kJ mol^{-1}
$C_2H_3O_2^- + H^+ + 2H_2 \rightarrow C_2H_6O + H_2O$	(1.10)	-9.1	1329
$C_2H_3O_2^- + C_2H_6O \rightarrow C_4H_7O_2^- + H_2O$	(1.11)	-40.6	2206
$C_2H_3O_2^- + H_2O \rightarrow CHO_3^- + CH_4$	(1.12)	-31.5	858

This high energy efficiency becomes clear, when the three reactions are compared to sugar fermentation to ethanol (ΔG_r^o= -226,1 kJ/mol). During sugar fermentation, 92% of the combustible energy in sugar (-2844 kJ/mol) is converted in ethanol. This shows that biohydrogenation and chain elongation are very efficient processes, but also that ΔG_r^o at standard conditions is close to the thermodynamical limit of 0 kJ. Thermodynamical calculations are needed to reveal under which conditions the reactions will be still spontaneous.

1.5 Scope of the thesis

1.5.1 Objective

This thesis is focused on the proof of principles of three biological conversions of VFA to precursors of liquid biofuels by mixed culture fermentation. The principle of mixed culture fermentation technology is based on the theory that application of the right process conditions in the bioreactor will select a microbial population that gives the desired product concentration, rate and selectivity. Process conditions and parameters that were tested in the fermentation experiments were substrate type, electron donor, pH and the implementation of temperature pretreatment. Table 1.3 outlines the content of each chapter in terms of biological conversion, VFA substrate, electron donor type and the research questions to be answered. The final aim was to determine the conditions at which VFA can be converted at a high rate to a liquid fuel in such a high concentration that the downstream processing can be performed efficiently.

Table 1.3 Overview of experimental research presented in chapters 2-6.

Biological Conversion		*VFA*	*E-donor*	*Research questions*
Biohydrogenation	2	Acetate, propionate and *n*-butyrate	H_2	-How does pH, substrate and product concentration influence the thermodynamics of biohydrogenation? -Can VFA be biohydrogenated by mixed cultures with hydrogen as an electron donor?
	3	Acetate	H_2	-How can methanogenesis be inhibited while acetate biohydrogenation is enhanced? -What is the influence of pH and heat shock on acetate biohydrogenation?
	4	Acetate	e^-	-What is the effect of mediators on acetate biohydrogenation? -Can acetate bioelectrochemically be reduced to ethanol?
Chain elongation	5	Acetate	H_2 and/or ethanol	-Can acetate be elongated to MCFA? -What is the effect of different pH and electron donors on product concentration, reaction rate and efficiency? -Which organisms are involved in the biological conversions?
	6	Acetate	H_2 and ethanol	-What is the effect of continuous flow reactor operation on MCFA production? -Can medium chain fatty acids be selectively separated from the fermentation broth?

1.5.2 Outline

In **chapter 2**, the aim was to experimentally show that ethanol, propanol and butanol could be produced from volatile fatty acids (VFA) with solely hydrogen as electron donor. The challenge was to establish the environmental conditions and bioreactor setup that allows a sufficiently high alcohol concentration at a reasonable rate with good efficiency. In chapter 2, methanogenesis appeared to be a side-process that consumed both substrate VFA and the electron donor hydrogen, which reduces the efficiency. The aim of the experimental research in **chapter 3** was to simultaneously avoid methanogenesis and enhance acetate reduction by varying pH and applying a thermal heat shock on the inoculum as pretreatment. As hydrogen is a valuable energy source, **chapter 4** was used to demonstrate the feasibility of using electricity via an electrode as the electron donor in the biohydrogenation of acetate to ethanol. The thesis continues with another anaerobic conversion, chain elongation of VFA to medium chain fatty acids. In **chapter 5**, biological conversion of acetate into medium chain fatty acids was demonstrated with the different electron donors hydrogen and ethanol. Subsequently, medium chain fatty acid fermentation was stimulated in presence of both electron donors in a controlled fed-batch reactor at different pH. The microbial population within the best performing reactor was characterized. Through continuous addition of substrate and nutrients, growth limitations could be avoided and the specific production rate of caproate and caprylate was calculated. In **chapter 6**, the performance of a medium chain fatty acids fermentation in continuous reactor operation was studied for the best functioning pH. Additionally, the products were selectively removed from the fermentation effluent to demonstrate that medium chain fatty acids can be produced at such high concentration that downstream processing is possible.

2 Biohydrogenation of VFA with hydrogen

Proof of principle study

Abstract

In this research we demonstrated a new method to produce alcohols. It was experimentally feasible to produce ethanol, propanol and butanol from solely volatile fatty acids (VFA) with hydrogen as electron donor. In batch tests, VFA such as acetic, propionic and butyric acids were reduced by mixed microbial cultures with a headspace of 1.5 bar of hydrogen. Observed alcohol concentrations were 3.69 ± 0.25 mM of ethanol, 8.08 ± 0.85 mM of propanol and 3.66 ± 0.05 mM of *n*-butanol. The conversion efficiency based on the electron balance was 55.1 ± 5.6% with acetate as substrate, 50.3 ± 4.7% with propionate and 46.7 ± 2.2% with *n*-butyrate. Methane was the most predominant by-product in each batch experiment, 33.6 ± 9.6% of VFA and hydrogen was converted to methane with acetate as substrate; which was 27.1 ± 7.1% with propionate and 36.6 ± 2.2% with *n*-butyrate. This VFA reducing renewable fuel production process does not require carbohydrates like fermentable sugars, but uses biomass with high water content or low sugar content that is unsuitable as feedstock for current fermentation processes. This so-called low-grade biomass is abundantly present in many agricultural areas and is economically very attractive feedstock for the production of biofuels.

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2.1 Introduction

Among renewable fuels, alcohols as ethanol are a viable option as it can be directly incorporated into the existing transport infrastructure in different blends with gasoline. The use of ethanol produced from biomass offers significant greenhouse gas benefits (Niven, 2005). At present, the ethanol production process depends on the use of raw materials containing high levels of fermentable sugars. Crops like sugar cane, beet, wheat and corn are typical feedstock materials. The use of these crops has a number of serious disadvantages: (i) it creates high feedstock prices (Ueno et al.); crop production has a high (fossil) energy consumption lowering system efficiency (Ye et al.); and competition with food production for arable soil raising ethical issues related to food scarcity (Granda et al., 2007). The use of a different feedstock as lignocellulose would offer significant benefits regarding the reduction of feedstock price, system energy efficiency and competition for arable land (Cardona and Sanchez, 2007). The difficulty of using lignocellulosic biomass is that a cost-effective pretreatment step to access the biomass still needs to be developed, therefore cellulosic ethanol production is still in pilot phase (Angenent, 2007).

Solid organic waste materials are abundantly present in many agricultural areas. The use of these materials for fuel production would be economically very attractive. The production of fuel from waste materials is considered sustainable as the waste is turned from an environmental burden into a benefit. Waste materials are, however, often unsuitable for ethanol production as their sugar content is low. In this chapter, we propose alcohol production from organic waste materials through biological reduction of volatile fatty acids derived from fermentative biomass acidification. Acidification of waste materials containing lipids, proteins and carbohydrates is a cheap reliable step known from anaerobic digestion. This step within anaerobic digestion produces an effluent containing volatile fatty acids (VFA) such as acetic, propionic and butyric acids together with a gas phase containing carbon dioxide and hydrogen (Metcalf & Eddy, 2003). As acidification is a well-known process, this study focuses on the biological reduction of the carboxylic group of a VFA to an alcohol. The reduction is driven by hydrogen as single electron donor. Hydrogen is a co-product of the same acidification process of organic material, in which VFA are produced (Li

and Fang, 2007). Lay et al. (1999) were one of the first to yield large amounts of hydrogen from the organic fraction of municipal solid waste, and to obtain a hydrogen headspace of 60%.

For the reduction of 1 mol of VFA, 2 mol of hydrogen are needed. Considering the fact that hydrogen is a fuel, it may sound contradictorily to use hydrogen for fuel alcohol production. But, ethanol production with hydrogen through VFA reduction recovers a larger share of the energy content of the organic material, compared to hydrogen production only from the same material; 1 mol of ethanol has two times higher heating value than 2 mol of hydrogen. Another advantage of ethanol over hydrogen, is the high energy density of 23.4 GJ m^{-3} of ethanol compared to compressed hydrogen at 200 bar of 1.95 GJ m^{-3}. Besides, using VFA and hydrogen instead of fermentable sugars for fuel production, diversifies the type of raw materials for the alcohol production process.

Hydrogen is used as single electron donor for mixed cultures to reduce the carboxyl group in VFA, being acetate, propionate and *n*-butyrate as they are the most common products of acidification process (Dinopoulou et al., 1988). The biological reduction of the carboxylic group of aliphatic and aromatic compounds has been described for several pure cultures for the synthesis of specialty chemicals (Arfmann and Abraham, 1993; Ban van den et al., 1999; Fraisse and Simon, 1988; Li and Rosazza, 2000; Simon et al., 1987). Our study differs considerably from these studies as we use firstly mixed cultures instead of a pure culture, and secondly hydrogen as electron donor instead of carbon monoxide or formate. Reduction of a VFA by mixed cultures has been mentioned earlier by Smith and McCarty (1989); they observed propionate reduction in a reaction coupled to ethanol oxidation. According to the authors this reaction was mediated by ethanol oxidizing organisms during high rates of ethanol utilization. To our knowledge, present study is the first attempt to produce alcohols from VFA by mixed cultures with solely hydrogen as electron donor. The objective of this paper was to study and examine whether it is feasible to convert acetate, propionate and *n*-butyrate into alcohol by mixed cultures with solely hydrogen as electron donor.

The biological reduction of acetate (Equation 2.1), propionate (Equation 2.2) and *n*-butyrate (Equation 2.3) yields little energy at standard conditions

(ΔG_r^0=-9.1, -9.4 and -5.4 kJ mol^{-1}, respectively at p_0= 1 atm. and pH 7). The value at standard condition is exothermic, but is close to 0 kJ compared to glucose fermentation (-225.5 kJ). Therefore the concentrations and partial pressure of reactants VFA, hydrogen and protons should be controlled such that a sufficient low actual Gibbs free reaction energy is created.

$$C_2H_3O_2^- + H^+ + 2\,H_2 \rightarrow C_2H_6O + H_2O \qquad \Delta G_r^0 = \text{-9.1 kJ mol}^{-1} \quad (2.1)$$

$$C_3H_5O_2^- + H^+ + 2\,H_2 \rightarrow C_3H_8O + H_2O \qquad \Delta G_r^0 = \text{-9.4 kJ mol}^{-1} \quad (2.2)$$

$$C_4H_7O_2^- + H^+ + 2\,H_2 \rightarrow C_4H_{10}O + H_2O \qquad \Delta G_r^0 = \text{-5.4 kJ mol}^{-1} \quad (2.3)$$

Thermodynamic calculations were first used to determine values for hydrogen pressure and pH to have a sufficient low actual Gibbs free energy to enable VFA reduction. Based on these values the experimental conditions were chosen and applied in the batch test so that VFA reduction would be at least thermodynamically feasible. Alcohol production capacity of the mixed cultures was evaluated on alcohol concentration, rate and reaction efficiency.

2.2 Materials and Methods

2.2.1 Thermodynamic calculations

An exothermic reaction has a reaction Gibbs free energy lower than 0 kJ. Schink (1997) and Thauer et al. (1977) reported that organisms are taking part of the metabolized energy to grow or to maintain cell functions. So the minimum energy quantity to have a microbial reaction, should be lower than 0 kJ. Schink and Thauer et al. discussed a minimal energy quantity in the range of -15 to -20 kJ mol^{-1} reaction. This last value -20 kJ mol^{-1} was used as upper limit for the thermodynamical calculations on VFA reduction. Process variables as VFA concentration, hydrogen pressure and pH were calculated at which the biological reaction would still be thermodynamically feasible. The reaction Gibbs free energy change of the reduction of VFA was defined by equation 2.4. The derivation of equation 2.4 is shown in the Supporting Information (Steinbusch et al., 2008):

$$\Delta G_r^{'} = \Delta G_r^0 + RT \ln \frac{[\text{Alcohol}]}{[VFA_t]pH_2^{\,2}} + RT \ln \frac{K_a + [H^+]}{K_a [H^+]} \qquad (2.4)$$

Standard Gibbs free energy change (ΔG_r^0) of components was given by Amend and Shock (2001). During the experiment, reactants are converted and ΔG_r^0 increases with time until an equilibrium has been reached at posed limit of -20 kJ. With Equation 2.4, we can calculate a maximum concentration of alcohol that can be produced. Then we assume that reactants are solely converted to alcohol and that the pH remains constant.

2.2.2 Experimental setup

Inoculum

Based on preliminary experiments (data not shown) granular sludge from up-flow anaerobic sludge blanket (UASB) reactors was selected as inoculum. The anaerobic granular sludge was obtained from an UASB reactors treating wastewater from a distillery (Royal Nedalco, Bergen op Zoom, the Netherlands, 2004). Granular sludge was washed with medium solution and sieved with a mesh of 500 mm.

Batch experiments setup

Serum bottles (120 ml) were filled with 37.5 mL medium having a concentration of 50 mM of either acetic, propionic or butyric acid. Medium was prepared according to Phillips et al. (1993), which has a low sulfate content to prevent sulfate reduction. To each bottle 0.5 g TS sludge was added. The pH of the liquid was set on 5 with 2 M of sodium hydroxide or hydrochloric acid, since thermodynamical calculations pointed out that a low pH value increases the ΔG_r^0. The bottles were sealed with rubber inlets and capped with aluminum crimp caps. The headspace was replaced five times with pure hydrogen to a final overpressure of 0.5 bar, since thermodynamical calculations pointed out that high hydrogen pressure decreases the ΔG_r^0. Four controls were included in the setup to identify the effect of hydrogen, VFA, sludge or a combination of them on the alcohol production capacity (Table 2.1). The bottles were incubated at 30°C in a rotating shaker (170 rpm). Samples of gas and liquid phase were taken with a syringe to determine the composition of both phases. The ratio of gas/liquid volume was corrected for sampling. The pH and pressure were measured at each sampling. The batches were performed in triplicate with exception of the controls of acetate which were done in duplicate.

Table 2.1 Composition of solution and gas phase in the experimental setup

	Nutrients	*VFA*	*Hydrogen*	*Nitrogen*	*Inoculum*
Experiment	x	x	x		x
Control 1	x		x		x
Control 2	x	x		x	x
Control 3	x			x	x
Control 4	x	x	x		

2.2.3 Analysis

Hydrogen, oxygen and methane were measured with a HP 5890A gas chromatograph by injecting 100 mL of gas-sample on a molsieve column (30m x 0.53mm x 0.25mm) with thermal conductivity detection (TCD). The oven temperature was 40°C, the injection gate 110°C and the TCD 150°C. The carrier gas was argon and had a flow rate of 20 mL min^{-1}. Carbon dioxide, methane and oxygen were measured with a Finsons Instruments GC 8340 gas chromatograph. Gas was splitted (1:1) over a molsieve column (30m x 0.53mm x 25 mm) and a porabond Q column (25m x 0.53mm x 10 mm). The oven temperature was 40°C, injection gate 110°C and the TCD 90°C. The carrier gas was helium and had a flow rate of 45 mL min^{-1}.

Alcohols (C_2–C_5) and VFA (C_2–C_5) were measured by gas chromatography (HP 5890 series II) with AT™ aquawax-DA glass column (30m x 0.32mm x 25 mm) and a flame ionization detector (FID). Liquid samples were centrifuged for 5 min at 10,000 rpm and diluted fivefold with 3% w/w formic acid water solution. Prepared sample (1.0 ml) was injected into the injection port at 280°C and was splitted into a ratio of 1:50 with a flow of 60 mL min^{-1}. The oven temperature was 5 min on 60°C then to 210°C at 25°C min^{-1} ramp and held for 2 min. FID had a temperature of 300°C. The carrier gas was nitrogen and had a flow rate of 2.6 mL min^{-1}. The pH was measured with a pH electrode Sentix 21 with pH range (0–14) stored in a 3 M KCl solution. Pressure of headspace of the bottles was measured with the GMH 3150, digital pressure meter from Greisinger electronic (Germany).

2.2.4 Electron equivalents balance

An electron balance gives insight into the direction of the electron flow from electron donor towards products. Moreover, the conversion efficiency of reduction can be defined with the electron balance. Following McCarty (1972), the electron balance was expressed in electron equivalents (in mol e)

which is based on concentration, carbon atoms and degree of reduction of each individual compound. The degree of reduction indicates the capacity of a compound to reduce other compounds. It is expressed in number of electrons that are involved in the half reaction of the compound with the compounds in the reference oxidation state. These components are HCO_3^-, NO_3^-, SO_4^{2-}, water and protons, and have by definition a degree of reduction zero. Knowing the degree of reduction, the electron equivalents of a compound were calculated by multiplying the degree of reduction with the concentration and number of carbon atoms of the compound.

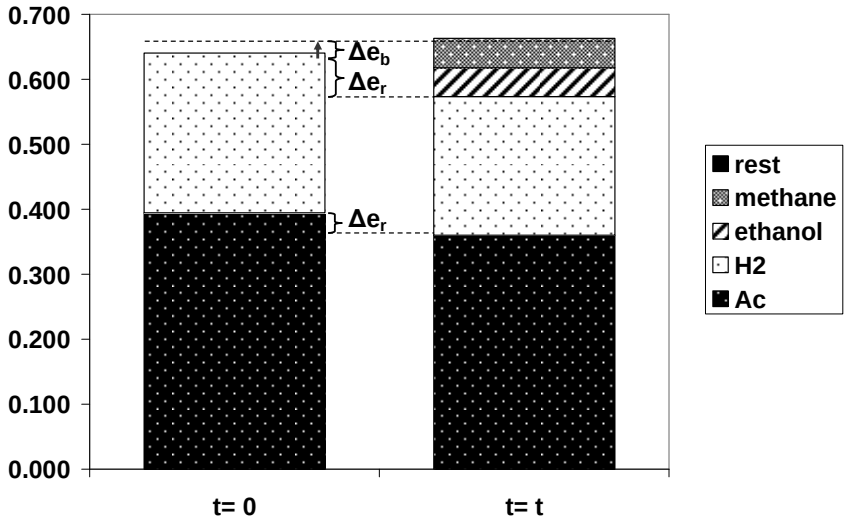

Figure 2.1 Distribution of electron equivalents (mol L^{-1}) among analyzed components at t=0 days and t=25 days with acetate and hydrogen as reactants and ethanol, methane and rest as products with Δe_b as the electron equivalents available from the sludge.

Electron equivalents in the experiment were initially present in the form of reactants (carboxylic acid and hydrogen) and biomass. At time greater than zero, the total amount of electron equivalents remained equal to the beginning situation, only the division among reactants, products and biomass may have changed (Figure 2.1). The total amount of electron equivalents (e_{total}) is defined as the summation of the electron equivalents of reactants (r) i (e_{ri}), of products (p) j (e_{pj}) and of the biomass (e_b) (Equation 2.5).

$$e_{total} = \sum_{i=1}^{m} e_{r,i}(t) + \sum_{j=1}^{n} e_{p,j}(t) + e_b(t) \quad (2.5)$$

The electron equivalents of reactants and products were known by analyzing VFA, alcohol and biogas concentrations. The absolute amount of electron equivalents in the sludge, however, could not be measured. Instead of the absolute amount, the change in electron equivalents of the sludge was estimated. The change in the electron equivalents (Δe) was defined as the value at time t compared to the time t= 0, such as Δe_b in Equation 2.6.

$$\Delta e_b = e_b(t) - e_b(0) \quad (2.6)$$

Solving 2.5 for $e_b(t)$ and substituting this result in Equation 2.6 gives the following result in Equation 2.7.

$$\Delta e_b = e_{total} - \sum_{i=1}^{n} e_{r,i}(t) - \sum_{j=1}^{n} e_{p,j}(t) - e_{total} + \sum_{i=1}^{n} e_{r,i}(0) + \sum_{j=1}^{n} e_{p,j}(0) = -\sum_{i=1}^{n} \Delta e_{r,i} - \sum_{j=1}^{n} \Delta e_{p,j} \quad (2.7)$$

The change in sludge electron equivalents (Δe_b) is defined consequently by the change in reactant (Δe_{ri}) and product ($\Delta e_{p,j}$) electron equivalents. Here we assumed that all reactants and products were detected in the gas and liquid phase and that the change in total electron equivalents was allotted to the change in electron equivalents of the sludge.

Regarding product formation, alcohol was produced either via VFA reduction or via sludge degradation. This implies that part of the electron equivalents in alcohol could derive from reactants (α_i) or from sludge (β_i) (Equation 2.8).

$$\Delta e_{pi} = \sum_{j=1}^{n} \alpha_i \Delta e_{rj} + \beta_i \Delta e_b \quad \text{with} \quad 0 \leq \alpha_i \leq 1 \wedge 0 \leq \beta_i \leq 1 \quad (2.8)$$

The fraction of converted VFA and hydrogen (α_i) that contributed to alcohol product was indirectly calculated using β_i, which derived from the controls without VFA (control no. 1). Products in these controls were assumed to derive only from the sludge as no VFA was added as reactant. The contribution of electron equivalents of hydrogen here was disregarded (α_{H2} = 0). Parameter β_i was calculated for each product i according to Equation 2.9.

$$\beta_{pi} = \frac{e_{pi}}{\Delta e_b} \quad (2.9)$$

To determine the occurrence of biological reduction of VFA, a conversion efficiency and a recovery of VFA and hydrogen into alcohol product were defined from Equation 2.8. The recovery is defined as the percentage of electron equivalents of the product that derived from VFA and hydrogen (Equation 2.10).

$$\mathrm{Recovery}(p_i) = \frac{\sum_{j=1}^{n} \alpha_i \Delta e_{rj}}{\Delta e_{pi}} * 100\% = \frac{\Delta e_{pi} - \beta_i \Delta e_b}{\Delta e_{pi}} \qquad (2.10)$$

The efficiency is defined as the fraction of consumed VFA and hydrogen that was converted to product i (Equation 2.11).

$$\mathrm{Efficiency}(p_i) == \frac{e_{pi} - \beta_i \Delta e_b}{\sum_{j=1}^{n} \Delta e_{rj}} * 100\% \qquad (2.11)$$

Equation 2.10 and 2.11 were applied to main products formed in the bottles as alcohol and methane, noting that for each product i, a specific β_i was used that was calculated from the controls.

2.3 Results and discussion

VFA reduction is thermodynamically feasible at elevated hydrogen pressure and low pH. According to the thermodynamic limit of Schink (1997), biological reduction of acetic, propionic and butyric acids would not be feasible at standard conditions with 1 M of all components and pH 7. As Figure 2.2 shows, $\Delta G_r'$ of reduction of acetic acid becomes more negative at higher hydrogen pressure and lower pH, though $\Delta G_r'$ leveled off when the pH becomes 5 or lower. The limit of -20 kJ at a hydrogen partial pressure of 1 bar is reached when the pH is 4.36. A further decrease of pH results finally in a minimal reaction energy of $\Delta G_r'$= -20.7 kJ, which is close to the required energy quantity for maintenance and growth. In other words, only low pH cannot contribute further to stimulate biological reduction, instead hydrogen pressure or the acetic acid/ethanol ratio should be increased. Equation 2.4 revealed that the hydrogen pressure has a stronger effect on the Gibbs free energy change than ethanol/acetate concentration ratio.

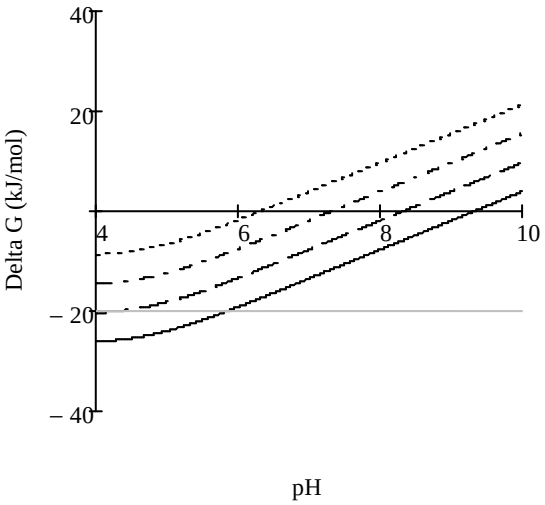

Figure 2.2 Gibbs free energy change of bio reduction of acetic acid as function of pH at different H_2 partial pressure of 0.01 bar (.....), 0.1 bar (_ . _), 1 bar (_ _ _) and 10 bar (____) at 1M acetic acid (sum of dissociated and undissociated) and ethanol and horizontally the thermodynamic limit of -20 kJ/mol (____).

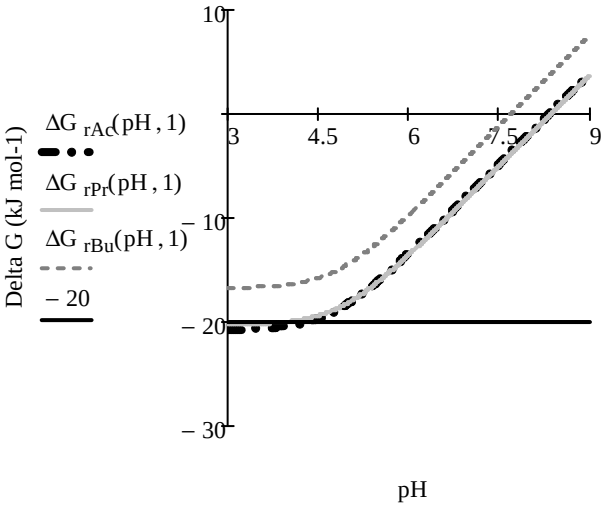

Figure 2.3 Gibbs free energy of biohydrogenation of acetate (.....), propionate (____) and *n*-butyrate (_ _ .) as function of pH at 1 bar of hydrogen and 1 M of ethanol and (un)dissociated acid, and the thermodynamical limit of -20 kJ/mol (____).

Similar trends were found for reduction of propionic and *n*-butyric acids as the reaction formula is similar to acetic acid reduction (Figure 2.3). Consequently, an elevated hydrogen partial pressure of 1.5 bar was used in the batch tests. With applied conditions as described in materials and methods section and a low pH of 5, a theoretical maximum ethanol concentration of 24.3 mM can be reached (Figure 2.4). In case of propionic acid reduction the maximum propanol concentration would be 24.7 mM and with *n*-butyric acid reduction it is 9.4 mM *n*-butanol.

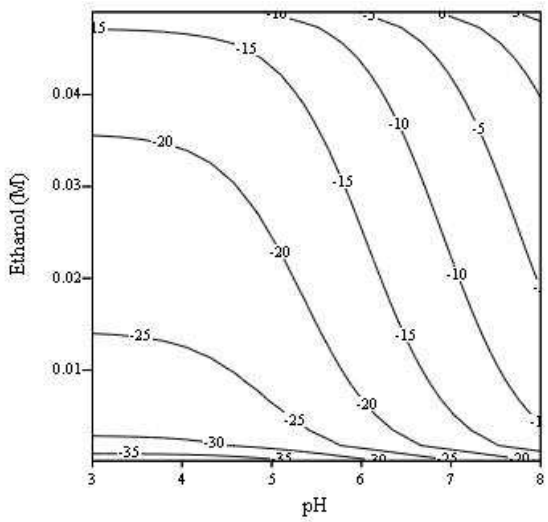

Figure 2.4 Contour plot of $\Delta G_r'$ (kJ mol^{-1}) of the reduction reaction of acetic acid to ethanol with hydrogen as electron donor as function of the pH (x-axis) and the molar ethanol concentration (y-axis).

Granular sludge with acetate, propionate or *n*-butyrate in the presence of hydrogen results in corresponding alcohol production. Figures 2.5, 2.6 and 2.7 and Table 2.2 all show a decrease in VFA and hydrogen concentration in each batch experiment together with an increase in alcohol concentration. The type of alcohol produced corresponds to the chain length of the VFA present in the medium (Table 2.2); only ethanol was detected with acetate as substrate (Figure 2.6), only propanol with propionate (Figure 2.7) and only *n*-butanol with *n*-butyrate (Figure 2.8). The highest measured alcohol concentration was 8.08 ± 0.85 mM propanol, which is 33% of the theoretical maximum. This concentration is high considering that reactants are not only used for the production of alcohol, but for by-products as well. By-product

formation lowered hydrogen pressure or caused an increase of pH, which consequently lowers the theoretical maximum alcohol concentration. Similar results were found for the butanol concentration which was 39% of the theoretical maximum. The obtained ethanol concentration was 15% of the theoretical maximum that could have been produced from present acetate and hydrogen.

Table 2.2 Molar concentration changes of products and substrates per liter medium with acetic acid as carbon source after 21 days, and with propionic or *n*-butyric acid after 25 days

System conditions	*Substrate*		
	Acetic acid	Propionic acid	*n*-Butyric acid
Products (mM)			
Acetate	*-4.33 ± 0.58	2.54 ± 0.45	1.59 ± 0.16
Propionate	-	*-11.06 ± 0.43	0.94 ± 0.08
i-Butyrate	-	-	0.09 ± 0.15
n-Butyrate	-	0.71 ± 0.12	*-4.33 ± 0.58
i-Valyrate	-	0.34 ± 0.05	-
n-Valyrate	-	0.61 ± 0.31	-
Ethanol	3.69 ± 0.25	-	-
Propanol	-	8.08 ± 0.85	-
n-Butanol	-	-	3.66 ± 0.05
Methane	5.65 ± 0.48	11.33 ± 1.15	9.68 ± 0.16
Hydrogen	*-16.33 ± 0.58	*-64.33 ± 6.43	*-48.00 ± 1.00
C recovery (%)	97.2 ± 1.3	110.4 ± 3.7	106.1 ± 1.0
E recovery (%)	103.5 ± 1.2	101.3 ± 2.5	100.8 ± 0.7
Recovery alcohol (%)	83.4 ± 4.6	98.1 ± 3.2	99.0 ± 0.9
Bioreduction efficiency (%)	55.1 ± 5.6	50.3 ± 4.7%	47.6 ± 2.2

* negative values indicates an overall consumption of the component during the experiment

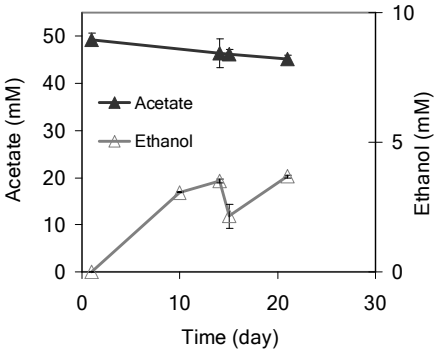

Figure 2.5 Concentrations of acetate as substrate (primary y-axis) along with product ethanol (secondary y-axis) in batch experiment with hydrogen as electron donor in time.

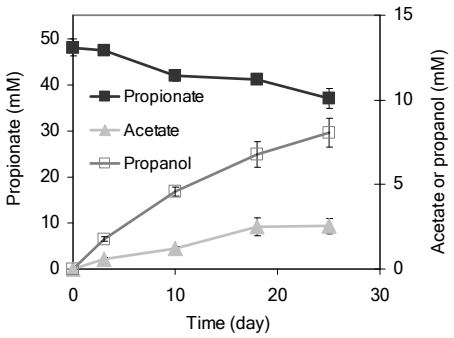

Figure 2.6 Concentrations of propionate as substrate (primary y-axis) along with products propanol and acetate (secondary y-axis) with hydrogen as electron donor in time.

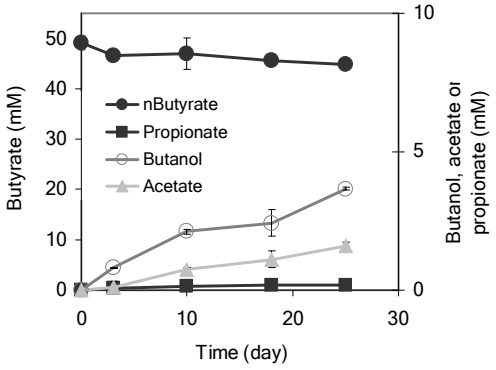

Figure 2.7 Concentration of *n*-butyrate as substrate (primary y-axis) along with products butanol and acetate with hydrogen as electron donor in time.

In the four controls, no alcohol was produced when hydrogen was left out. In another control without VFA, small amounts of propanol and *n*-butanol were detected. Propanol concentration (1.2 ± 0.02 mM) was, however, eight times lower than in the presence of 50 mM propionate. *n*-Butanol concentration (0.34 ± 0.02 mM) was 10 times lower than in the presence of 50 mM *n*-butyrate. Alcohol production in the control is assumed to attribute fully to the release of electron equivalents from the sludge, which was used to calculate β_i. Parameter β_i was 0.334 for ethanol, 0.223 for propanol and 0.084 for *n*-

butanol. Parameter β_i was determined under experimental conditions of the control no.1, though the conditions differ somewhat from the experiment. In the absence of VFA (control 1), 10-31 times more electron equivalents were measured in the aqueous and gas phase than in the presence of VFA. These electron equivalents are estimated to derive from the change in electron equivalents of the sludge. Without substrate, the sludge decay is higher as the release of electron equivalents was larger. Given this, β derived from the control might not be fully representative, but gives an overestimation of the influence of sludge decay on alcohol production. The mass balance based on electron equivalents was almost closed, while the carbon recovery was in some cases much higher (110.4 ± 3.7%). It is known that granular sludge in anaerobic wastewater treatment systems can contain high amount of calcium carbonate precipitates even up to an ash content of 90% (Van Langerak et al., 1998). Release of the carbonates present in sludge causes a positive carbon balance and while the electron balance remains unaffected by the carbonates.

The highest alcohol production rate during the first 10 days was observed for propanol amounting to 0.459 ± 0.026 mmol L^{-1} d^{-1} compared to ethanol with 0.309 ± 0.042 mmol L^{-1} d^{-1} and *n*-butanol with 0.211 ± 0.009 mmol L d^{-1}. The production rate was not limited by hydrogen transport from gas to liquid phase as the transport rate even by diffusion in a stagnant medium would be higher. The highest conversion efficiency of reactants to alcohol was observed for propionate and hydrogen. It was found that minimally 50.3 ± 4.7% of the converted propionate and hydrogen is reduced to propanol based on the electron balance (Table 2.2). When taking β=1 the reduction efficiency is underestimated, and would be 32.5 ± 5.2% for ethanol, 46.6 ± 5.3% for propanol and 42.2 ± 2.7% for *n*-butanol. Thus even with an overestimation of the influence of electron equivalents of sludge, one-third of the electron equivalents of the carboxylic acid and hydrogen are converted to alcohol product.

Positive value for alcohol recovery indicates reduction of VFA. High recovery was observed in alcohol products: at least 83.4 ± 4.6% of the electron equivalents in alcohol derived from VFA and hydrogen (Table 2.2). The high positive values for recovery of reactants in alcohol give evidence that the majority of the alcohols were products of biological reduction. The residual

percentage of the total electron equivalents in alcohol products might originate from the sludge. When taking β=1, the recovery would be 50.2 ± 13.6% for ethanol, 91.6 ± 14.5% for propanol and 88.2 ± 10.3% for *n*-butanol.

Methane was the most predominant by-product in the presence of acetate, propionate and *n*-butyrate (Table 2.2). The conversion efficiency of reactants to methane was 33.6 ± 9.6% with acetate as substrate (Equation 2.11), while 27.1 ± 7.1% for propionate and 36.6 ± 2.2% for *n*-butyrate. Contribution of released electron equivalents from the sludge to methane formation is calculated to be maximum (β =1) for all controls. Methane formation with granular sludge as inoculum is not remarkable as the sludge was taken from a UASB reactor treating brewery wastewater that converted VFA among others into methane daily. The substrates in present experiment are either direct or indirect substrates for methanogenesis: hydrogen for hydrogenotrophic and acetate for acetoclastic methanogenesis. In addition to that, from a thermodynamical point of view, the most favorable reaction to occur spontaneously is hydrogenotrophic methanogenesis (ΔG_r^0= -110 kJ mol^{-1}) compared to acetic acid reduction (ΔG_r^0= -37 kJ mol^{-1}) calculated for initial experimental conditions. Propionic and *n*-butyric acids, however, cannot directly be converted to methane, but first need to be oxidized to acetic acid. The oxidation of propionic and *n*-butyric acid is suppressed by the high hydrogen partial pressure applied in the experiments consequently. Here the actual free energy for propionic acid oxidation (+13.9 kJ mol^{-1}) is endothermic and higher than reduction of propionic acid (-37.5 kJ mol^{-1}).

Comparably, oxidation of *n*-butyric acid (-9.73 kJ mol^{-1}) has a higher actual Gibbs free energy than reduction of *n*-butyric acid (-33.7 kJ mol^{-1}). This could explain the high efficiency for propionic and butyric acid reductions compared to acetic acid reduction especially in the beginning of the experiment. Although we can explain methane formation it was not likely to occur at a low pH, for the reason that Kim et al. (2004) found that a pH below 5 sincerely inhibit hydrogenotrophic methanogenesis. With the aim of preventing methanogenesis, the medium of present study had initially a pH of 5. Nevertheless, during the experiment the pH slowly increased to 5.6 with acetate as substrate, to 6.1 with propionate and to 5.7 with *n*-butyrate. The pH increase canceled the inhibiting effect on methanogenesis and made

simultaneously VFA reduction energetically less favorable. The pH increase was not expected regarding the buffer capacity of the remaining VFA in the medium. An explanation for the increase would be carbonate release from the granular sludge. As described earlier, granular sludge contains metal precipitants as metal carbonates that will dissolve at pH shocks. A shock of pH 5, as done in the initial phase of the experiment, already strongly affects metal content as iron in the anaerobic sludge was concluded by Zandvoort et al. (2005).

2.4 General discussion

Acetate, propionate and *n*-butyrate were reduced to alcohols by mixed anaerobic cultures with solely hydrogen as electron donor. As far as we could find in literature this is for the first time demonstrated. The propionate reducing capacity of mixed cultures is also reported by Smith and McCarty (1989). They observed propanol formation in methanogenic ethanol- and propionate-fed CSTR after sudden increase in ethanol concentration in the effluent. After perturbation with ethanol, the hydrogen partial pressure increased, where after propanol was formed and a shift towards more reduced products occurred. The highest observed pH of 2 ($7.6 \cdot 10^{-3}$ atm.), however, is thermodynamically still too low to initiate propionate reduction with hydrogen. Smith and McCarty attributed propanol formation to a coupled ethanol oxidation/ propionate reduction, whereas we demonstrated direct propionate reduction with hydrogen.
Maintaining a high hydrogen pressure for reduced product formation seems crucial as Smith and McCarty observe that the reduced products were oxidized as soon as hydrogen pressure decreased again. In the present study partial pressure of hydrogen decreased in the batch tests and might even have become limited for VFA reduction. Further research could study the influence of maintaining a high hydrogen partial pressure on VFA reduction and increase alcohol concentration and production rate.

Reduced organic products as alcohols might substantially contribute to sustainable bioenergy production from waste even if hydrogen is used as electron donor. The heating value of the fuel is higher with alcohol production from waste than with hydrogen production from the same waste. This is demonstrated by a calculation based on the research of Fang et al. (2006)

who determined the hydrogen production potential of a carbohydrate-rich food waste with mixed cultures at pH 5; it was calculated that acidification of 1 kg of rice food waste yields 3.0 mol *n*-butyrate, 2.8 mol acetate and 10.4 mol of hydrogen. When assuming that all produced hydrogen is used to bio catalytically reduce acetate and *n*-butyrate, it was estimated that 2.7 mol butanol and 2.5 mol ethanol can be produced from the same kilogram of food waste. Compared to the production of only hydrogen, this process would recover 4.3 times more energy from the food waste. The energy requirement to separate the fuel from fermentation liquid is not included in this calculation.

More research is needed to determine whether this new method to produce alcohols can substantially contribute to the mixed-culture biotechnology for bioenergy purposes. Measured concentrations and production rates in the batch test are still very low compared to conventional alcohol production. Further research should focus on inhibition of methane formation to increase the conversion efficiency, e.g. by pretreatment of the inocolum to decrease carbonate release. Further attention should be paid on increasing alcohol concentrations and production rate for example by maintaining a high hydrogen pressure and by optimizing pH.

2.5 Conclusions

- Results of batch experiments showed that acetic, propionic and butyric acids could biologically be reduced to alcohols with hydrogen as electron donor in the presence of granular sludge. Highest measured alcohol concentration was 3.69 ± 0.25 mM ethanol, 8.08 ± 0.85 mM propanol and 3.66 ± 0.05 mM *n*-butanol produced from the reactants with an efficiency of 55.1 ± 5.6%, 50.3 ± 4.7% and 47.6 ± 2.2%, respectively. As far as reported in literature this is the first research that showed that alcohol was produced with solely acetate, propionate or *n*-butyrate as substrate and hydrogen as electron donor using mixed cultures.
- Methane was the largest by-product. The conversion efficiency of acetate and hydrogen to methane was 33.6 ± 9.6%, of propionate and hydrogen it was 27.1 ± 7.1% and of *n*-butyrate and hydrogen it was 36.6 ± 2.2%.
- Alcohol production from VFA allows the use of a wide diversity of organic raw materials, though the alcohol concentrations and reaction rate should be increased to become competitive with current alcohol production.

3 Selective inhibition of methanogenesis during acetate biohydrogenation

Abstract

Acetate reduction is an alternative digestion process to convert organic waste into ethanol. Using acetate for fuel ethanol production offers the opportunity to use organic waste materials instead of sugar-containing feedstock. Methanogenesis, however, competes with acetate reduction for acetate and hydrogen and lowers the final efficiency. The aim of this research is to selectively inhibit methanogenesis and to enhance acetate reduction. Acetate reduction was stimulated in batch tests at pH between 4.5 and 8; and at pH 6 with and without thermal pre-treatment. It was found that methanogenesis was selectively inhibited while acetate reduction was enhanced after thermal pre-treatment incubated at pH 6. Initially the acetate reduction yielded 7.7 ± 3.2 mM ethanol with an efficiency of 60.2 ± 8.7%, but later on it was consumed to form 7.02 ± 0.85 mM *n*-butyrate with an efficiency of 76.2 ± 14.0%. It was the first time demonstrated that *n*-butyrate can be produced by mixed cultures from only acetate and hydrogen.

A modified version of this chapter was published as:

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3.1 Introduction

Organic waste is abundant in society and represents a large source of renewable energy if suitable technology to convert this source into valuable energy-carriers would be in place. The use of specially grown crops for energy production gives only a limited or no greenhouse gas emissions reduction because of the energy use in agriculture and changed land use (Khosla et al., 2008; Searchinger et al., 2008). There is also a concern that production of biomass for energy will compete with food production (Doornbosch and Steenblik, 2007). The use of waste has no such disadvantages, it leads to a substantially higher greenhouse gas emission and would not compete for land with food production. Organic waste consists of a mixture of solid and dissolved organic polymers as lipids, proteins and carbohydrates. Because of this versatile composition and the often high water content, it is difficult to recover the chemical energy of organic waste streams in one single product. An established process that recovers energy from those diluted complex organic waste streams is anaerobic digestion. Digestion is an efficient mixed culture process in which mixed cultures convert the composition of wet waste into biogas. Biogas is easy to separate from the liquid slurry. The demand for liquid sustainable biofuels are a drive to innovate anaerobic digestion in such a way that other energy carriers than methane can be produced from waste streams (Angenent, 2007; Kleerebezem and van Loosdrecht, 2007)

Alcohols like ethanol and butanol are an example of alternative energy carriers that can be obtained from organic waste materials. Steinbusch reports production of these alcohols from acidification products through reduction of VFA with hydrogen using mixed cultures (Steinbusch et al., 2008). This newly exploited ethanol production process consists of two biological conversion steps. In the first step, solid organic waste materials are acidified to acetate and hydrogen similar to the acidification step in the digestion process. In the second step, the produced acetate and hydrogen are converted to ethanol Equation 3.1.

$$C_2H_3O_2^- + H^+ + 2\,H_2 \rightarrow C_2H_6O + H_2O \qquad \Delta G_r^0 = -9.1 \text{ kJ mol}^{-1} \qquad (3.1)$$

The standard Gibbs free reaction energy of equation 3.1 is close to zero and therefore a high hydrogen pressure is needed to enhance the reaction.

Although hydrogen is a fuel by itself, using it to produce ethanol recovers twice as much energy from the organic waste; 1 mol of ethanol contains twice as much energy as the two moles of hydrogen that are needed to reduce acetate. Yet, the separation of ethanol from the fermentation broth is more energy consuming than the separation of hydrogen. Hydrogen can be generated from organic waste during aforementioned acidification or by microbial electrolysis. The yield of hydrogen during acidification depends on process parameters and substrate composition, this has been extensively reviewed by (Li and Fang, 2007). Microbial electrolysis is a recently developed process that converts biodegradable material almost stoichiometrically into hydrogen using modified microbial fuel cells (Logan et al., 2008). Previous work yielded in 20 days 3.69 ± 0.25 mM ethanol through acetate reduction at a pH of 5 with a reaction rate of 0.50 ± 0.03 mM d^{-1}. The ethanol conversion efficiency, defined as the share of consumed acetate and hydrogen that was converted into ethanol, was 55.1 ± 5.6% (Steinbusch et al., 2008). The remainder of the converted substrate ended up mainly in methane via methanogenesis, resulting in a methane production efficiency of 33.6 ± 9.6%. To become competitive with digestion, the acetate reduction process should have at least conversion efficiency comparable to digestion. For now, methane formation takes away a significant portion of the reactants acetate and hydrogen, so methanogenesis should be prevented. The aim of this research was therefore to selectively inhibit methanogenesis to increase the efficiency of acetate reduction by mixed cultures. How to reduce hydrogen consumption by methanogenesis, is extensively studied in research of dark fermentation by mixed cultures (Li and Fang, 2007). One of the most effective ways to inhibit methanogenesis is to apply a low pH (Chen et al., 2002; Kim et al., 2004; Oh et al., 2003). Moreover a simple heat shock as pre-treatment of the inoculum removed any hydrogen consuming non-spore forming bacteria (Oh et al., 2003).

We study the effect of pH and heat shock treatment of the inoculum on the inhibition of methanogenesis under acetate reducing conditions. The effectiveness of these treatments has not yet been studied before under these conditions. Furthermore we try to understand the dynamics of mixed culture conversions by using energetic calculations with the experimental work.

3.2 Materials and method

3.2.1 Experimental setup

Granular sludge from up-flow anaerobic sludge blanket reactors (UASB) was selected as inoculum. The anaerobic granular sludge was obtained from an UASB treating distillery wastewater (Royal Nedalco, Bergen op Zoom, The Netherlands, 2004). The inoculum was selected based on previous results (unpublished results). Preparation of sludge included washing with phosphate buffer (20 mM potassium phosphate) of pH 5, 6 or 7 and sieving the granules with a mesh of 500 µm for three times. The granular sludge washed at pH 6 had after the last washing step a dry matter content of 4.87 ± 0.43%, which had an ash content of 10.6 ± 0.3%.

3.2.2 Batch experiments setup

pH experiment

For these experiments, an experimental setup was build similar to that of Steinbusch et al. (2008). Different was the size of the serum bottle to decrease the pH variation in the bottle. Serum bottles (250 ml) were filled with 75 mL medium with 50 mM of acetic acid. Medium with low content sulfate was prepared according to Phillips (1993) to prevent sulfate reduction. The pH of the medium in the bottles was set on 4.5, 5, 6, 7 and 8 with 2 M of sodium hydroxide or hydrochloric acid. Controls without acetic acid, at pH 5 and 7, were included. To each bottle 5.0 g of wet weight granular sludge was added; the washed sludge with pH 5 buffer was added to the media with pH 4.5, 5 and 6; the washed sludge disregarding other with pH 7 buffer to the media of pH 7 and 8. The bottles were sealed with rubber inlets and capped with aluminum crimp caps. The headspace was replaced five times with pure hydrogen to a final pressure of 1.5 bar. The bottles were incubated under mesophilic conditions at 30°C in a rotating shaker (170 rpm) for 30 days. Samples of gas- and liquid-phase were taken with a syringe to determine the composition of both phases. The pressure and pH were measured at each sampling; and the pH was controlled with sodium hydroxide or hydrochloride solution. Both gas and liquid volume were corrected for liquid sampling. The batches were performed in triplicates. To refine the pH optimum for acetate reduction and inhibition of

methanogenesis, the experiment was repeated at pH 5, 5.5 and 6. The batches were prepared according to the same method as described above.

Pre-treatment experiment

The experimental setup was equal to the pH experiment; only the working volume of the serum flasks was 120 ml, which were filled with 35 mL of medium. To each bottle 2.5 g of wet weight granular sludge was added with a final content of 3.48 g L^{-1} total solids and 3.11 g L^{-1} total volatile solids. Finally the pH of each bottle was adjusted to 6. The bottles were sealed with rubber inlets and capped with aluminum crimp caps. The headspace was replaced five times with pure hydrogen to a final pressure of 1.5 bar. One triplicate was exposed to thermal pre-treatment and was boiled in water for 15 min. The bottles were incubated at 30°C in a rotating shaker (170 rpm) for 34 days. Sampling was done according to the same method as the pH experiment.

3.2.3 Analysis

Gas analyses of hydrogen, oxygen, nitrogen, methane and carbon dioxide; and alcohols (C_2-C_5) and VFA C_2-C_6) were analyzed with gas chromatography (Steinbusch et al., 2008). Pressure of headspace of the bottles was measured with the GMH 3150, digital pressure meter from Greisinger electronic (Germany).

3.2.4 Calculations

Efficiency

The effectiveness of biological conversions was described by the efficiency, which is defined as the share of the consumed reactants acetate and hydrogen that is converted into ethanol. The efficiency was calculated with the electron balance and was corrected for a minimal share of products deriving from the granular sludge (Steinbusch et al., 2008). The correction was made to exclude the contribution of granular sludge as reactant. Due to bacterial cell decay, sludge could break down in degradation products as acetate, hydrogen and/or ethanol. The share of electrons that might derive from the sludge via degradation was expressed in parameter β and was calculated from the controls without acetate addition at pH 5 and 7. The control at pH 5 was used for calculation of β of the batches at pH 4.5, 5 and

6; whereas the control at pH 7 was used for β calculation of the batches at pH 7 and 8.

Thermodynamical calculations

Acetate reduction is hydrogen driven reaction of which the Gibbs free energy is close to zero in Equation 3.1. The thermodynamical limit for an exothermic reaction is 0 kJ. An organism that catalyzes this reaction uses part of the reaction energy for maintenance and growth. So the thermodynamical limit for an exothermic reaction catalyzed by organisms is lower than 0 kJ and is assumed to be around -15 and -20 kJ (Amend and Shock, 2001; Schink, 1997). Based on the upper and lower thermodynamical limit, being 0 and -20 kJ, respectively, a maximum ethanol concentration was calculated disregarding other side-reactions consuming acetate and hydrogen (Steinbusch et al., 2008). The Gibbs free reaction energy is calculated with the Standard Gibbs free energy change (ΔG_r^0) of components given by Amend and Shock (2001).

3.3 Results

Ethanol, methane and *n*-butyrate were the three main products of the mixed culture fermentation experiments (Table 3.1). The three products were present at each pH also after heat pre-treatment and contributed together for at least on average 73.6 ± 13.2% to the total product electron equivalents. Because of this large product share of ethanol, methane and *n*-butyrate, only the formation of these three products will be described in the next part.

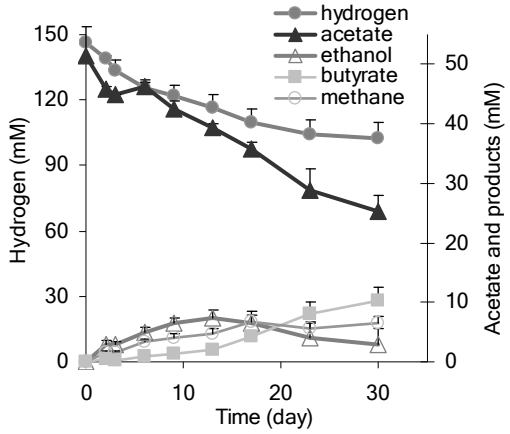

Figure 3.1 Conversion of hydrogen (primary y-axis) and acetate (secondary y-axis) at pH 6 as function of time with the main products ethanol, *n*-butyrate and methane (secondary y-axis).

Table 3.1 Molar concentration changes of products and substrates per liter medium during acetate reduction at different pHs after 30 days.

pH	4.5	5.0	6.0	7.0	8.0
Products (mM)					
Acetate	-8.94 ± 2.64*	-10.3 ± 5.0*	-26.3 ± 6.6*	-48.4 ± 1.54*	-8.93 ± 1.39*
Propionate	0.13 ± 0.02	0.30 ± 0.03	0.27 ± 0.06	0.98 ± 0.39	1.54 ± 0.23
i-Butyrate	0.23 ± 0.05	0.29 ± 0.01	0.25 ± 0.03	-0.01 ± 0.01	0. 46 ± 0.07
n-Butyrate	2.47 ± 0.44	3.73 ± 0.32	10.3 ± 2.18	0.06 ± 0.10	1.00 ± 0.18
i-Valyrate	0.40 ± 0.05	0.45 ± 0.01	0.44 ± 0.06	-0.01 ± 0.01	0. 79 ± 0.12
n-Valyrate	-0.01± 0.01	0.01± 0.01	-0.02 ± 0.01	-0.02 ± 0.01	0. 17 ± 0.04
n-Caproate	0.07± 0.03	0.26± 0.11	0.02 ± 0.02	0.01 ± 0.00	0.04 ± 0.00
Ethanol	0.28 ± 0.05	1.70 ± 0.13	2.82 ± 2.76	0.02 ± 0.01	1.40 ± 0.15
Propanol	0.01 ± 0.01	0.02 ± 0.01	0.05 ± 0.05	0.02 ± 0.01	0.04 ± 0.03
n-Butanol	0.01 ± 0.00	0.06 ± 0.01	1.38 ± 0.25	0.00 ± 0.01	0.03 ± 0.01
Hydrogen	-17.5 ± 0.7*	-20.6 ± 1.6*	-45.8 ± 7.2*	-141.2 ± 0.6*	-67.5 ± 9.0*
Methane	3.87 ± 0.43	3.53 ± 0.76	6.42 ± 1.08	105.3 ± 1.5	29. 2 ± 3.0
CO2	0.13 ± 0.23	0.85 ± 0.06	0.03 ± 0.05	10.8 ± 0.41	0.00 ± 0.00
C recovery (%)	100.4 ± 3.5	108.7 ± 10.4	111.1 ± 12.7	123.2 ± 1.6	130.7 ± 2.3
E recovery (%)	100.3 ± 2.0	105.6 ± 6.0	107.4 ± 6.9	130.4 ± 0.9	119 .2 ± 1.8

* negative values indicates an overall consumption of the component during the experiment

Figure 3.1 shows the course of the concentrations of substrate and the three main products in the batch at pH 6: ethanol is first produced and later consumed again, while methane partial pressure and *n*-butyrate concentration gradually increased in time. This typical product formation in time was seen for all the batches, it only differed in concentration and rate. Other products as propionate, *i*- and *n*-valerate are formed in smaller amounts.

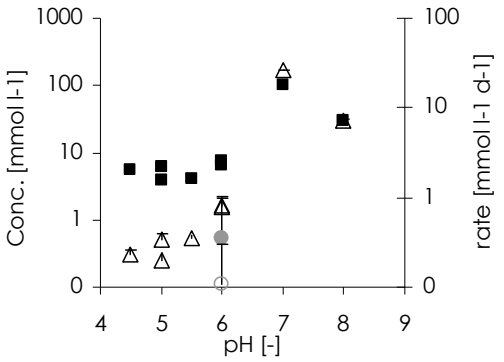

Figure 3.2 Highest methane concentration (■) in log scale and rate (Δ) expressed in mmol per liquid medium as function of applied pHs; the heat pre-treated triplicate are indicated in bullets (● as concentration and ○ as rate).

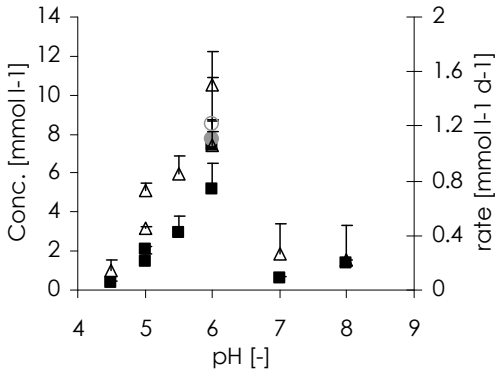

Figure 3.3 Highest ethanol concentration (■) and rate (Δ) as function of applied pHs in batches of the pH experiment; the heat pre-treated triplicate are indicated in bullets (● as concentration and ○ as rate).

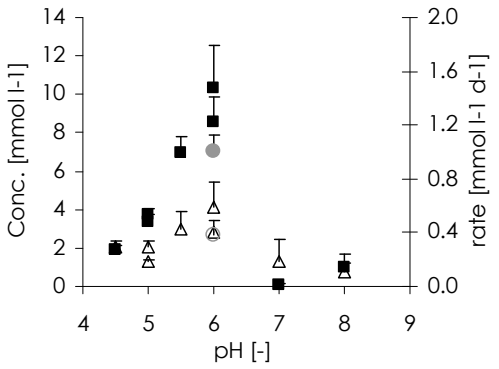

Figure 3.4 Highest *n*-butyrate concentration (■) and rate (Δ) as function of applied pHs in batches of the pH experiment; the heat pre-treated triplicate are indicated in bullets (● as concentration and ○ as rate).

3.3.1 Effect of pH on methanogenesis and acetate reduction

Figure 3.2 shows in log scale the methane production at each pH from 4.5 to 8. Methane production differed strongly between the various pHs; methane production at pH 7 and 8 was more than 4 times higher than at pHs 4.5, 5 and 6. The highest methane partial pressure was 1.02 bar measured in the batches at pH 7 after 7 days, which was 97.8 ± 0.5% of the total headspace. At this pH, all acetate and hydrogen were consumed after 7 days and converted mainly to methane, while at other pH's there was still acetate and hydrogen present. After 30 days, 58.0 ± 5.8% of the reactants was left at pH 6 and 83.3 ± 4.3% at pH 5. The highest methane production rate of 26.6 ± 1.8 mmol L^{-1} d^{-1} was also observed at pH 7, which is 33 times higher compared to the rate at pH 6.

The maximum ethanol concentration at each pH increased with increasing pH up to 6 (Figure 3.3). At a pH 7 and 8, the maximum ethanol concentration is lower compared to pH 6. The highest alcohol concentration is measured at pH 6 amounting to 7.40 ± 1.36 mM after 13 days of incubation. In Figure 3.3, the pattern of the ethanol production rate curve is similar to the curve of the maximum ethanol concentrations as function of pH. So just like the concentration, the production rate increased with increasing pH to a maximum of 1.50 ± 0.25 mmol ethanol L^{-1} d^{-1} at pH 6. At pH 7 the ethanol production was remarkably lower than at other pHs.

n-Butyrate was also found at every pH from 4.5 to 8 as shown in Figure 3.4. The maximum *n*-butyrate concentration at different pH values has a similar pattern as the curve of the maximum ethanol concentration curve in Figure 3.2. The highest *n*-butyrate concentration was also obtained at pH 6 amounting to 10.4 ± 2.19 mM. The electron and carbon balances were closed for the batches at all pHs; except for pH 7 and 8 with a higher methane production (Table 3.1). At pH 7 and 8, the electron as well as the carbon balance was positive. The excess of carbon and electrons equivalents most certainly derived from the solid phase due to sludge decay. This seems in agreement with Veeken et al. (2000), who concluded that the hydrolysis rate is pH dependent and was higher at higher pH for solid organic waste. The electron access is taken into account during efficiency calculations. Table 3.2 shows the efficiencies of the main products ethanol, methane and *n*-butyrate calculated with the highest concentrations in the batch. The highest methane

Table 3.2 Efficiencies of ethanol, *n*-butyrate and methane formation calculated for the highest concentration of product in the pH and the thermal pre-treated experiment.

pH	Ethanol %	Butyrate %	Methane %
4.5	5.2 ± 1.1	47.8 ± 7.0	23.8 ± 5.1
5	23.4 ± 9.7	57.7 ± 8.4	4.7 ± 8.1
6	55.0 ± 12.2	65.3 ± 6.7	5.2 ± 4.6
7	0.0 ± 0.0	0.0 ± 0.0	97.8 ± 0.5
8	0.7 ± 0.5	1.5 ± 1.1	53.8 ± 1.0
5	21.0 ± 2.9	58.5 ± 4.8	22.7 ± 5.4
5.5	7.3 ± 7.3	87.2 ± 3.5	5.7 ± 0.6
6	30.9 ± 7.8	61.0 ± 9.6	3.8 ± 3.4
Therm. 6	58.6 ± 7.4	76.2 ± 14.0	0.0 ± 0.0

production efficiency was calculated for pH 7 and the lowest efficiency was calculated for low pH 5 and 6. The highest efficiency for ethanol as well as *n*-butyrate was at most at pH 6, while the methane production efficiency was one of the lowest values 5.2 ± 4.6%. In the batch at pH 6, 55.0 ± 12.2% of consumed acetate and hydrogen was converted into ethanol at 13 days, where after ethanol concentration decreased. At that moment *n*-butyrate production increased to a total conversion efficiency of 65.3 ± 6.7% at 30 days.

3.3.2 Effect of thermal pre-treatment on methanogenesis and acetate reduction

The influence of heat pre-treatment on the fermentation was determined at the best performing pH. The best results were achieved at pH 6 at which the ethanol production was the highest in concentration, rate and efficiency, while methane formation remained low. Methane formation was almost completely inhibited in the heat pre-treated batches. Methanogenesis was excluded in two of the heat pre-treated triplicate. The remaining methanogenic activity in one of the triplicate can be explained by infection with methanogens during sampling. Ethanol was still produced after thermal pre-treatment of the batches. The ethanol production was even higher in concentration and production rate than in the untreated batch at the pH 6 (Figure 3.3). The ethanol production efficiency of 58.6 ± 7.4% was higher compared to the non-treated batches which had an efficiency of 30.9 ±

7.8%. *n*-Butyrate formation was not inhibited by thermal pre-treatment. The efficiency of *n*-butyrate formation was even higher with 76.2 ± 14.0% due to the methane inhibition than the non-treated batch at pH 6 with 61.0 ± 9.6%.

3.3.3 Thermodynamics and acetate reduction

The Gibbs free reaction energy of acetate reduction to ethanol at different pH rises with increasing ethanol concentration (Figure 3.5). At a certain level of ethanol concentration, the Gibbs free reaction energy is higher than -20 kJ and becomes biological limited, or is higher than 0 kJ and becomes exothermic. For both Gibbs free energy values, the maximum achievable ethanol concentration is calculated.

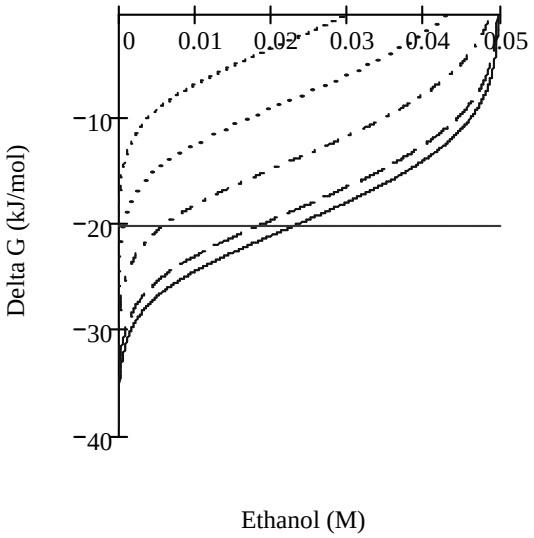

Figure 3.5 Actual Gibbs free reaction energy of acetate reduction as function of the ethanol concentration (in mol) calculated starting with 50mM acetate and 1.5 bar hydrogen at pH 8 (.....),7 (.....), 6(— .), 5(— .), and 4.5 (——) and the horizontal thermodynamic limit of -20 kJ/mol (——).

Table 3.3 shows those theoretical maximum ethanol concentrations together with the experimental ethanol concentrations at the different pHs. At pH 6 and 8, the experimental ethanol concentrations exceeded the theoretic maximum calculated for the limit of -20 kJ mol^{-1}. At pH 4.5, 5 and 7, the actual ethanol concentrations remained below the theoretical maximum concentrations calculated with both limits. Figure 3.6 shows the actual Gibbs free reaction energy of the three dominant reactions; acetate reaction with

Table 3.3 Highest ethanol concentrations that are experimental determined and that are theoretical maximally at a reaction Gibbs free energy of 0 and -20kJ.

pH	*Max. ethanol conc.(mM)*			
	Experimental	Experimental	Theoretical with -0kJ	Theoretical with -20kJ
4.5	0.41 ± 0.04		49.9	23.2
5	2.09 ± 0.13	1.50 ± 0.32	49.8	18.7
5.5		2.95 ± 0.57	48.8	12.2
6	7.40 ± 1.36	5.20 ± 1.17	48.7	5.8
Therm 6		7.72 ± 3.17		
7	0.63 ± 0.01		43.5	0.8
8	1.62 ± 0.16		30.5	0.1

hydrogen to ethanol, acetate reduction with ethanol to *n*-butyrate and acetoclastic methanogenesis. Hydrogenotrophic methanogenesis was not considered as only little CO_2 is available. The Gibbs free energy values were calculated with data of a batch run at pH 6. Methane formation was energetically the most favorable reaction during the whole run. Ethanol formation was more favorable than *n*-butyrate production when if only little ethanol was present in the batch. Only as soon as ethanol was produced, from the second day on, it was thermodynamically more favorable to produce *n*-butyrate as end-product.

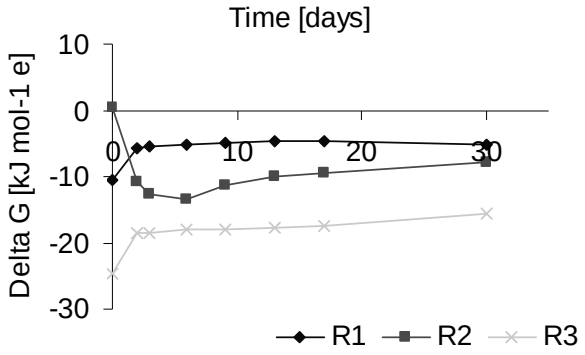

Figure 3.6 Gibbs free reaction energy per mol electron of the three product formations as function of time with experimental data of the batch at pH 6 without thermal pre-treatment: R1(♦) for ethanol (Equation 1.10) and R2 (■) for *n*-butyrate (Equation 1.11) and R3 (x) for methane via acetoclastic methanogenesis (Equation 1.12).

3.4 Discussion

Three dominant biological conversions of hydrogen and acetate were observed in all experiments: methanogenesis, ethanol production and *n*-butyrate formation. Methanogenesis was selectively inhibited while ethanol production was enhanced at a controlled pH of 6 or lower and after a heat pre-treatment of 15 minutes before incubation at pH 6.

3.4.1 Effect of pH

The pH influenced all three anaerobic processes; methanogenesis was inhibited by a pH 6 or lower, ethanol production and *n*-butyrate production from ethanol were optimal at pH 6. Observed inhibition of methanogenesis at pH 6 corresponds to literature values of Van Haandel and Lettinga (1994). According to them, the methanogenesis rate decrease or stops at a pH lower than 6.3. Similar findings were found in this study as the methanogenesis rate was 33 times smaller at pH 6 compared to that at pH 7. So lowering the pH decreased the methane production, however, it also affected eventually the ethanol production. Ethanol production increased with increasing pH between 4.5 and 6 and decreased again at a pH higher than 6. Considering the effect of pH on ethanol production, it was not possible to compare the results with previous research on ethanol by acetate reduction by Steinbusch et al. (2008), wherein, despite the buffer, the pH had risen from 5 to 5.6 in 21 days. But it was clearly shown that from the perspective of ethanol yield and production rate, acetate reduction at pH 6 was optimal for ethanol production, while acetate and hydrogen consumption towards methane was prevented.

Methanogenesis could be inhibited by lowering the fermentation pH, though the final ethanol production efficiency remained low due to production of *n*-butyrate. *n*-Butyrate production was not reported in the similar set up by Steinbusch et al. (2008). They did not detect *n*-butyrate during 21 days of fermentation at uncontrolled pH starting at 5. Whereas in this experiment, *n*-butyrate was the most dominant product to be found at pH 4.5, 6 and 7 after 30 days. Apparently, *n*-butyrate producing populations were present in this research or were earlier active in the experiment. That *n*-butyrate production started 9 days later than the ethanol production, which was formed directly after starting-up the experiment in Figure 3.1. The formation of *n*-butyrate

seems though, related to ethanol production. First of all, *n*-butyrate production started when ethanol was already present, and progresses as ethanol was consumed over time. Second, there is a relation between ethanol and *n*-butyrate formations, regarding the product concentration and rate. The highest *n*-butyrate and ethanol concentrations and production rates were both observed at pH 6. Third, the high deviation in ethanol and *n*-butyrate concentrations can be explained by the fact that ethanol consumption/n-butyrate production in each batch was initialized at a slightly different time. Based on this, we assume that ethanol was consumed to produce *n*-butyrate. The influence of pH on *n*-butyrate production is hard to predict as the production rate is most likely limited by the ethanol production rate. This assumption is supported by the fact that the *n*-butyrate conversion efficiency is higher than the ethanol conversion efficiency at pH 4.5, 5 and 6.

3.4.2 Effect of thermal pre-treatment

The thermal pre-treatment almost completely inhibited methane formation, while ethanol formation remained unaffected. As noted by Schlegel, methanogens are heat sensitive and are together with many other heat sensitive microorganisms killed when they are exposed to temperatures over 80°C (1986). The exposure to heat did not inhibit ethanol and *n*-butyrate production. On the contrary, the heat pre-treated batches yielded even 1.5 times higher ethanol concentrations after thermal pre-treatment compared to without pre-treatment. It is therefore very likely that both the organisms producing ethanol and the organisms producing *n*-butyrate are heat-resistant bacteria that can survive high temperature by for example forming spores. A reasonable explanation for the high ethanol concentration in the experiment with heat pre-treatment is the difference in hydrogen partial pressure. The hydrogen pressure in the heat pre-treated batch was higher ($1.18 \cdot 10^5 \pm 0.09 \cdot 10^5$ Pa) than the final pressure in the batch without heat treatment ($0.99 \cdot 10^5 \pm 0.03 \cdot 10^5$ Pa). As hydrogen pressure is a driving force of acetate reduction, it means that the higher the pressure is, the higher ethanol concentrations can be reached. Not all can be explained by thermodynamics: a decrease of pH decreases the Gibbs free reaction energy and therefore higher final ethanol concentration would be expected at lower pH. From Figure 3.3, however, it is clearly that the ethanol concentration decreased with a decreasing pH from 6 to 4.5. Most likely also other phenomena limit

the ethanol production at low pH such as toxicity and a different internal pH (Schlegel, 1986).

3.4.3 n-Butyrate production

The *n*-butyrate production dominance can be explained by thermodynamics. First, ethanol production through acetate reduction becomes thermodynamically limited by the ethanol concentration itself. That may have happened in the experiments at pH 6 or 8, in which the Gibbs free energy for acetate reduction reaction exceeds the lower limit of -20 kJ mol^{-1} reaction. Second, it was thermodynamically more favorable to produce *n*-butyrate from acetate and hydrogen over ethanol. Thermodynamics determine the limit of the reactions and within the boundaries of constraints the process can be steered by regulating environmental conditions like pH, temperature or concentrations. For example, of the three dominant processes, actually methane formation was energetically the most favorable process, but could be prevented by removing methanogens physically with heat.
A known *n*-butyrate producing bacterium using acetate and ethanol was first described by Barker et al. (1945) who reported the discovery of the anaerobic soil bacterium *Clostridium kluyveri*. A typical fermentation balance of *C. kluyveri* is described by Gottschalk (1986):

$$6C_2H_6O + 3C_2H_3O_2^- \rightarrow 3C_4H_7O_2^- + C_6H_{11}O_2^- + 2H_2 + 4H_2O + H^+ \qquad (3.2)$$

Caproate was also detected in this research only in very little amounts to 0.26 ± 0.11 mM. As a result, the production ratio caproate/n-butyrate was with 0.002 far lower than the 0.33 of the typical fermentation balance of Gottschalk. So, *n*-butyrate production in this research seems comparable to the fermentation of *C. kluyveri* only less caproate is produced. However, to produce *n*-butyrate from only acetate and hydrogen has not been demonstrated before, by combining the acetate bio-reduction to ethanol and *n*-butyrate fermentation. This opens new perspectives for the conversion of organic wastes that not only alcohols can be formed, but pure products as *n*-butyrate as well. The product *n*-butyrate can be used as chemical or food: in the food, chemical and pharmaceutical industry as pure acid or in the form of esters as a food additive to increase fruit fragrance (Zigova and Šturdík, 2000). According to them, *n*-butyric acid plays also an important role in the plastic materials and textile fibers industries. The production of pure *n*-butyrate from organic waste materials would have the biggest advantage that

uses, instead of sugars, raw material that is cheap and abundantly present. Further research on ethanol should focus on how to prevent losses of hydrogen and acetate via *n*-butyrate production. Ethanol producing organisms can be enhanced by applying different process conditions such as temperature and SRT, and by finding inhibiting factors for *n*-butyrate production organisms. Laboratory studies can be combined with the use of molecular techniques to selective remove other hydrogen or ethanol consuming bacteria. Besides, if *n*-butyrate production increase from acidified waste, it can be harvested and purified and used as chemical. Purification of organic acids determines their costs. The potential of both ethanol and *n*-butyrate production conversion from organic waste can be studied by coupling an acidification tank to acetate reduction reactor.

3.5 Conclusions

Methanogenesis was selectively inhibited at pH 6 or lower and with a thermal pre-treatment of the inoculum, while the production of ethanol and of *n*-butyrate from waste products acetate and hydrogen was enhanced. After heat pre-treating the inoculum, the ethanol concentration was reached of 7.7 ± 3.2 mM at a production rate of 1.22 ± 0.04 mmol L^{-1} d^{-1} with an efficiency of 58.6 ± 7.4%. Ethanol was converted into *n*-butyrate, which was finally the most dominant reaction during acetate reduction at all pHs. Highest *n*-butyrate concentration after pre-treatment was reached at pH 6 and was 7.02 ± 0.85 mM at a production rate of 0.38 ± 0.02 mmol L^{-1} d^{-1} with an efficiency of 76.2 ± 14.0%. The dominant effect of *n*-butyrate over ethanol production was supported by thermodynamical data. With this research, a potential new method to produce *n*-butyrate from acidified organic waste material was demonstrated.

4 Bioelectrochemical acetate reduction

Proof of principle

Abstract

Biological acetate reduction with hydrogen is a potential method to convert wet biomass waste into ethanol. Since the ethanol concentration and reaction rates are low, this research studies the feasibility of using an electrode, in stead of hydrogen, as an electron donor for biological acetate reduction in conjunction of an electron mediator. Initially, the effect of three selected mediators on metabolic flows during acetate reduction with hydrogen was explored; subsequently, the best performing mediator was used in a bioelectrochemical system to stimulate acetate reduction at the cathode with mixed cultures at an applied cathode potential of -550 mV. In the batch test, methyl viologen (MV) was found to accelerate ethanol production 6-fold and increased ethanol concentration 2-fold to 13.5 ± 0.7 mM compared to the control. Additionally, MV inhibited *n*-butyrate and methane formation, resulting in high ethanol production efficiency (74.6 ± 6%). In the bioelectrochemical system, MV addition to an inoculated cathode led directly to ethanol production (1.82 mM). Hydrogen was coproduced at the cathode (0.0035 normalized m^3 hydrogen m^{-2} d^{-1}), so it remained unclear whether acetate was reduced to ethanol by electrons supplied by the mediator or by hydrogen. As MV reacted irreversibly at the cathode, ethanol production stopped after 5 days.

A modified version of this chapter has been published:

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4.1 Introduction

Sustainably produced biofuel is of great demand in our society. The sustainability of biofuel production is substantially determined by the feedstock choice (Tilman et al., 2009). The chosen raw material has implications for food production as well as for the eventual reduction of greenhouse gas emissions of the biofuel as compared to traditional fossil fuel. Using biomass waste for biofuel production potentially emits less greenhouse gases than using cultivated energy crops and does not compete for land with food production (Searchinger et al., 2008). Furthermore, exploiting waste as biomass feedstock is estimated by the US Department of Energy to have the largest potential for biofuel production (Perlack et al., 2005), as it is a cheap resource and is abundantly present in all rural areas. Biomass waste can be converted by two general conversion types: biological and thermal conversion (Cantrell et al., 2008). Thermal conversion is considered to be only applicable to dry waste, whereas biological conversion can also be applied to waste with a high moisture content. Biological conversion such as anaerobic digestion that converts wet biomass waste into biogas is a well established technology, whereas conversion of biomass waste to liquid fuels as ethanol is only in the exploratory research phase (Kleerebezem and van Loosdrecht, 2007). Ethanol has been produced by microbial reduction of acetate as the main intermediate of anaerobic digestion with hydrogen as electron donor (Steinbusch et al., 2009). Hydrogen can be produced by acidification of wet biomass but also by bioelectrolysis in a bioelectrochemical system (Logan, 2004). A BES oxidizes acetate at the anode and biologically reduces protons at a cathode to hydrogen. Hydrogen production in such a system has been shown to be energy efficient and highly selective. Here we propose to reduce the acetate to ethanol in the cathode compartment of a BES. Our objective is to study the feasibility of using the cathode as electron donor for biological acetate reduction by mixed cultures. Mediators are used namely to accelerate electron transport from the cathode to organisms; moreover, they can influence the metabolism of organisms and block parasitic reactions such as methanogenesis. Thus, initial work studied the influence of three selected mediators on the metabolic flows of mixed cultures during acetate reduction. Here, hydrogen was used as electron donor so that the electron transfer capacity of each mediator did not influence the outcome of the experiment. Subsequently, the best performing mediator was used in the cathode of a

BES to stimulate acetate reduction at the cathode mixed cultures to prove the principle that the cathode is used as electron donor.

4.2 Materials and Methods

Inoculum

Acetate-reducing inoculum was obtained from an upflow anaerobic sludge blanket reactor treating distillery wastewater (Royal Nedalco). Before inoculation, the sludge was washed three times with tap water and three times with medium.

Mediator Selection

Mediators for biological acetate reduction were selected on the basis of the criteria of Fultz and Durst (1982). They compiled a list of appropriate electron mediators that are soluble, completely reversible, stable, and do not interact with other molecules. Mediators from the list should be selected that have an electrochemical potential (118 mV from the electrochemical potential of studied reaction and involved biocomponent. In this way, the ratio between reduced and oxidized form of mediator is in the range of 0.01-100 (according to the Nernst equation), in which the mediator is assumed to be applied most effectively. The actual potential of acetate reduction is -433 mV calculated with the Nernst equation using actual conditions of previous experiments: pH 5.5, acetate 50 mM, 2 mM ethanol at 30°C (Steinbusch et al., 2008). The biocomponent that is most likely involved in biological reduction reactions is NADH with-320 mV. All potentials are reported against normal hydrogen electrode (NHE). Three different mediators that comply with the described criteria and cover the potential of acetate reduction and NADH were chosen: anthraquinone-2,6-disulfonate (AQDS, E^0= -184 mV), neutral red (NR, E^0= -325 mV), and 1,1'-dimethyl-4,4'-bipyridyl dichloride (methyl viologen, MV, E^0= -440 mV). All mediators were demonstrated to be biologically active in strict or facultative anaerobic conversions (Günther and Simon, 1995).

Mediator Tests

Serum bottles (120 mL) were filled in triplicate with 0.24 g of VSS sludge and 50 mL of medium containing 50 mM acetic acid and 1 mM mediator of each mediator: NR (Sigma), AQDS (Aldrich), or MV hydrate (Aldrich). To identify the mediator effect on ethanol production, a control triplicate without

mediator was included in the setup. The pH of the final medium was adjusted to pH 5.5 with 2M sodium hydroxide. The bottles were sealed with rubber stoppers and capped with aluminum crimp caps. The headspace was purged five times with pure hydrogen to a pressure of 1.5 bar. The bottles were incubated at 30°C in a rotating shaker (170 rpm) for 22 days. At day 0, 1, 2, 5, 8, 16, and 22, the gas pressure was measured by a pressure meter GMH 3150 (Greisinger Electronics) and a gas sample (0.1 mL) was taken with a gas syringe to analyze the gas composition directly at the GC. Additionally, a liquid sample (0.5 mL) was taken anaerobically with a syringe and collected in a reaction tube that was centrifuged for 5 min at 10 000 rpm. After spinning down the solids, the liquid was diluted with formic acid solution to 1.5% (v/v) for VFA analysis and with water for alcohol analysis.

Setup of BES

The BES was a flat plate reactor of Plexiglas described by Ter Heijne et al. (2006). Both cathode and anode were a graphite felt electrode (21.9 × 21.9 × 0.3 cm, FMI Composites Ltd.) with a projected effective surface area of 290 cm^2. The reference electrode was a Ag/AgCl, 3MKCL electrode (QM 711, 4×50 mm PVC, Qis). The compartments were separated by a monovalent selective anion-exchange membrane (Neosepta ACS; Tokuyama Co.). The cathode compartment was connected to a MilliGascounter type MGC-1 (Ritter) via an injection port and an overflow bottle by Marprene tubings. The anode compartment was closed by a waterlock. Including the gas outlet, injection parts, and tubes, the cathode volume was 0.870 L and the anode volume was 0.802 L.

Operation of the BES

The anode compartment was filled with 0.2 M hexacyanoferrate [$K_4(Fe(CN)_6)\cdot 3H_2O$] and the cathode compartment with growth medium as described in the mediator tests. The pH of the cathode medium was regulated at 6.0 with 2 M NaOH and 2 M HCl solutions with a pH controller. The BES was operated in batch mode as a three-electrode system with a working electrode (cathode), a reference electrode in the cathode compartment, and a counter electrode (anode). The BES was operated at constant cathode potential of -550 mV vs NHE. The cell voltage, that is the working electrode compared to the counter electrode, was adjusted by a DC power supply (HP 6632A, Hewlett-Packard Development). Cell voltage,

cathode potential (working electrode compared to reference electrode), and current were recorded every 30 s on a PC via a data acquisition unit (HP 3421A, Hewlett-Packard Development). Data were collected using LabVIEW, which was programmed to maintain the cathode potential stable at -550 mV by correcting cell voltage with the power supply in an internal loop. The BES was cleaned with 6% hydrogen peroxide water solution to oxidize biological material before operation (Rozendal et al., 2006). Three days after filling both compartments the cathode potential was controlled at -550 mV. The voltage of the applied potential was adapted from a polarization test of the BES. Four days after filling the reactor, inoculum was added. After 1 day more MV was added to 1 mM. In the control run, the potential was applied directly after filling the reactor and MV was added after 3 days. Two pumps (505U Watson Marlow) recycled anode and cathode media through the BES at 30 rpm. Sludge concentration in the cathode was 9.1 g VSS L^{-1} and 4.0 g VSS L^{-1} in the control run.

MV exposure test

Batch tests were performed to see if MV reacted with one of the components it was subjected to in the BES. These components were medium, light, air, N_2, H_2, hexacyanoferrate (0.1 M), inoculum, tubing, membrane, electrode, and Plexiglas. In total, 11 different combinations of agents and a control with demineralized water were exposed to MV in triplicate in 120 mL serum bottles, as described in Table 4.1. Each bottle contained 80 mL of liquid and 40 mL of gas and was closed with a rubber stopper. In all batches, MV concentration at t=0 d was 1.0 mM and the pH was adjusted to 6. The bottles were shaken at 100 rpm at 30 °C. At day 8, 20, and 46, 0.5 mL samples were taken to analyze MV concentration.

Chronoamperometry

The cathode potential for the BES was selected on the basis of the results of chronoamperometry. With chronoamperometry, the electrochemical activity of the system with and without microorganisms was measured at different cathode potentials with an IviumStat (type 10 V/5A, Ivium Technologies). The cathode potential was decreased every 200 s by 50 mV starting from -200 to -600 mV and increased again to -200 mV again by 50 mV. At each potential, the current production was averaged over the last 15 s.

Table 4.1 Addition scheme of the exposure test with the agents or combinations of agents to which MV was subjected to in the BES.

Medium	*Light*	*N2*	*Air*	*H2*	*$K_4Fe(CN)_6$*	*Biomass*	*Tube*	*Membrane*	*Electrode*	*Plexiglas*
		+								
		+				+				
		+					+			
		+						+		
		+							+	
		+								+
+		+								
			+							
				+						
					+					
	+	+								
+	+	+				+				
+	+		+			+				

Analyses

Hydrogen, methane, carbon dioxide, nitrogen, oxygen, C2-C5 alcohols, and C2-C5 VFA were measured by gas chromatography (Steinbusch et al., 2008). $MV^{+\bullet}$ and MV^{2+} were measured spectrophotometrically according to AOAC method 969.09 (1996). Small adaptation was made: the dilution step with sodium dithionite solution was directly performed in a cuvette of 1 mL, so that prepared sample could immediately be analyzed, as rapid oxidation of the MV occurred.

Calculations

Conversion efficiencies were described as the efficiency of electron flow from reactants to products. Following McCarty, the electron flow was expressed in electron equivalents (in mol e) based on concentrations, carbon atoms, and degree of reduction of each individual compound (1972). Conversion efficiency in the mediator test is defined as the proportion of consumed electron equivalents of acetate and hydrogen that was converted to electron equivalents of ethanol or other products as *n*-butyrate. The efficiency was corrected for electron equivalents that entered the liquid phase due to sludge decay (Steinbusch et al., 2008). The Coulombic efficiency in the BES is

defined as the percentage of supplied electrons that was converted to P product in the cathode:

$$e = \frac{([P]_{t2} - [P]_{t1}) \quad V \quad n \quad F}{\int_{t1}^{t2} Idt} \quad 100\% \qquad (4.1)$$

with $[P]_t$ product concentration at time = t (mol L^{-1}), n number of electrons involved in the reduction, F the Faraday constant (C mol^{-1}), and I the current (A). The electron equivalents that might derive from the inoculum were disregarded in the calculation.

4.3 Results

MV and NR increased ethanol concentration and production rate.

Addition of MV and NR in batch experiments resulted in higher ethanol concentration and production rate compared to the control (Figure 4.1 and 4.2). The highest ethanol concentration was obtained with MV was 2.0 times higher than in the control and with NR this was 1.2 times higher. Ethanol concentrations in the presence of NR decreased again after 8 days due to formation of *n*-butyrate, and experiments with MV showed increased ethanol concentrations. The concentration of the major products of this batch experiment, especially ethanol, *n*-butyrate, and methane, were dependent on the type of mediator added (Figure 4.3). Methane was only formed in the

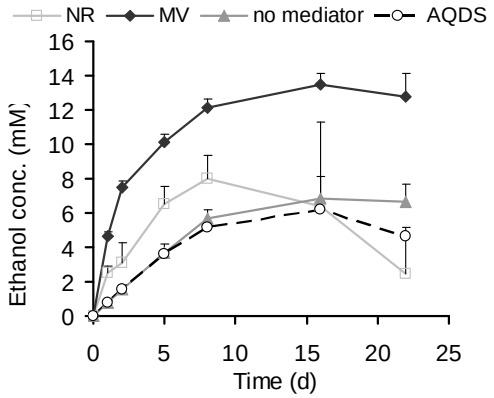

Figure 4.1 Ethanol production in presence of mediators NR, MV, AQDS and without mediator (control) over the course of 22 days.

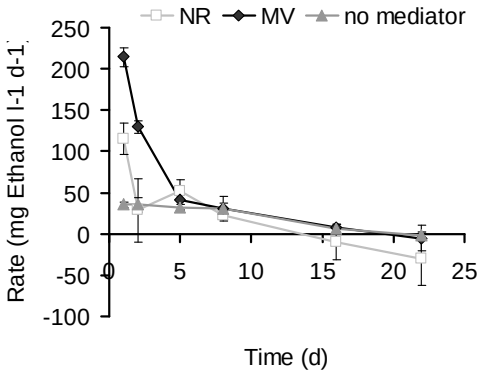

Figure 4.2 Ethanol production rate in presence of mediators NR, MV, AQDS and without mediator (control) over the course of 22 days.

control and with AQDS. The ethanol production rate in the presence of MV and NR was also higher compared to the control; the rate over the first two days was 6.0 times higher with MV and 3.2 times higher with NR. Addition of mediator AQDS did not affect ethanol production in concentration nor in production rate compared to the control. Hydrogen was consumed in all batches. The highest hydrogen consumption rate occurred in MV experiments over the first two days and was 2.4 times higher than the control.

High ethanol production efficiency with MV due to inhibition of n-butyrate production and methanogenesis

Table 4.2 shows the conversion efficiency of the three dominant processes in the mediator test after 22 days: ethanol and *n*-butyrate production and methanogenesis. Ethanol production efficiency in the presence of MV was the highest at 74.6 ± 6.3%, an improvement of 3.4 times over the control. This

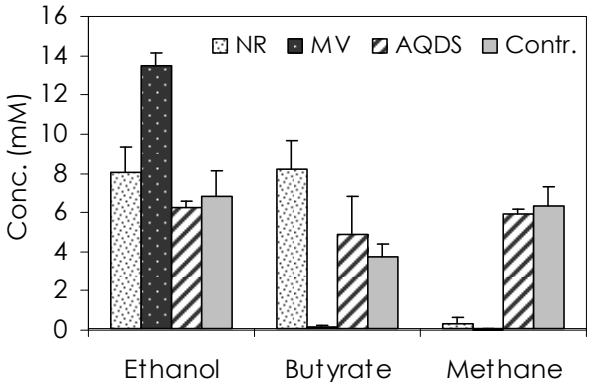

Figure 4.3 Highest ethanol concentrations during the experiment of ethanol, *n*-butyrate and methane with mediators NR, MV, AQDS and without mediator (control).

can be explained by the fact that in the presence of MV no significant quantity of side products was found. Methanogenesis was inhibited by addition of MV as well as NR. In contrast, in control batches and experiments with AQDS, high efficiencies of 41.3 ± 8.3% and 47.3 ± 25.4%, respectively, were observed for the side products *n*-butyrate and methane. Methanogenesis was inhibited by addition of NR and MV and had a conversion efficiency of nearly 0. *n*-Butyrate production was the most dominant reaction in the presence of NR, but was hardly observed in the presence of MV experiments. The conversion efficiency of ethanol production in the presence of NR was first 82.5 ± 2.6% after 8 days but declined to 38.3 ± 16.7% after 22 days when *n*-butyrate was produced with a conversion efficiency of 58.9 ± 8.2%. *n*-Butyrate production in the presence of NR was even higher in concentration, rate, and efficiency than in the controls.

The conversion efficiencies were corrected for the amount of electrons or carbons that were derived from the sludge, since the carbon recovery (107.6-110.4%) and the electron recovery (98.2-120.6%) were higher than 100% except for the batches with MV. This indicates that more carbon and electrons were recovered in the gas and liquid phase than was initially present in hydrogen and acetate.

Table 4.2 Conversion efficiency (%) of products in batch experiments with or without mediator after 22 days

Products	*Efficiency per mediator (%)*			
	NR	MV	AQDS	Control
Ethanol	38.3 ± 16.7	74.6 ± 6.3	8.7 ± 7.9	21.8 ± 5.2
Methane	0.4 ± 0.3	1.5 ± 0.1	21.5 ± 2.8	20.8 ± 1.1
Butyrate	58.9 ± 8.2	0.5 ± 0.5	25.8 ± 22.6	19.5 ± 7.2

Ethanol and H_2 produced by mixed cultures at the cathode in presence of MV

In the BES containing inoculum, ethanol and current production started directly after MV addition. Initial rapid ethanol production during the first 2 days was followed by a slower phase to a concentration of 1.82 mM (Figure 4.4a). A similar trend was observed for the current, which peaked at 1.33 A m^{-2} (Figure 4.5). The decrease in current production may be related to the decline in MV concentration from initially 1 mM to <0.05 mM in 5 days (Figure 4.5). At the moment MV dropped below the detection limit, current

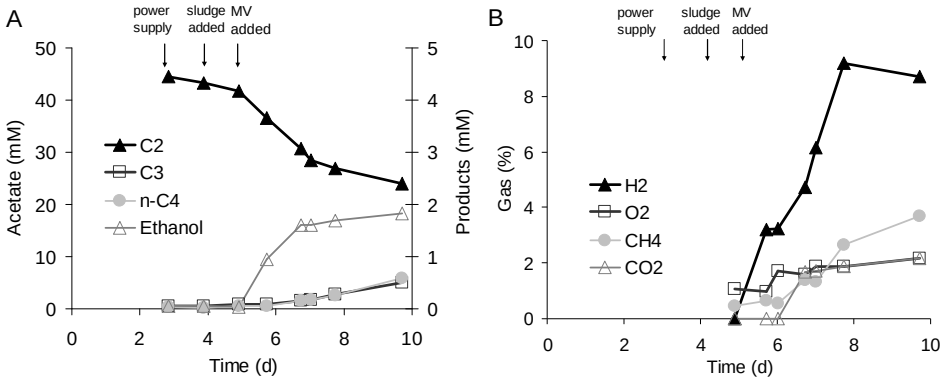

Figure 4.4 (a) Ethanol, propionate and *n*-butyrate production and (b) biogas production in time during acetate consumption at the cathode.

density was nearly zero and ethanol production stagnated, whereas VFA production as *n*-butyrate started at low MV concentration. MV addition affected the gas composition, as shown in Figure 4.4b. Hydrogen production started also directly after MV addition, peaked at $9.2 \cdot 10^3$ Pa in 3 days, and then gradually decreased until finally a vacuum was created. In the abiotic control run, MV addition caused neither ethanol nor hydrogen production.

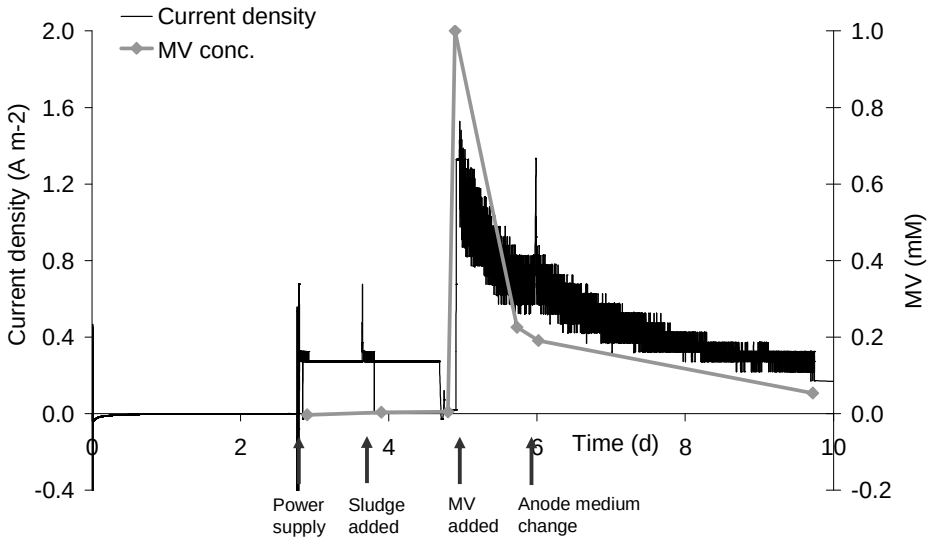

Figure 4.5 Current density (primary y-axis) and MV concentration (secondary y-axis) as a function of time both changed as soon as MV was added to the BES.

Moreover, the current density in the first 25.3 h after MV addition was 3 times lower than in the BES with inoculum. It most be noted that in the exposure tests MV concentration remained the same before and after exposure to the materials and agents of the BES.

Performance of the BES with cathode potential of - 0.55 V

In the polarization test, it was found that the current density increased as the cathode potential decreased from -450 to -600 mV (Figure 4.6). Increasing the potential from -600 to -200 mV, it was found that until -550 mV the current density was still substantial. At a cathode potential higher than -500 mV, the current density decreased fast. Therefore, the cathode potential in the BES was set at -550 mV for both reactor runs. The peak current density was 1.33 A m^{-2} directly after MV addition and decreased to 0.27 A m^{-2} after 10 days, whereas the cathode voltage was kept stable at -550 mV. The current density was high compared to the current density for a biocathode producing hydrogen, which has been shown to be less than 0.2 A m^{-2} at a similar over potential (Jeremiasse et al.). The highest production rate of ethanol was 1.26 mmol m^{-3} cathodic compartment d^{-1} or, expressed per projected effective cathode surface area, was 0.377 mmol m^{-2} d^{-1}. Hydrogen was produced in 0.012 normalized (N)m^3 m^{-3} cathodic compartment d^{-1} or in 0.0035 Nm^3 hydrogen m^{-2} d^{-1}.

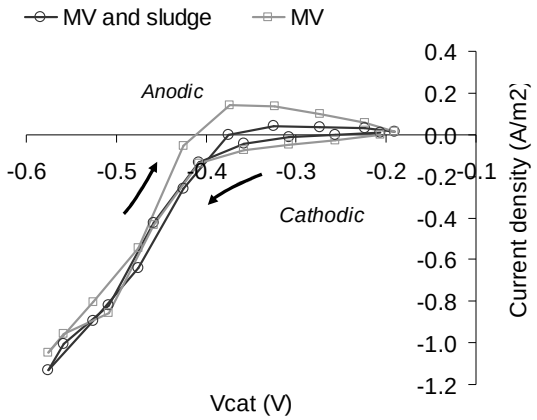

Figure 4.6 Polarization curve of the a cathode potential with 1 mM MV with or without sludge as function of the current density in the chronoamperiometry test.

Ethanol production had a coulombic efficiency of 49%.

In total, four major products were formed at the cathode: ethanol, hydrogen, *n*-butyrate, and the nonreversible reduced MV^{2+}. All products were detected simultaneously with the start of current production directly after MV addition. Therefore, it is assumed that the four products are a result of reduction reactions, as shown in Equation 4.2-4.5, in which the electrons are supplied directly by the cathode or indirectly via MV, hydrogen, or ethanol.

$$C_2H_3O_2^- + 5H^+ + 4e^- \rightarrow C_2H_6O + H_2O \quad (4.2)$$

$$2H^+ + 2e^- \rightarrow H_2 \quad (4.3)$$

$$MV^{2+} + 2e^- \xrightarrow{reversible} MV^{+\bullet} + e^- \xrightarrow{irreversible} MV^0 \quad (4.4)$$

$$2C_2H_3O_2^- + 5H^+ + 4e^- \rightarrow C_4H_7O_2^- + 2H_2O \quad (4.5)$$

On the basis of these reactions, the distribution of electrons among the products is calculated and shown in Figure 4.7. Ethanol took the largest product share of total amount of electrons, which was 49% on the first day after MV addition and decreased to 12% after 5 days of batch operation.

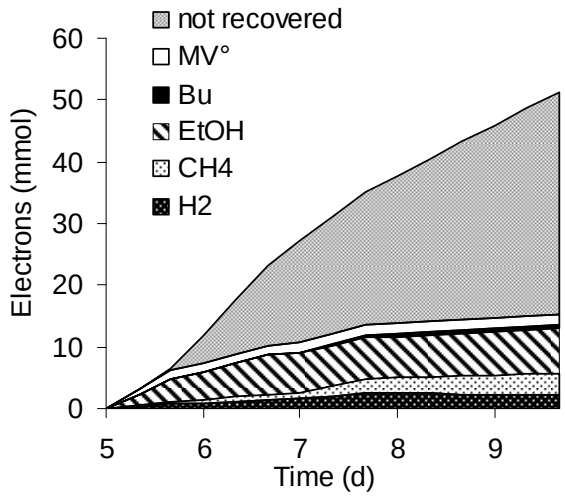

Figure 4.7 Distribution of electrons supplied to the cathode among products during the first 5 days after MV addition.

4.4 Discussion

MV enhanced ethanol production compared to the control with respect to concentration, production rate, and efficiency, while NR enhanced ethanol production in rate and efficiency only in the short term. The high efficiency of MV could be reached due to the fact that two dominant side processes, methanogenesis and *n*-butyrate production, were inhibited by MV. Inhibition of methanogenesis by MV was reported earlier by Wolin et al. (1964). Working with *Methanobacillus omelianskii*, they showed that methane formation from ethanol or hydrogen and carbonate was suppressed with 1.6 µM MV. The inhibiting mechanism of MV on *n*-butyrate production was also claimed for *Clostridium acetobutylicum*. Peguin et al. (1994) reported that MV addition to the fermentation directly stopped growth and shifted product formation from *n*-butyrate to butanol production. Ethanol production enhancement of NR was short-term, because the mediator seemed to inhibit only methanogenesis and not the ethanol-consuming reaction to *n*-butyrate. So MV seemed to be a suitable mediator to transport electrons for acetate reduction at the cathode, as it inhibited side reactions and it enhanced ethanol production.

High electron and carbon recovery is explained by the increase of electrons and carbons from decomposing sludge. Also the solubilization of carbonate salts from the granular sludge may be a plausible explanation for the higher carbon recovery than the electron recovery (Van Langerak et al., 1998). This research showed that it was feasible to produce ethanol from acetate at the cathode of a BES in the presence of mixed cultures and MV. MV initiated ethanol production at the inoculated cathode in the BES at an applied cathode potential of -550 mV. Ethanol production was only achieved when the cathode was inoculated and MV was added. Ethanol production lasted 2 days and reached a concentration of 1.82 mM. The Coulombic efficiency decreased from 49% on the first day to 12% after 5 days of operation. This decline can be related to biomass growth or product loss by diffusion. The diffusive hydrogen loss over the membrane, for example, plays also a dominant role as an electron sink (Rozendal et al., 2006). At current densities lower than 0.5 A m^{-2}, electron lost via hydrogen diffusion over the membrane increases. Still, the measured Coulombic efficiencies in similar BES design are found to be comparable to Rozendal et al. (2008). Leakage of hydrogen might have also occurred. In both the experiment and the control,

oxygen was present in small amounts in the gas phase. Presumably, the BES was not a completely closed system, and oxygen is entering the cell between the plates.

Both MV and mixed microbial cultures were not only necessary for the production of ethanol but also for the production of hydrogen. As hydrogen and ethanol were only produced after the addition of the inoculum, it is concluded that hydrogen is biologically produced in the cathode and was induced by MV. Biological hydrogen production in a biocathode without mediator was first reported by Rozendal et al. (2008). They produced more hydrogen, 0.63 Nm^3 H_2 m^{-3} at pH 7, but at a lower cathode potential of -700 mV and at a current density of 1.1 A m^{-2}. With the presence of a mediator in this study, a higher current density of 1.33 A m^{-2} at a lower potential was achieved to produced 0.035 Nm^3 H_2 m^{-2} and other products at pH 6. So, the presence of the mediator increases the reaction speed (current) and it avoids a long start-up period of 30 days (Jeremiasse et al.).

The exact electron donor of ethanol formation by acetate reduction remains unknown, since both reduced MV and hydrogen are present in the cathode compartment and both reduction mechanisms are possible. MV can diffuse into bacterial cells, where it can substitute natural electron donor NADH in enzymatic reactions with, for example, hydrogenases (Günther and Simon, 1995); hydrogen can serve as electron donor in acetate reduction by mixed cultures (Steinbusch et al., 2008). MV was obviously not stable in the cathode compartment, considering that after 8 days only 1% was left and the dark blue color of reduced MV was not visible anymore. The disappearance of MV stagnated ethanol and hydrogen production, allowing methanogenesis and *n*-butyrate production to become dominant. Whereas MV completely inhibited methanogenesis and *n*-butyrate production in the mediator experiment, most likely, the low residual MV concentration was not sufficient here. Loss of MV^{2+} cannot be explained by adsorption to any of the materials or chemicals in the BES, as was shown in the exposure test. An untested yet possible factor is the chemical potential. Previous work indicates that soluble MV was irreversibly converted into its most reduced form, MV^0 (Bird and Kuhn, 1981; Peguin et al., 1994). It is difficult to prevent MV^0 formation during MV^{2+} reduction to $MV^{+\bullet}$, because of the small difference in potential between the first and the second reduction reactions. Peguin used MV and a

graphite electrode during Acetone, Butanol, and Ethanol fermentation and observed complete MV loss after 100 h at a redox potential of -560 mV (Peguin et al., 1994). We applied a potential of -550 mV at pH 6, which is lower than the redox potential of MV^{2+} to $MV^{+\bullet}$. Higher redox potential may impede MV losses. Another explanation for the MV loss at the cathode is given by Bowden and Hawkridge, who claimed that $MV^{+\bullet}$ strongly adsorbs to nickel and gold in pH 6-8 electrolytes (1981). This is also a plausible mechanism of MV removal, as three golden wires connected the graphite felt inside the BES to the electric wire outside.

About 93% of the electrical current on the first day was recovered in products such as ethanol, reduced MV, and hydrogen. The MV concentration decrease coincided with electron recovery, which decreased to 42% after 10 days. It should be noted that products such as ethanol, hydrogen, or *n*-butyrate could have been lost via transport through or absorption at the anion exchange membrane. However, the anode medium was changed the day after MV addition, so products that were transported through the membrane to the anode compartment were removed and disregarded in the efficiency calculations. Biomass growth is also an important electron sink for the "nonrecovered" 36.2 mmol of e. This amount of electrons would be involved during biomass growth at a biomass concentration of 9 g VSS/L and a net growth rate of 0.006 d^{-1}, which is a conceivable rate for anaerobic reactions (Rittmann and McCarty, 2002). In the present feasibility study, product concentrations and rates are still very low in the BES compared to other waste conversion processes, such as anaerobic digestion. To improve the ethanol production process in a BES, further research should focus on nonmediated reduction of acetate at the cathode itself by growing microorganisms at the electrode or on immobilization of methyl viologen on the electrode (Aulenta et al., 2007).

5 Chain elongation of acetate

Proof of principle

Abstract

There is an increasing global demand for sustainably produced biodiesel. Expansion of the present biodiesel production, which is mainly based on seed oil production, is limited by crop yields and sufficient arable land. Preferably, the growing biodiesel demand should be met by sustainable feedstocks that are cheap and widely available, and do not interfere with food production or nature conservation. This research introduces a new fermentation that converts agricultural residues into precursors for biodiesel or chemicals. It was found that acetate, a main intermediate of anaerobic conversion, can be elongated to medium chain fatty acids with 6 and 8 carbon atoms in a simple mixed-culture fermentation. Mixed microbial cultures were able to produce 8.17 g L^{-1} caproate and 0.32 g L^{-1} caprylate under methanogenesis-suppressing conditions in a stable reactor run. The highest production rate was 25.6 mM C caproate per day with a product yield of 0.6 mol C per mol C. This elongation process was performed with two electron donors, ethnol and hydrogen, demonstrating the flexibility of the process. Microbial characterization revealed that the microbial populations were stable and dominated by relatives of *Clostridium kluyveri*.

This chapter has been submitted.

5.1 Introduction

The present biodiesel production from seed oil crops will not be able to meet the increasing global biodiesel demand in the next decennia (Ohlrogge et al., 2009). Expansion of seed oil production is limited by a shortage of suitable arable land, high feedstock prices, as well as the competition with food production and nature conservation (Durrett et al., 2008). To overcome the growth limitations of the biodiesel supply on the one hand, but lower greenhouse gas emissions with little or no competition with food production on the other hand, different feedstock sources than seed oils should be explored (Tilman et al., 2009). In a recent technical feasibility study of the US (Perlack et al., 2005), waste and agricultural residues were addressed as high-potential feedstock for sustainable biofuel and chemical production due to their large availability (Perlack et al., 2005). The conversion techniques for processing waste and agricultural residues into diesel are limited and should be further exploited (Fortman et al., 2008).

Diesel fuels are long hydrocarbons (C_{12}-C_{20}) characterized by their cetane number (around 45) and a boiling range of 250-360°C (Petrus and Noordermeer, 2006). Converting biomass like hemicellulose $[C_5(H_2O)_4]_n$ to hydrocarbons requires removal of oxygen and addition of hydrogen. Thermic processes as gasification and Fischer-Tropsch process have demonstrated to convert biomass to diesel fuels. This technology is rather expensive and energy inefficient at small scale applications and can not deal with wet biomass. Biological conversion can deal with a diversity of wet biomass and has already demonstrated to be energy-efficient and cheap on small scale for anaerobic digestion. In this study, we investigate the possibility to convert biomass biologically to precursors of biodiesel or chemicals.

Biological conversions of biomass to liquid fuel or chemicals by an mixed cultures process have been described earlier by Levy *et al*. (1981) and by Holtzapple (1997). They use volatile fatty acids (VFA) as precursor for fuel alcohol, which can be blend with gasoline. VFA, carboxylic acids with 2 to 5 carbon atoms, are key intermediates in anaerobic digestion of organic matter and are produced during acidification of biomass. VFA can not be used as fuel directly because of the high oxygen-to-carbon ratio. Levy *et al.*(1981) also mentioned another possibility to produce medium-chain fatty acids (MCFA)

from the same organic matter. MCFA with 6 or 8 carbon atoms have a higher energy density than VFA because of their longer carbon chain and a lower oxygen/carbon ratio of 0.33 or even 0.25. Caproic and caprylic acids are interesting as they can be further processed to diesel or kerosene-like components by ketonization (Gaertner et al., 2009). In this way, residual biomass is converted to a long-chain ketone $C_{15}H_{30}O$, in three steps in which the number of carbons in the chain is increased to 15 and oxygen is almost completely eliminated. Based on these properties, MCFA would be superior intermediates for further processing to diesel (better than VFA). However, until now, no experimental data have been published for mixed-culture fermentations to produce these desired components for biotechnological application.

Some anaerobic bacteria are able to ferment acetate plus ethanol by the fatty acid synthase complex, also called reversed β-oxidation to fatty acids with longer chains. Butyric and caproic acid are produced from ethanol and acetate by pure cultures of, for example, *Clostridium Kluyveri* (Barker et al., 1945) or *Eubacterium pyruvatiorans* (Wallace et al., 2003) or from ethanol and cellulose by co-cultures of *Clostridium kluyveri* with ruminal cellulolytic bacteria (Kenealy et al., 1995). In a mixed-culture fermentation, not only ethanol can be used as electron donor (Equation 5.1), but also hydrogen (Equation 5.2), as hydrogen and acetate together are also converted to ethanol (Steinbusch et al., 2008).

$$3C_2H_3O_2^- + 2H^+ + 4\,H_2 \rightarrow\ C_6H_{11}O_2^- + 4H_2O \qquad \Delta G'_r = -177\ \text{kJ} \qquad (5.1)$$

$$C_2H_3O_2^- + 2C_2H_6O \rightarrow\ C_6H_{11}O_2^- + 2H_2O \qquad \Delta G'_r = -\ 79\ \text{kJ} \qquad (5.2)$$

Ethanol and hydrogen are products of anaerobic conversions of biomass. Hydrogen is co-produced during acidification of biomass (Li and Fang, 2007), or by bio-electrolysis (Logan et al., 2008), whereas ethanol can be generated from biomass by either acetate reduction with hydrogen (Steinbusch et al., 2008) or sugar fermentation of lignocellulose. Ethanol and hydrogen are fuels themselves, but using them to produce MCFA increases the energy content; 1 mol of caproic acid of 3452 kJ contains more energy than 2 moles of ethanol of 2638 kJ that is needed to produce caproic acid. An expected advantage of MCFA production from biomass is most likely the easy separation. Owing to the aliphatic tail, MCFA are not very soluble in water; the solubility of

undissociated caproic and caprylic acid in water is 10.6 g L^{-1} and 0.68 g L^{-1}, respectively (Yalkowsky, 2003). Production of MCFA above the solubility level allows product removal by simple phase separation. The combination of higher energy content and an easy phase separation makes MCFA an economically attractive and sustainable precursor for biodiesel.

The objective of the research described in this paper was to demonstrate the feasibility of MCFA production by mixed-culture fermentation from acetate, the main intermediate in organic waste treatment. The production of MCFA such as caproic and caprylic acid was stimulated using mixed cultures in a suppressed-methane anaerobic environment. To investigate the feasibility, two different experimental setups were chosen. The first experiment was a batch test in which MCFA production was stimulated with acetate with or without the electron donors ethanol and hydrogen. The second experiment was performed with a controlled (fed-)batch reactor in which MCFA production was stimulated with both electron donors at pH 5.5 and 7.0. From the best MCFA-producing bioreactor, the microbial population was characterized using Denaturing Gradient Gel Electrophoresis (DGGE), combined with cloning and sequencing.

5.2 Materials and Methods

Inoculum

The inoculum was an enrichment derived from granular sludge of a UASB reactor treating brewery wastewater.

Medium

The medium for the batch experiment contained substrate (see next paragraph) with additionally per liter: 3600 mg $NH_4H_2PO_4$, 330 mg $MgCl_2 \cdot 6H_2O$, 200 mg $MgSO_4 \cdot 7H_2O$, 150 mg KCl, 200 mg $CaCl_2 \cdot 2H_2O$, 1 mL of vitamin B solution (Phillips et al., 1993), 1 mL of trace element solution (Phillips et al., 1993) and 10 g of 2-bromoethanosulfonic acid as inhibiter of methanogenesis (Soubes et al., 1994). For the fed-batch run, medium was prepared as described above, but the 330 mg $MgCl_2 \cdot 6H_2O$ and 200 mg $MgSO_4 \cdot 7H_2O$ were replaced by 600 mg $MgCl_2$.

Batch experiment

MCFA production from acetate was stimulated with hydrogen, ethanol or with a combination of hydrogen and ethanol. The experiment was performed in triplicate and one control was left without electron donor. In total 12 serum flasks of 125 mL with rubber stoppers and aluminum caps were used. Each flask contained 37.5 mL of growth medium plus 50 mM acetate and 6 flasks contained also 50 mM ethanol. Before inoculation, the pH was adjusted to pH 5.5 with 2 M NaOH solution. After inoculation with 4.8 g of wet sludge, the flasks were closed and capped. The headspace was flushed 8 times between 500-1500 mbar with nitrogen or hydrogen to finally 1500 mbar. The bottles were incubated at 30°C in a rotating shaker (170 rpm) for 105 days. At day 0, 2, 7, 14, 21 and 105 the gas pressure was measured by a GMH 3150 meter (Greisinger Electronics, Germany) and a gas sample (0.1 mL) was taken to analyze the gas composition directly at the GC. Meanwhile, a liquid sample (0.5 mL) was anaerobically taken and collected in a reaction tube. The pH was measured in the liquid sample with a pH microelectrode BlueLine 16 pH (Schott, Germany) and corrected manually in the bottles with either 2M HCl or 2M NaOH solution to maintain its value between 5.35 and 5.65. Then the liquid sample was centrifuged for 5 min at 10.000 rpm and diluted with formic acid solution to 1.5% (v/v) for FA analysis and with water for alcohol analysis.

Setup of controlled (fed-)batch reactors

The fermentation was performed in controlled (fed-)batch reactors in an experimental setup as shown in Figure 5.1. Each reactor was made of glass with a total volume of 1 liter. Reactors 1 and 2 were filled with respectively 471 and 541 mL medium containing 50 mM acetate and 50 mM ethanol. The reactors were positioned on a magnetic stirrer for agitation of medium at 200 rpm. Medium was continuously flushed with hydrogen (200 mL h^{-1}) using a mass flow controller (Brooks 5850E, PA, USA) with a small pore size sparger. Effluent gas volume was determined using a gas meter type MilliGascounter MGC-1 (Ritter, Germany). The redox potential was monitored by a radiometer type PHM210 (Meterlab, France) with an Ag/AgCl redox electrode type QR480x (QIS, the Netherlands). Reactor temperature was controlled at 30°C. The pH was measured and controlled at pH 5.5 or 7 by a pH controller with 2 M NaOH or HCl solutions.

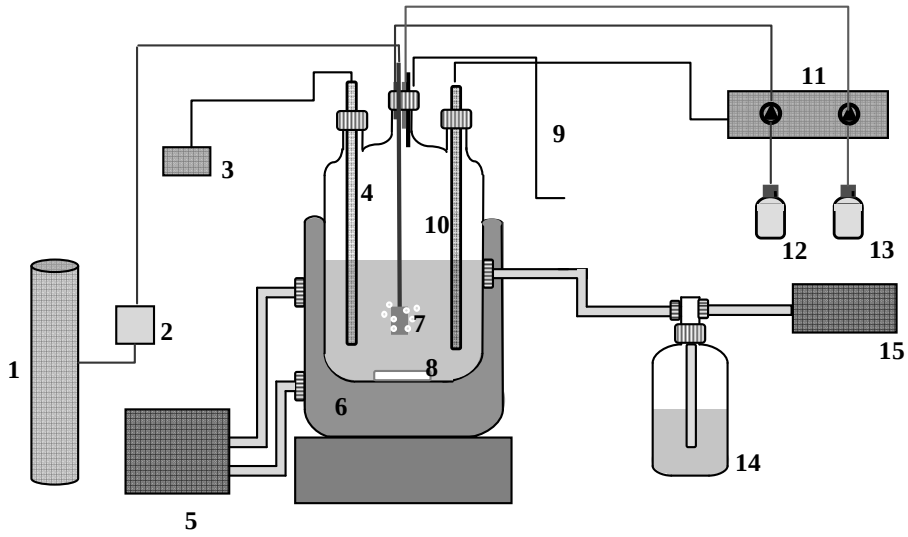

Fig. 5.1 Schematic representation of setup of the bioreactor: 1) Hydrogen gas bottle, 2) Mass flow controller, 3) Redox potential meter, 4) Redox electrode, 5) Water bath, 303K, 6) Water mantle, 7) Gas inlet, 8) Magnetic stirrer, 9) Sampling, 10) pH electrode, 11) pH controller, 12) Base stock (NaOH, 2M), 13) Acid stock (HCl, 2M), 14) Overflow and gas valve, 15) Gas volume meter.

Operation of the reactors

Reactors 1 and 2 were operated over a period of 116 days. Liquid and gas phases were sampled regularly and analyzed for alcohol, VFA and headspace gas composition using similar method as described above; and for DNA composition. At each sampling instant the redox potential, the pH and the total volume of gas effluent were measured. Whenever the ethanol concentration was lower than 100 mg L^{-1}, concentrated ethanol solution (40 g L^{-1}) was added to the reactor to obtain a concentration of 2.5-3 g L^{-1}. The yield calculations are based on the consumed amount of substrate. This amount was corrected for the mol amount of ethanol evaporated from the reactor fluid by the continuous gas flow. The calculation is based on the vapor pressure of ethanol and the gas flow using the ideal gas law. The vapor pressure was calculated by multiplying the mol fraction of ethanol in the water times the saturation pressure of ethanol of $1.03 \cdot 10^4$ Pa at 30°C.

Chemical analysis

Hydrogen, oxygen, nitrogen, methane and carbon dioxide were analyzed by gas chromatography (Steinbusch et al., 2008). Fatty acids (C_2-C_8) were

analyzed by gas chromatography using a Hewlett Packard 5890 series II equipped with a glass column (2m x 6mm x 2mm) packed with 10% Fluorad 431 on Supelco-port 100-120 mesh. Prepared samples (1.0 µL) were injected directly on the column at 200˚C. The carrier gas was nitrogen saturated with formic acid at 40 mL min^{-1}. The column temperature was 130°C for 4 min, raised linearly at 12°C min^{-1} ramp to 160ºC for 6.5 min. FA were detected with a FID at 280˚C using hydrogen at 30 mL min^{-1} and air at 400 mL min^{-1}. Alcohols (C_2-C_6) were eluted using the same settings, but at constant column temperature of 70ºC.

Protein concentration determination with modified Hartree-Lowry method was used to quantify bacterial cell concentration in a small sample volume (Caprette, 1995). One mL of reactor liquid was transferred in a 2 mL vial and centrifuged for 5 min at 10.000 rpm. Supernatant was removed and the pellet was resuspended with 1 mL of 1 M NaOH. The vial was left for 30 min at 50ºC in a water bath to hydrolyze cells. Accordingly, protein concentration of the hydrolyzed cells was quantified using modified Hartree-Lowry method. It was experimentally found that 1.0 g of protein equals 3.0 g of volatile suspended solids (VSS).

DNA extraction, amplification and DGGE

The microbial population of reactor R1, with the highest MCFA production, was characterized. Bacterial genomic DNA was extracted from weekly taken reactor liquid samples using a FastDNA® SPIN Kit for Soil (Qbiogene, Carlsbad, CA). DNA concentration and integrity were measured with the NanoDrop® spectrophotometer. From extracted DNA, bacterial 16S rRNA was amplified with primers U968-f and L1401-r using the GoTaq polymerase kit (Promega, Madison, WI) with 5x Green buffer as described by the Promega instructions. The fragments were amplified with using the following PCR program: 94°C for 2 min, 35 cycles of 94°C for 30 s, 56°C for 40 s, and 72°C for 60 s, and 72°C for 5 min. All primers used were synthesized commercially by Biolegio (Nijmegen, the Netherlands). Bacterial amplicons were subsequently separated by DGGE as described by Zoetendal (2001) using the D-code system (Bio-Rad, Hercules, CA) with 8% (v/v) polyacrylamide gels having a denaturant gradient of 30-60%.

Cloning and sequencing

On basis of the DGGE, two out of thirteen DNA samples of the reactor run R1 were selected for further analysis. Before cloning, bacterial 16S rRNA was amplified from the genomic DNA using primers 27f en 1492r. The fragments were amplified with using the following PCR program: 94°C for 2 min, 35 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 90 s, and 72°C for 5 min. The amplicons were purified using a DNA Clean & Concentrator™-5 kit (Zymo Research, Orange, CA). The purified amplicons were ligated in pGEM-T easy vector (Promega, Madison, WI) and cloned into E. coli JM109 by transformation. After blue white screening, 96 white colonies of each bioreactor sample were transferred on medium containing 200 µl of LB plus 20 µg ampicillin and grown overnight on a shaker at 37°C. For the lysis, 45 µl Tris-EDTA buffer was added to 5 µl of each culture. After centrifuging for 1 min at 10,000 rpm, the cells were lysed at 95°C in a PCR. The inserts were amplified with primers T7 and Sp6 using the following PCR program: 94°C for 2 min, 35 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 90 s, and 72°C for 7 min. The PCR products were screened with ARDRA using restriction enzymes AluI, CfoI and MspI (Promega, Madison, WI) according to Sousa(Sousa et al., 2007) to check the distribution and the number of phylotypes present. Amplicons with unique ARDRA patterns were purified using DNA Clean & Concentrator™-5 kit again and subjected to DNA sequence analysis. The analyzed 16S rRNA sequences were compared with sequences in the GenBank database using the NCBI Blast search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Closest relatives and cultured relatives were retrieved from the database, with the degree of similarity.

5.3 Results

5.3.1 Effect of substrate on MCFA production

In the batch experiment, fatty acid to a chain length of 8 carbon atoms and alcohols to a chain length of 6 carbon atoms were produced from acetate with hydrogen and/or ethanol. Depending on the substrate combination, different products were formed, which all had an even number of carbon atoms. Figure 5.2 shows the exact product distribution after 116 days. Caproate was formed in all experiments with electron donor and not in the control. Caprylic acid, the product with the highest caloric value, was formed in two experiments: containing acetate with hydrogen; and acetate with

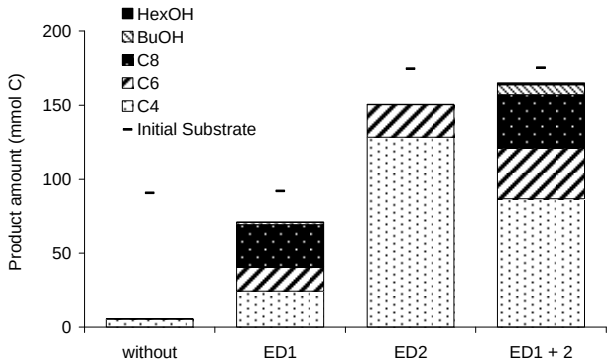

Fig. 5.2 Product distribution in moles carbon of converted carbon during acetate fermentation to MCFA and alcohols with different electron donor: hydrogen (ED1) and/or ethanol (ED2) — indicates the initially added amount of substrate in carbon.

ethanol and hydrogen. Table 5.1 shows the stoichiometrie for the production of 1 mol of caproate in each of the three experiments. From an economical point of view, the reaction mixture that uses the highest acetate/ethanol ratio is most interesting to study further. Moreover, we selected the substrate combination acetate, hydrogen plus ethanol to sufficient electron supply.

5.3.2 Effect of pH on MCFA production

In the controlled reactor experiment, the substrate combination of acetate with hydrogen and ethanol was fermented at pH 5.5 and 7. Caproate was produced at both pH 5.5 and 7, while caprylate was produced surprisingly only at pH 7. Table 5.2 shows that the MCFA concentration and production

Table 5.1 Balance of mixed-culture fermentation of acetate and/ or ethanol with or without hydrogen normalized to caproate

	Acetate, H_2	Acetate, ethanol	Acetate, ethanol, H_2
Hydrogen	-19.3	0.0	-5.1
Acetate	-11.8	-10.3	-8.1
Butyrate	1.8	7.2	3.1
Caproate	1.0	1.0	1.0
Caprylate	1.3	0.0	0.8
Ethanol	0.1	-11.4	-7.5
Butanol	0.1	0.0	0.3

[a] Negative values indicate overall consumption of the component

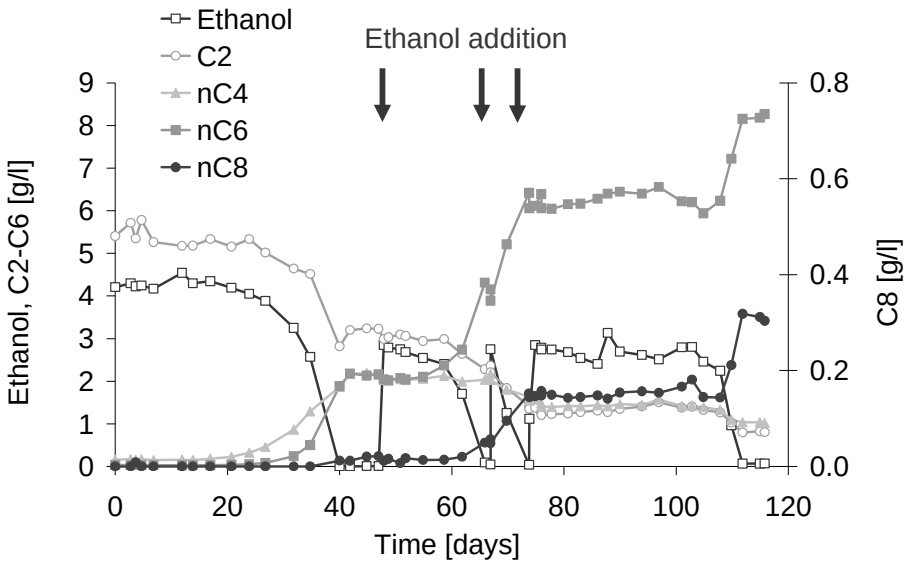

Figure 5.3 Concentration of ethanol and C2-C8 fatty acids in time of the fed-batch reactor operation at pH 7, with initially acetate and ethanol as substrate. The red arrow indicates the times of ethanol addition.

rate were higher at pH 7 than at pH 5.5. Figure 5.3 shows the substrate and product concentrations of the best performing reactor at pH 7. Caproate and caprylate were produced in three active periods. In the active periods, ethanol was consumed fast and extra ethanol was added 3 times to prevent depletion. Production of *n*-butyrate started after a lag time of 20 days. During that period, the redox potential decreased from initially 39 mV to -500 mV vs. Ag(s)/AgCl. When the *n*-butyrate concentration reached 450 mg L^{-1}, caproate production started and increased exponentially. When caproate reached 501 mg L^{-1}, caprylate production started. After 40 days, ethanol was completely consumed, and the production of *n*-butyrate and caproate stopped. Ethanol dosage did not lead directly to a continuation of the fermentation. It took 7 days before production of caproate and caprylate was restored. Remarkable is that the *n*-butyrate concentration did not increase further, but remained 2.0 g L^{-1}. After the second time that ethanol was almost consumed completely, ethanol was added immediately, which avoided a lag in conversion. A third ethanol addition after 75 days, however, was too late as it took 19 days until the microbial activity was recovered. After 115 days, the reactor run was stopped.

Table 5.2 Concentration and production rate of caproic and caprylic acid in controlled batch reactors

Substrate	*pH*	*Concentration (g l^{-1})*		*Rate (mmol C l^{-1} d^{-1})*	
		C6[a]	C8[a]	C6	C8
acetate, ethanol, H_2	5.5	0.12	0	3.9	
acetate, ethanol, H_2	7	8.27	0.318	25.6	2.98

Figure 5.4 shows the carbon distribution over the products of the total converted substrate. Caproate was the main product of the fermentation. After each ethanol addition, this yield increased from 0.36, 0.51, 0.58, to finally 0.60 mol C caproate per mol C of the converted substrates; the product caprylate yield was 0.02 mol C caproate per mol at the end. Whereas the caproate yield increased in time, the *n*-butyrate yield decreased in time. This suggests that *n*-butyrate was further converted to caproate. At the end of the run, 29% of the converted substrate carbon was not recovered as VFA or MCFA product, but had been converted to biomass and CO_2. A remaining 9% of the converted carbon could not be assigned to any product. Biomass production contributed for 6.5% of the converted carbon. The biomass yield was at maximum 0.064 mol C per mol C, based on protein concentration. CO_2 was measured at a very low percentage around 0.01%. However, this value is close to the detection limit. In this way, CO_2 would account for approximately 14% of the converted carbon. For one mol of caproate, 3.8 moles of ethanol and 1.09 moles of acetate were consumed, and 0.14 mol *n*-butyrate, 0.03 mol caprylate and 0.45 mol biomass were formed.

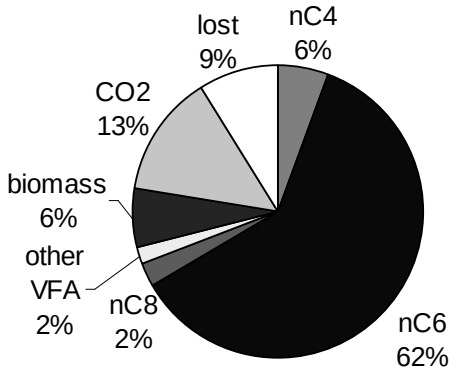

Figure 5.4 Product distribution in carbon among *n*-butyrate (nC4), caproate (nC6), caprylate (nC8), other VFA (such as propionate, i-butyrate and (i/n)-valerate); biomass and CO_2 at the end of the fermentation at pH 7, with initially acetate and ethanol as substrate.

5.3.3 Growth and microbial characterization

Microbial growth was determined by the increase in DNA concentration in the reactor. The growth rate was 0.06 d^{-1} on average and was highest on day 32 at 0.35 d^{-1}. The growth yield was difficult to determine over the three active periods as the active periods were sometimes shorter then the time between biomass measurements. Figure 5.5 shows the bacterial population dynamics in time by means of a DGGE gel of 16S rRNA. Based on the ARDRA pattern of all clones and the sequencing of part of the clones, 15 different phylotypes among a total 86 detected phylotypes are shown in Table 5.3. Relatives of *Clostridium kluyveri* were most abundant in both samples: 57% and 40% of all clones analyzed showed high homology with bands of no. 1. All sequenced clones with their closest relative and the closest cultured relative together with the GenBank accession number are listed in Table 5.4. Comparison of the DGGE pattern of the reactor samples with patterns of the sequenced clones revealed that bacteria closely related to *Clostridium kluyveri* (98% similarity) and *Azospira oryzae* (99% similarity) were the dominant species. Visual comparison of the DGGE profiles of each sample over time revealed that after 17 days in all samples bands

Table 5.3 Phylogenetic affiliation and number of bacterial 16S rRNA gene clones generated from 2 selected reactor samples.

Closest bacterial relative 16S rRNA phylotypes	*Amount*	
	S1 [a]	S2 [a]
1 Clostridium kluyveri	26	16
2 Azospira oryzae	18	14
3 Uncultured Rhodocyclaceae from MFC-B162-F06		2
4 Uncultured Rhodocyclaceae		1
5 Clostridiaceae bacterium FH052	1	
6 Uncultured Clostridiales from MFC-B162-F04	1	
7 Uncultured clone LC30	1	
8 Propionibacterium acidipropionici	1	
9 Propionivibrio limicola		1
10 Uncultured clone 16saw35-1b12.p1k		1
11 Uncultured clone ANTLV1_C11		1
12 Uncultured clone MES_rTCB90		1
13 Unidentified ZF3		1
14 Bacterium Te16R		1
15 Sedimentibacter sp. C7		1
Total clones	46	40

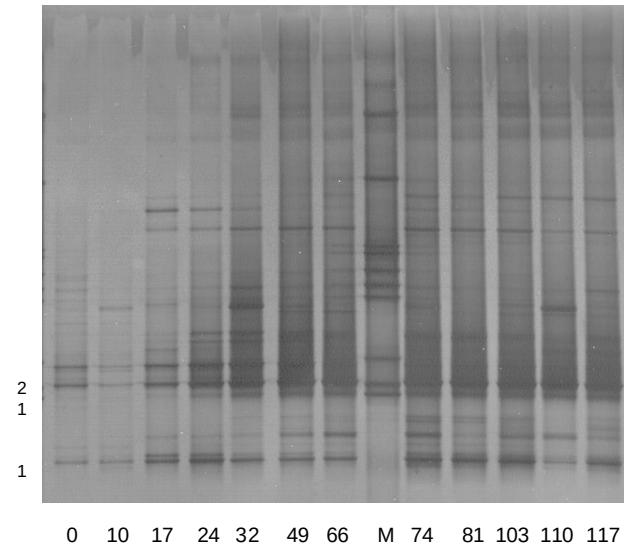

Figure 5.5 DGGE band patterns of bacterial 16S rRNA gene amplicons from 12 reactor samples taken at different time (days). M is a Marker. The numbers along the vertical boarder indicating DGGE bands correspond to clones listed in Table 5.3.

Clostridium kluyveri and *Azospira oryzae* proliferated. After these 17 days, only small shifts in numbers of bands and their intensity were observed, indicating a quite stable microbial population in the bioreactor.

5.4 Discussion

This research clearly demonstrates that it is technical feasible to produce MCFA by mixed-culture fermentation from acetate, the main intermediate in organic waste treatment. For the caproate and caprylate production, both ethanol and/ or hydrogen can be used as electron donor. Since hydrogen and ethanol are produced from different biomass feedstocks, MCFA and ultimately diesel is not depending on the availability of solely one type of feedstock. As the feedstock price determines a large portion of the costs, flexibility in electron donor choice and therefore in biomass feedstock choice can help to reduce costs. In the long term experiment, both electron donors were used to produce MCFA to avoid growth limitations. It was found that ethanol was preferred by the organisms over acetate and hydrogen as substrate and

Table 5.4. Sequenced bacterial clones with GenBank accession number and similarity with their closest relative (uncultured and cultured)

Clone #	Length seq (bp)	GenBank acc #	Closest relative	% Sim.	GenBank acc # Closest relative	Closest cultured relative	% Sim.	GenBank acc # cultured relative
8259101	1000	GU216627	*Azospira oryzae*)*	99		*Azospira oryzae*)*	99	DQ863512 (Strain N1) AY277622 (Strain GR-1)
111	967	GU216619	*Azospira oryzae*)*	99		*Azospira oryzae*)*	99	
114	1003	GU216622	*Azospira oryzae*)*	99		*Azospira oryzae*)*	99	
115	991	GU216617	*Azospira oryzae*)*	98		*Azospira oryzae*)*	98	
118	1006	GU216610	*Azospira oryzae*)*	99	DQ863512	*Azospira oryzae*)*	99	
134	996	GU216607	*Azospira oryzae*)*	99		*Azospira oryzae*)*	99	
121	1001	GU216613	Bacterium Te16R	92	AY587806	*Clostridium aminovalerum*	92	M23929
103	1004	GU216629	*Clostridium kluyveri*	97		*Clostridium kluyveri*	97	CP000673(DSM555) AP009049 (NBRC12016)
104	1008	GU216623	*Clostridium kluyveri*	98		*Clostridium kluyveri*	98	
106	998	GU216624	*Clostridium kluyveri*	98		*Clostridium kluyveri*	98	
107	825	GU216625	*Clostridium kluyveri*	98		*Clostridium kluyveri*	98	
113	947	GU216621	*Clostridium kluyveri*	98	AB298768	*Clostridium kluyveri*	98	
119	982	GU216611	*Clostridium kluyveri*	97		*Clostridium kluyveri*	97	
120	1013	GU216612	*Clostridium kluyveri*	99		*Clostridium kluyveri*	99	
122	1007	GU216614	*Clostridium kluyveri*	98		*Clostridium kluyveri*	98	
123	998	GU216615	*Clostridium kluyveri*	98		*Clostridium kluyveri*	98	
124	1005	GU216630	*Clostridium kluyveri*	98		*Clostridium kluyveri*	98	
125	859	GU216598	*Clostridium kluyveri*	98		*Clostridium kluyveri*	98	
130	972	GU216603	*Clostridium kluyveri*	88		*Clostridium kluyveri*	88	
112	1012	GU216620	*Clostridium sp.*	91	AB298768	*Clostridium kluyveri*	90	
110	989	GU216618	*Propionibacterium acidipropionici* DH42	99	AY360222	*Propionibacterium acidipropionici* DH42	99	
127	991	GU216600	*Sedimentibacter* sp.	99	AY766466	*Sedimentibacter* sp.	99	
102	1006	GU216628	Uncult bact clone LC30	95	FJ715993	*Clostridium mesophilum*	94	DQ521480
128	988	GU216601	Uncult. Rhodocyclales clone MFC	98	FJ823876	*Azospira oryzae*)*	94	
116	996	GU216616	Uncultured Azospira clone	99	FN436195	*Azospira oryzae*)*	99	
117	991	GU216609	Uncultured Azospira clone	99	FN436195	*Azospira oryzae*)*	99	
132	1009	GU216605	Uncultured clone	94	DQ521480	*Clostridium mesophilum*	93	EU037903
133	998	GU216606	Uncultured clone HAW-R60-B745	95	FN436123	*Anaerotruncus* sp.	93	EU815226
126	1004	GU216599	Uncultured clone MES_rTCB90	95	AB275987	*Proteiniborus ethanoligenes*	92	EF116488
108	1020	GU216626	Uncultured Clostridiales from MFC	97	FJ393118	*Clostridium* sp.	96	EU815224
131	991	GU216604	Uncultured Rhodocyclales	99	FJ393120	Perchlorate reducing bacterium CR	96	AY530552
135	964	GU216608	Uncultured Rhodocyclales	99	FJ393120	Perchlorate reducing bacterium CR	93	AY530552
129	996	GU216602	Unidentified bacterium ZF3	99	AJ404681	*Clostridium leptum*	95	AJ305238

electron donor. In the controlled reactor that was operated with acetate and addition of both electron donors (to avoid growth limitation), 3.8 times more ethanol was consumed than acetate. This ratio is not the stoichiometric value of the MCFA formation, since ethanol is most likely being consumed by another metabolic process to CO_2.

Very likely, a relative of *Clostridium kluyveri* is responsible for the MCFA production in the experiment as it dominated the microbial population in the MCFA producing bioreactor. Besides genetic similarities, also the product formation during the fed-batch operation is a strong indication that *Clostridium kluyveri* is involved in the fermentation. The ethanol-acetate metabolism of *C. kluyveri* can be described by three coupled reactions (Seedorf et al., 2008). First, ethanol is converted to acetate, the only reaction where ATP can be generated. This reaction is endergonic, and is coupled by two exergonic reactions. First, *n*-butyrate is produced from acetate and ethanol through the coupling of two acetyl CoA moieties to butyl CoA. Second, caproate is formed from *n*-butyrate and ethanol in a similar loop by coupling butyl CoA with acetyl CoA. A similar pattern can be seen in Figure 5.1. First *n*-butyrate was detected, then caproate and finally caprylate. Caprylate formation has not been reported before for *C. kluyveri*. Most likely, in our experiment, caprylate was formed in a similar loop with hexyl CoA and acetyl CoA.

Besides *Clostridium kluyveri* species, the microbial population in the bioreactor was dominated by a species related to *Azospira oryzae*. *Azospira oryzae* (also known as *Dechlorosoma oryzaes*), is a nitrogen-fixing beta-proteo bacterium that is also able to reduce chlorate or selenate (Hunter, 2007). Presumably, *Azospira* in the experiment converted ethanol to CO_2 with the inhibiter BES as electron acceptor in the absence of other electron acceptors. With the tetrahedral structure with three oxygen bonds in the sulfonate part, BES is comparable to the chemical structure of electron acceptor selenate. Ye *et al*. showed that long-term exposure to BES not only eliminated Archaea but also altered the bacterial community structure by acting as a competing electron acceptor for sulfate-reducing bacteria or dehalogenating microorganisms (Chiu and Lee, 2001; Ye et al., 1999). Leaving out the inhibiter will probably out compete *A. oryzae*, which as a result increases the product yield and the ethanol:acetate consumption ratio

as less ethanol is converted to CO_2. The shift in microbial population can affect MCFA formation, but it is expected that MCFA producing bacteria will be active in mixed culture fermentation, since caproate production was also observed without inhibiter where methanogenesis was prevented by heat pre-treatment (Steinbusch et al., 2009).

MCFA product formation was pH dependent. It was found that MCFA production was higher at pH 7 than at pH 5.5 in the controlled reactor. At pH 5.5 even no caprylate was formed, whereas at the same pH in the batch experiment the caprylate concentration was almost equal to that of caproate. Since the experimental set-ups were different, the results are difficult to compare. An explanation for the different product formation might be the difference in ethanol and acetate concentration or hydrogen partial pressure; in the controlled batch hydrogen was continuously sparged into the stirred reactor, whereas in the batch experiment hydrogen headspace was limited. We know that hydrogen limits the metabolic step of *Clostridium kluyveri* in which ATP is formed: the oxidation of ethanol to acetate. It might be the case that the partial pressure of hydrogen was too high in the controlled batch experiment at pH 5.5, that the first reaction became exergonic, and consequently, growth was inhibited.

On the one hand, a high hydrogen partial pressure inhibits growth of *Clostridium kluyveri*, on the other hand a certain hydrogen partial pressure is needed to prevent oxidation of MCFA by other organisms in the mixed culture fermentation. The high hydrogen pressure or ethanol concentration makes it energetically favorable for microorganisms to use VFA as electron acceptors (equations 5.1 and 5.2). At these reduced conditions, coupling of fatty acids is presumably the best mechanism for an organism to release its electrons (Kunau et al., 1995; Seedorf et al., 2008). The redox potential of the NADH/NAD in *C. kluyveri* is around near -300 mV vs. NHE, about the same value that is measured during the controlled batch run.

The highest MCFA concentration obtained in the controlled reactor operation at pH 7 was 8.2 g caproate L^{-1}. Such a high concentration has never been reported before; Kenealy *et al*. (1995) produced 2.6 g *n*-butyrate L^{-1} and 4.6 g caproate L^{-1} from 4.4 g L^{-1} ethanol and 6.0 g L^{-1} cellulose with a yield of 0.647 mol C per mol C. Since the production of MCFA in the fed-batch reactor

was ethanol-limited, further research may show what concentration can be achieved if ethanol is present in abundance. Sufficient ethanol present may show the maximum achievable concentration in the bioreactor and the potential of this fermentation technology. High caproate concentration is important as it makes selective removal of caproate possible. Selective removal of MCFA together with recirculation of substrate and *n*-butyrate into the bioreactor could increase the yield because recycled *n*-butyrate might be converted to MCFA again in the bioreactor. This way, it may be possible to obtain an efficiency higher than 60%.

Of the converted substrate carbon, 9% was neither recovered as *n*-butyrate, caproate and caprylate, nor as biomass or CO_2. Possibly, this can be attributed to inaccurate CO_2 measurements below the detection limit. Another explanation might be that not all grown biomass was taken into account by calculating the growth yield. Only suspended biomass was measured, while in the reactor also a biofilm on the glass was formed, which could not be taken into account in the overall calculations. Improvements in experimental setup may resolve these uncertainties.

A major drawback of our MCFA fermentations as shown here is that methanogenesis was inhibited by the addition of BES. Further studies should emphasize on the prevention of methanogenesis in mixed culture fermentations (Steinbusch et al., 2009). This could be achieved by heat pre-treatment of the inoculum. In contrast to methanogens, the *Clostridia* in our process, will survive heat treatment due to their capacity to form spores. From a concentration and rate point of view, this anaerobic conversion shows possibilities for application in a biotechnological process to produce biodiesel or chemicals. The peak caproate production rate we found was 2.6 g COD g^{-1} VSS d^{-1}, which is comparable to that of other applied anaerobic processes such as anaerobic digestion. The conversion rate of methanogenesis from acetate at a similar temperature of 30ºC is only two times higher: 5.12 g COD g^{-1} VSS d^{-1} (Rittmann and McCarty, 2002). As our fermentation process is not optimized yet, the caproate production rate as shown here may still increase more.

5.5 Conclusions

In the present study, medium chain fatty acids were produced from acetate with hydrogen and/or ethanol as electron donor. At pH 7, mixed microbial cultures that were dominated by relatives of *Clostridium kluyveri* were able to produce 8.17 g L^{-1} caproate and 0.32 g L^{-1} caprylate under methanogenesis suppressed conditions in a stable reactor run. The highest production rate was 0.485 g L^{-1} caproate d^{-1} with a product yield of 0.6 mol C per mol C.

6 Chain elongation of acetate: continuous flow with selective product removal

Abstract

By producing medium chain fatty acids (MCFA), acidification products of low grade biomass such as acetate can be converted with hydrogen or ethanol to precursors of biodiesel or chemicals. To avoid long lag times and substrate limitations associated with batch fermentations, this research investigated MCFA production in a continuous flow operating reactor followed by MCFA separation out of the effluent. Two CSTR were operated for 61 and 67 days continuously at an HRT of 22 days with stable growth conditions. During the operation period, the specific MCFA production activity increased to 2.9 g caproate and 0.09 g caprylate per gram VSS d^{-1}, independent of the different start-up methods of the CSTR reactors. Final caproate concentration in the fermentation broth was 10.5 g L^{-1} and caprylate was 0.48 g L^{-1}, which were demonstrated to be selectively removable by calcium precipitation and solvent extraction with ethyl hexanoate and petroleum ether.

6.1 Introduction

There is a high demand for large quantities of sustainably produced fuel and chemicals from biomass resources to decrease the dependency on fossil fuel and reduce carbon dioxide emissions. The sustainability of fuel and chemical production is substantially determined by the type of biomass feedstock chosen (Tilman et al., 2009). Using biomass waste as feedstock for fuel production prevents competition with food production while simultaneously reducing carbon dioxide emissions most effectively (Searchinger et al., 2008). Furthermore, employing biomass waste as feedstock is estimated by the US Department of Energy to have the largest potential for biofuel production (Perlack et al., 2005) as it is a cheap resource and is abundantly present in rural areas. Recently, we described a fermentation to convert wet biomass waste into medium chain fatty acids like caproic acid (MCFA) (Chapter 5). With a higher carbon/oxygen ratio and a longer aliphatic tail, MCFA have a higher energy density than the original biomass. Additionally, MCFA can easily be processed into diesel-like components by ketonization (Gaertner et al., 2009; Renz, 2005). It was found that MCFA could be produced from acetate, an abundant intermediate in anaerobic conversions. By combining the anaerobic conversion steps hydrolysis and acidification with MCFA fermentation, residual biomass can be transformed in a simple non-sterile process to precursors of biodiesel. The three step process involves, (1) hydrolysis and acidification of biomass to volatile fatty acids (VFA) such as acetate and hydrogen, (2) conversion of VFA with the electron donors hydrogen or ethanol to medium chain fatty acids (Chapter 5) and (3) MCFA separation with a potentially more energy efficient removal process than distillation.

The proof of principle of MCFA fermentation was demonstrated in a fed-batch reactor. Here, mixed microbial cultures were able to produce 8.17 g L^{-1} caproate and 0.32 g L^{-1} caprylate with a yield of 0.63 mol C per mol C under methanogenesis suppressed conditions. The volumetric production rate peaked at 3.0 g caproate L^{-1} d^{-1}, but could not be sustained due to rapid substrate depletion. However, the combination of a significant lag time of 20 days and the long recovery time after substrate depletion, makes the fed-batch system unsuitable for studying the actual potential of the fermentation. To further access the potential of MCFA production for biotechnological

applications, knowledge is needed about (i) the specific MCFA production rate, and (ii) the ease of caproic and caprylic acid removal from the fermentation broth. The specific production rate and the biomass growth rate can be easily determined in a constantly operating CSTR. Continuous addition of fresh medium with nutrients and substrate enables stable growth conditions which therefore stimulate natural selection of the MCFA producing bacteria.

Employing such a MCFA production process is only of use if the product can be separated from the fermentation broth. Separation methods that were described for the removal of mixed acid fermentation products with mainly acetate are ion exchange, precipitation, extraction, and membrane diffusion (Levy et al., 1981). It is expected that the aliphatic tail of MCFA makes removal from water easier than, for example, from acetate or ethanol, as reflected in the low water solubility of 10.6 g L^{-1} for caproic acid and 0.68 g L^{-1} for caprylic acid as compared to full solubility of acetate and 101 g L^{-1} of *n*-butyrate. Techniques that take advance of the low solubility in water are liquid-liquid extraction and precipitation.

The objective of this study is to continuously produce and selectively remove caproic and caprylic acid from acetate and ethanol by mixed culture fermentation in a CSTR. The specific sludge activity was determined in two reactors that were started up in a different manner: one operated continuously directly from the beginning, whereas the other operated first in batch mode, and was then switched to continuous mode. From the effluent of one of the reactors, two different separation techniques were experimentally tested to selectively remove caproic and caprylic acid. The two fermentations were compared based on the MCFA concentration, the production rate per volume and per gram cells and the yield.

6.2 Materials and methods

6.2.1 Biological MCFA production

Inoculum

Enrichment out of a caproate producing bioreactor, that originally came from a UASB reactor treating brewery wastewater, was used to inoculate. Reactor 1 was filled under anaerobic conditions with 0.33 g VSS L^{-1} and reactor 2 started with a biomass concentration of 0.30 g VSS L^{-1}.

Medium

1 liter of medium was prepared by adding 3600 mg $NH_4H_2PO_4$, 330 mg $MgCl_2 \cdot 6H_2O$, 200 mg $MgSO_4 \cdot 7H_2O$, 150 mg KCl, 200 mg $CaCl_2 \cdot 2H_2O$, 1 mL of trace element solution (Phillips et al., 1993) and 10 g of sodium 2-bromoethanosulfonate as a methanogenesis inhibiter to just under one liter of demineralized water. The medium was boiled for 5 minutes and cooled down under a deoxygenated nitrogen atmosphere. Subsequently, 50 mM of acetate, 50 mM of ethanol and 1 mL of vitamin B solution (Phillips et al., 1993) were added to the medium, which was then filled up to 1 liter with demineralized water. In the continuous mode, medium with a higher substrate concentration was added to both reactors containing 350 mM ethanol and 150 mM acetate in a ratio of 2.3: 1, according to the consumption ratio observed in chapter 5. Reactor 2 already contained medium that was used in a prior continuous operation, so the starting substrate concentrations were different from reactor 1, 90 mM ethanol and 36 mM acetate.

Experimental setup of reactors

The fermentation was performed in two CSTRs made of glass with a total volume of 1 liter and a liquid volume of 471 and 457 mL for reactor 1 and 2, respectively. The reactors were positioned on a magnetic stirrer for agitation of the medium. The medium was continuously flushed with hydrogen (200 mL h^{-1}) using a mass flow controller (Brooks 5850E, PA, USA) with a small pore size sparger. The effluent gas volume was determined using a gas meter type MilliGascounter MGC-1 (Ritter, Germany). In continuous mode, medium addition was computer controlled to allow a stable low medium feed with the

Watson Marlow 101U/R pumps (Cornwall, England). The weight of each medium vessel was logged by connecting the balance to computer program control EG. Every 4 hours the pumps were switched on until the medium vessels were 4 grams lighter. Redox potential was monitored by a radiometer type PHM210 (Meterlab, France) with an Ag/AgCl redox electrode type QR480x (QIS, the Netherlands). The reactor temperature was controlled at 30°C by a recirculating water jacket. The pH was controlled at pH 7 by a pH controller with 2 M NaOH or HCl solutions. Liquid and gas phases were sampled and analyzed for alcohol, VFA, and protein concentrations as well as headspace gas composition. Also, at each sample point, the redox potential, pH and the total volume of effluent gas were measured.

Operation of the reactors

Reactor 1 was first flushed with hydrogen for several hours, then filled with the anaerobic medium and finally inoculated. After inoculation, the reactor was operated continuously for 67 days. Reactor 2 operated first 49 days in batch mode, after which it was switched to continuous mode for an additional 61 days. The medium composition of reactor 2 was similar to the composition of reactor 1, but had a higher ethanol (90 mM) and lower acetate (36 mM) concentration because of the crash in the previous continuous run experiment. In continuous mode, concentrated medium was added at an average flow of 22 mL d^{-1} to a HRT 21 days.

6.2.2 Calculations

The volumetric and specific production rates were calculated by averaging the cumulative caproate production. The cumulative caproate production was averaged, using the supsmooth function of the computer program Mathcad (Mathsoft version 13). The function returns a vector created by the piecewise use of a symmetric nearest neighbor linear least-squares fitting on each element in the cumulative caproate production vector, for which the number of nearest neighbors is adaptively chosen.

6.2.3 MCFA separation

Two separation techniques, liquid-liquid extraction and precipitation, were tested for their capacity to selectively remove caproate and caprylate from the effluent of reactor 2. Effluent was collected throughout the whole fermentation experiment. The effluent contained per liter 9.4 g caproic acid

and 0.26 g caprylic acid and had a pH of 6.52. The pH determines the efficiency of each separation technique; extraction only separates undissociated acids from the water phase, whereas precipitation only occurs with the dissociated form. Therefore, the separation tests were performed with the fermentation medium at pH 5, 6.52 and 7, which was adjusted by titrating with 2 M NaOH or HCl solutions.

Extraction

Liquid-liquid extraction is a separation technique in which the target component is transported from one liquid, namely the fermentation broth, into another liquid, specifically a solvent that can be removed easily. Two different solvents, ethyl hexanoate and petroleum ether, were tested for their suitability to selectively remove caproate and caprylate from the fermentation broth by extraction. The extraction was performed in 50 mL separation funnels of glass (Schott, Germany), which were washed 3 times with acetone before usage. After filling the funnels with 27 mL of fermentation broth and 6 mL of solvent, they were shaken for 15 min at 150 rpm. After shaking, the funnels were left for 15 minutes to separate into two phases. Then 1 mL samples were taken from the fermentation broth to analyze the residual VFA concentration. All extractions were performed in duplicate.

Distribution coefficient calculations

After extraction, the undissociated acid in the solvent phase $[HA]_s$ is in equilibrium with the undissociated acid in the water phase $[HA]_w$. The distribution of the undissociated acid among both phases is expressed in the distribution coefficient. We calculated the effective distribution coefficient based on the total acid concentrations as in Equation 6.1. The solvent concentration (HA_s^{Tot}) is calculated from the difference in acid concentration before (initial) and after the extraction per volume fermentation broth (V_w), divided by the solvent volume (V_s). The effective distribution coefficient was calculated for each extraction with initial pH values of 5, 6.52 and 7.

$$D = \frac{[HA]_s^{Tot}}{[HA]_w^{Tot}} \quad (6.1) \qquad \text{whereas } [\mathrm{HA}]_s^{\mathrm{Tot}} = \frac{\mathrm{V_w}\ [\mathrm{HA}]_{\mathrm{w\ initial}}^{\mathrm{Tot}} - [\mathrm{HA}]_{\mathrm{w\ final}}^{\mathrm{Tot}}}{\mathrm{V_s}}$$

Activity test in extracted medium

After extraction, the fermentation medium was tested for the ability to act as a substrate again for bacterial growth. Residues of the solvent in the medium could be toxic for bacteria and thus impede fermentation with recycled medium. Bottles were filled with the extract and with non-extracted fermentation medium (control), sealed with rubber inlets and capped with aluminum crimp caps. After purging the headspace 8 times with pure hydrogen to a final pressure of 1.5 bar, 1 mL of inoculum was injected anaerobically. At day 0 and day 58, the pressure of the headspace was measured and a liquid sample was taken to analyze the VFA and alcohol concentrations.

Precipitation

The separation technique precipitation relies on differences in solubility products, with the requirement that the target component has a lower solubility product compared to other components in the system. Precipitation with a divalent cation was preferred over a monovalent cation in order to reduce the amount of salt addition and to maintain a lower solubility product. Of the common divalent cations, copper, magnesium, barium and calcium, calcium was selected as ligand for the precipitation of caproate and caprylate. Calcium, like the divalent cation copper, had a lower solubility product with caprylate than Mg and Ba salt (Papageorgiou, unpublished results), and, unlike copper, is not a highly toxic ion.

Precipitation was performed with fermentation broth at pH 5, 6.52 and 7 with different amounts of calcium to estimate the solubility product and the amount of calcium needed to separate caproate and caprylate as calcium carboxylates from the fermentation broth. To a 100 mL bottle containing 50 mL of fermentation broth. 0.05, 0.1, 0.5, or 1 g of calcium chloride dihydrate was added. Additionally, in the case of the fermentation broth at an unaltered pH 6.52, experiments adding 2, 3 and 4 g were performed. After gentle mixing, the bottles were kept in a 20ºC room for 15 hours. After the suspension was filtrated by gravity, the filtrates were analyzed for residual FA concentration. Before and after precipitation experiments, the pH of solutions and filtrate was measured.

6.2.4 Analysis

Hydrogen, oxygen and methane were analyzed by gas chromatography as previously described (Steinbusch et al., 2008). Fatty acids (C_2-C_8) were analyzed by gas chromatography using a HP 5890 serie II with a glass column (2m x 6mm x 2mm) packed with 10% Fluorad 431 on Supelco-port 100- 120 mesh. Prepared samples (1.0 µl) were injected directly on the column at 200°C. The carrier gas was 40 mL min^{-1} nitrogen saturated with formic acid. Oven temperature was 130°C for 4 min, raised at 12°C min-1 ramp to 160ºC for 6.5 min. Fatty acids were detected with a FID at 280°C using hydrogen at 30 mL min^{-1} and air at 400 mL min^{-1}. Alcohols (C_2-C_6) were measured using the same column, but at a constant oven temperature of 70ºC.

Protein concentration determination with modified Hartree-Lowry method (Caprette, 1995) was used to quantify bacterial cell concentration in a small sample volume. One mL of reactor liquid was transferred in a 2 mL vial and centrifuged for 5 min at 10.000 rpm. The supernatant was removed and the pellet was resuspended in 1 mL of 1 M NaOH. The vial was left for 30 min at 50ºC in a water bath to hydrolyze cells. Subsequently, protein concentration of the hydrolyzed cells was quantified using modified Hartree-Lowry method. It was experimentally found that 1.0 g of protein equals 3.0 g of volatile suspended solids (VSS).

6.3 Results and discussion

6.3.1 Continuous caproate production

Caproate and caprylate were produced in both continuous flow CSTRs. The reactors were operated anaerobically: no oxygen was detected and the redox potential in reactor 2 remained below -500 mV. Results of both reactors operations are discussed separately.

Reactor 1

Caproate production was detected first at day 6 with 0.1 g g^{-1} VSS d^{-1} (Figure 6.1). During a continuous operation of 62-days, the specific caproate production rate increased from 0.1 to 1.6 g g^{-1} VSS d^{-1}. Caproate was produced up to a concentration of 10.5 g L^{-1} and caprylate to 0.48 g L^{-1} within 47 days, while ethanol and acetate were consumed (Figure 6.2). The

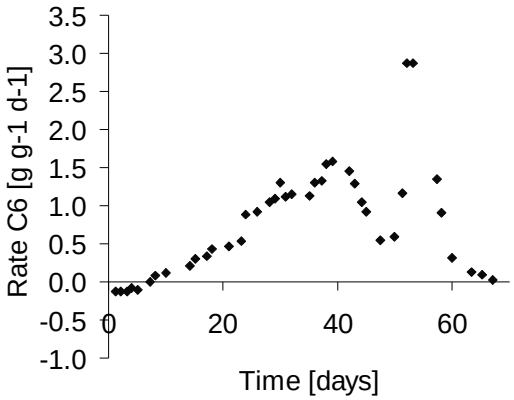

Figure 6.1 Caproate production rate per gram VSS in a continuous flow CSTR R1 (left) operating 67 days.

consumption ratio of ethanol versus acetate to products *n*-butyrate, caproate and caprylate changed over time, but was calculated to be 4.0: 1 over the course of the total experiment. The main product of the fermentation is caproate, for which the product yield increased in time to 0.6 mol C per mol C. The *n*-butyrate and caprylate production yield were 0.10 and 0.03 mol C per mol C, respectively. Initially, the *n*-butyrate yield was higher, at 0.26 mol C per mol C, but part of the *n*-butyrate was converted to caproate.

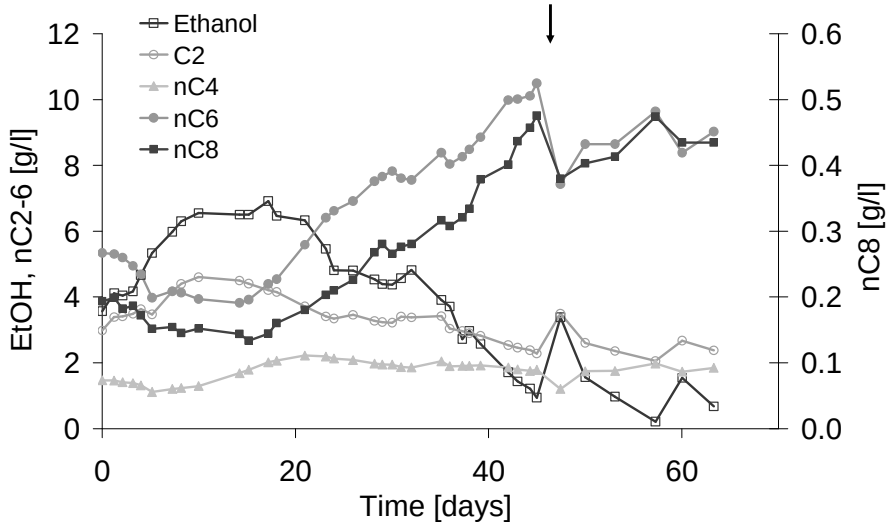

Figure 6.2 Concentration of substrate ethanol and acetate (C2) and products *n*-butyrate (nC4), caproate (nC6) on the primary y-axis and caprylate (nC8) on the secondary y-axis in a continuous flow CSTR of 67 days. The arrow indicates the timing of the pump failure.

On day 47, the medium pump was not switched off by the computer due to power failure. Instead of 4 grams, the pump added 73 grams of medium during half an hour. This event caused a sudden increase in substrate concentration and a decrease in MCFA concentration and production rate. To maintain a HRT of 21 days the pump remained switched off for the following 62 hours. Nevertheless, the sudden high medium dosage caused the biomass concentration to decrease from 0.32 to 0.013 g VSS L^{-1}, far more then was expected based on the dilution. In principle, the biomass dilution would only affect the volumetric production rate. However, the specific caproate production rate was also significantly lower than before the pump failure. The sudden high ethanol and acetate concentrations might have negatively influenced the bacterial population.

Reactor 2

Starting on the first day, caproate was produced and ethanol and acetate were consumed in reactor 2 (Figure 6.3). During the first 48 days, reactor 2

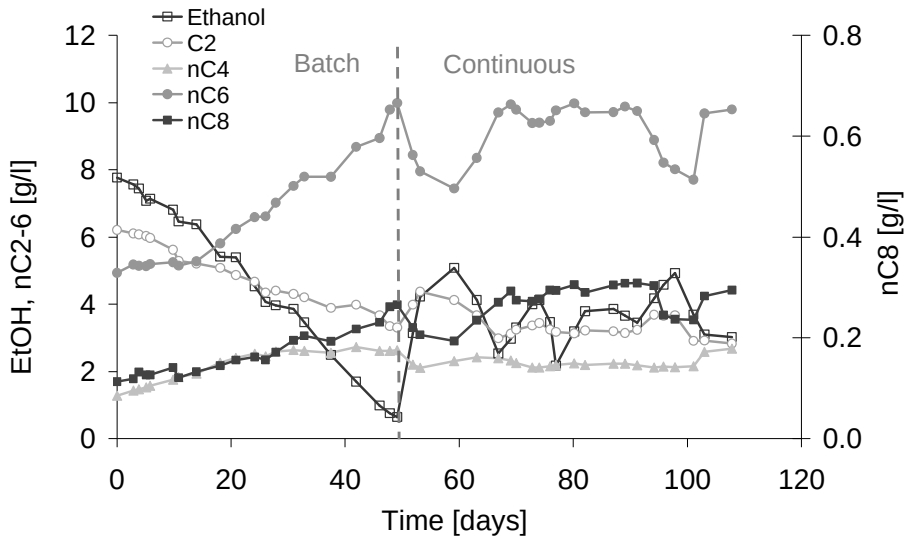

Figure 6.3 Concentration of substrate ethanol and acetate (C2) and products *n*-butyrate (nC4) and caproate (nC6) on the primary y-axis and caprylate (nC8) on the secondary y-axis in a CSTR reactor that was operated in batch for 49 days, followed by 61 days of continuous operation.

operated in batch mode until the ethanol was almost completely consumed. The specific caproate production rate increased slowly in the first 50 days, but remained below 1.0 g caproate g^{-1} VSS d^{-1} (Figure 6.4). As soon as the ethanol concentration was 650 mg L^{-1}, the pump was switched on and the reactor operated continuously for 62 days. After this mode switch, it took 10 days until the rate increased enough to convert all incoming ethanol and acetate. Then the specific production rate increased to a final rate of 2.94 g caproate g^{-1} VSS d^{-1}. After 80 days, 2 M NaOH solution was added by the pH controller, which caused a pH shock up to 8.3. Caproate production continued, though the specific production rate decreased to a level similar to the average production level previously observed in batch mode, namely 1.1 g caproate g^{-1} VSS d^{-1}. The consumption ratio of ethanol versus acetate over the total experiment was 4.4: 1. Overall caproate yield increased during the experiment from 0.28 to 0.60 mol C per mol C, whereas the *n*-butyrate yield decreased from 0.36 to 0.14 mol C per mol C. The caprylate yield was 0.02 mol C per mol C. The biomass concentration was stable at an average of 0.1 g VSS L^{-1}. When the reactor was operated in batch mode without biomass wash out, the biomass concentration did not increase, which might indicate a lack of nutrients. After switching the reactor operation from batch to continuous run, the biomass growth increased together with the specific caproate production rate.

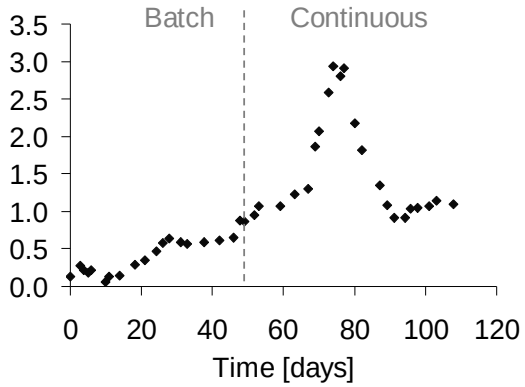

Figure 6.4 Caproate production rate per gram VSS in batch followed by a continuous flow CSTR R2 operating 116 days.

The performance of both reactors runs is summarized in Table 6.1. The start-up of the fermentation is not a point of concern, since the continuous operation on similar medium in the end resulted in comparable caproate concentrations and yields.

Table 6.1 Characteristics of caproate (C6) and caprylate (C8) production in two continuous flow CSTR runs.

	R1		*R2*	
	C6	*C8*	*C6*	*C8*
Conc. (g l^{-1})	10.5	0.48	10.6	0.26
Rate (g l^{-1} d^{-1}) $_{max}$	0.57	0.03	0.30	0.01
Rate (g g^{-1} VSS d^{-1}) $_{max}$	2.86	0.09	2.94	0.09
Yield in carbon	0.5-0.71	0.03	0.6	0.03

6.3.2 MCFA Separation

Extraction

Solvent extraction with petroleum ether and ethyl hexanoate could selectively remove caprylic and caproic acid from the fermentation broth. The effective distribution coefficient of caprylic and caproic acid between water and the solvents ethyl hexanoate as well as petroleum ether were significant higher than those for acetic and butyric acid (Figure 6.5). Caprylic acid had the highest effective distribution coefficient in both solvents and was thus most easily removed from the fermentation broth. The pH influenced the effective distribution coefficient strongly by influencing the amount of undissociated acid. Decreasing the pH of the broth before the extraction to 5 increased the effective distribution coefficient for both solvents with all the fatty acids, though the distribution coefficient of acetate and *n*-butyrate remained around or below 1.0 for both solvents. Extrapolating the results at pH 5, the real distribution coefficient for both solvents for caprylic acid is around 300 and for caproic acid 22. The high distribution coefficient of the target compound and low coefficient for the other compounds, combined with a low solubility of the solvent in the water phase, makes solvent extraction a suitable method to remove MCFA from the fermentation broth. The actual quantity of MCFA ultimately removed depends on the pH and on the water/solvent volume ratio. For example in the extraction experiment at pH 5, caproic and caprylic acid were concentrated 2.4 and 4.0 times, respectively, in the solvent.

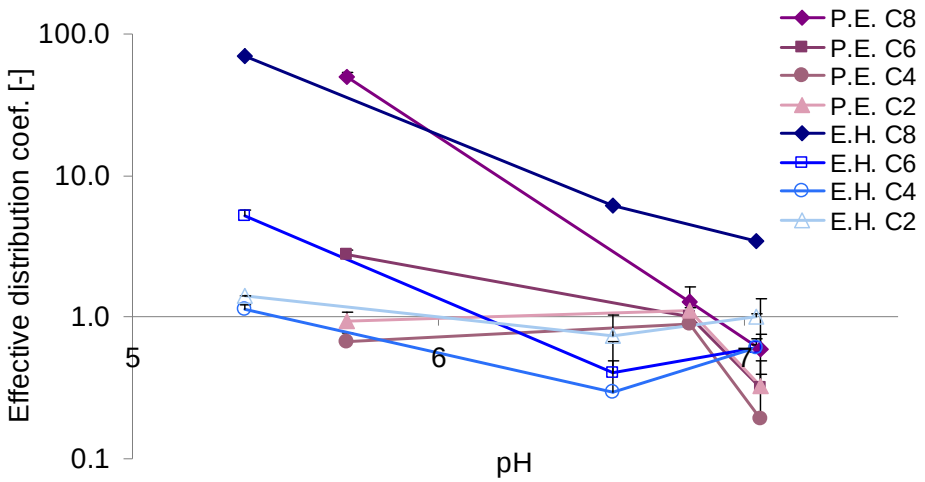

Figure 6.5 Distribution of acetic acid (C2▲), butyric acid (C4●), caproic acid (C6 ■) and caprylic acid (C8 ◆) among ethyl hexanoate (E.H.) and petroleum ether (P.E.) and water as function of pH.

Precipitation

Caprylate was selectively removed from the fermentation broth by calcium chloride addition. Adding calcium to the fermentation broth decreases caprylate concentration in the water phase, but hardly influences caproate concentrations (Figure 6.6). Addition of calcium in concentrations up to 22 g L^{-1} removed 64% of caprylate from the water phase, 8% of caproate, but no *n*-butyrate and acetate. In order to achieve an efficiency over 90%, much more calcium is needed than would be expected based on the solubility product. From earlier experiments, the calciumcaproate solubility product was determined to be $6.31 \cdot 10^{-07}$ mol^{-3} L^{-3} with an ionic strength of 0.1 M. In the present study, the solubility product was higher, between 2.24 - $4.48 \cdot 10^{-05}$ mol^{-3} L^{-3} at pH 6.52. This phenomenon is explained by the fact that addition of Ca influences the pH. Calcium carbonate precipitation upon calcium addition competes with caprylate reactions. The reduction in carbonate in the system associated with the aforementioned reaction decreases the buffer capacity and thus the pH of the system. At this lower pH, less caprylic acid dissociates, which reduces calcium caprylate

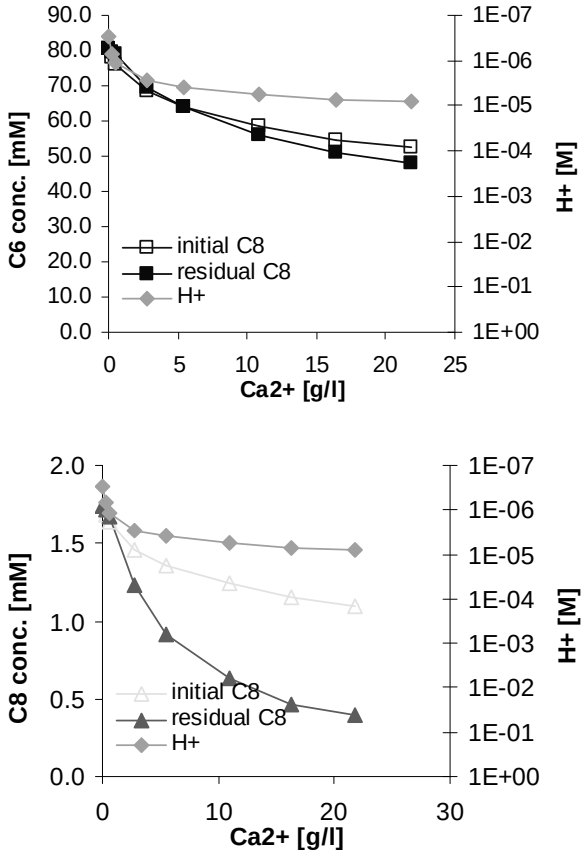

Figure 6.6 Calcium addition lowered the residual dissociated caproic acid (upper graph) and caprylic acid (lower graph) concentrations in fermentation broth, with an initial pH of 6.52, through both precipitation and by lowering the initial dissociated acid concentration through indirectly decreasing the pH.

precipitation. In summary, both separation techniques demonstrated selective removal of caprylic acid and caproic acid. Caprylic acid with its longer hydrophobic tail, is less soluble in water and was therefore easier to separate than caproic acid. Depending on the pH, the amount of calcium salt or the volume of extraction liquid, a higher removal efficiency can be achieved. Whereas calcium precipitation does provide higher product purity than solvent extraction, the effluent contains elevated calcium concentrations. Although solvent extraction requires an additional step to remove the desired compound, the solvents are either, like petroleum ether, insoluble in water or, in the case of ethyl hexanoate, moderate soluble in water (0.63 g L^{-1}), but can be removed by wastewater treatment.

6.3.3 MCFA fermentation and separation in general

The proof of principle of MCFA production by mixed cultures was demonstrated in a fed-batch operating CSTR (Chapter 5). Comparing the fed

batch operation mode of the CSTR with the continuous flow, more caproate was produced in the continuous flow than in the fed-batch operation. Although the volumetric caproate production rates were similar in both modes, this high rate was maintained over a longer period in the continuous operation, whereas the high rate in the fed-batch operation represents a peak during two days in one of the three active periods (Chapter 5). The specific production rate during continuous operation in both reactors was significantly higher than during batch operation. The better performance of the continuous flow operation can be explained by the stable growth conditions obtained by continuously adding medium and nutrients. In this way, substrate depletion, suspected of limiting caproate production in the fed-batch, was prevented. The yield in the continuous flow reactor was similar to the fed batch reactor.

Increasing yield by combining process production and separation

Based on the results of the activity test in the extracted medium, it was demonstrated that the extracted fermentation broth was fermentable after re-inoculation. In the effluent, significant amounts of *n*-butyrate were present, 1.7-2.1 g L^{-1} in reactor 1 and 2.1-2.6 g L^{-1} in reactor 2. If the *n*-butyrate and the non-extracted MCFA could be recycled back into the elongation reactor, a higher efficiency could be achieved. Similarly, increased efficiency can be achieved by improving the efficiency of the carbon balance. Part of the converted substrate (acetate and ethanol) was not detected, neither in products as VFA, MCFA, and alcohols nor in biomass. A share of 20-25% of carbon was "missing" in the carbon balance of both reactors. The "missing" carbon can be attributed to bacterial growth that was not suspended in the liquid phase. Another explanation is that part of the carbon in the form of ethanol was converted to CO_2 by dechlorinating bacteria using 2-bromoethanosulfonic acid as an electron acceptor (Chapter 5). There we saw that in addition to the MCFA producing relatives of *Clostridium kluyveri*, the microbial population in a MCFA producing reactor was dominated by a dechlorinating bacterium closely related to *Azospira oryzae*.

Implications

This research shows that MCFA as caproate and in small amounts caprylate were produced in higher concentrations, at higher specific production rates and with similar but more stable volumetric rates during continuous flow

operation than in fed-batch operation. Higher MCFA production could be achieved 1) by increasing influent concentrations, or 2) by increasing the flow rate. In case of higher influent concentration, ethanol concentration needs to be monitored, because as soon as the MCFA production activity stops the ethanol concentration will reach values that are toxic for organisms. Anaerobic conversion with low biomass growth on the order of 0.006 d^{-1}, demand high sludge retention times in order to have sufficient biomass for high production rates. Further research, MCFA fermentation can be performed in anaerobic systems with high biomass retention and a low growth rate while processing a high flow such as gas lift reactors or membrane bioreactors.

6.4 Conclusion

Caproate and caprylate were produced in a continuous flow CSTR by mixed cultures. Biological conversion of ethanol and acetate led finally to 10.5 g L^{-1} caproate and 0.48 g L^{-1} caprylate. The production yield was 0.6 mol C per mol C for caproate and 0.03 mol C per mol C for caprylate and the biomass growth was estimated to be 0.01 g g^{-1}. The conversion rate was found to be stable at 3.2 g caproate and 0.09 g caprylate g^{-1} VSS d^{-1}. Both caproate and caprylate were demonstrated to be selectively removed by calcium precipitation and solvent extraction with ethyl hexanoate and petroleum ether.

7 General discussion

7.1 Introduction

This thesis describes three new processes to convert volatile fatty acids to precursors of liquid compounds with a higher energy content by using mixed culture fermentations. VFA are involved in many anaerobic conversions. They are intermediates in the degradation processes of organic materials such as carbohydrates, proteins and lipids. Using VFA as building blocks for fuel or chemical production enables municipal and industrial waste to be utilized as sustainable biomass feedstock. VFA cannot directly be used as fuel, but an energy efficient biological conversion can decrease the quantity of oxygen to create a product with a higher energy content than VFA. Fermentation is a method to decrease the oxygen content of VFA without sacrificing the carbon or energy present in the starting material. During fermentation, bacteria release their electrons and protons using their organic products as acceptors, or using the hydrogenase enzymes to produce hydrogen (Rittmann and McCarty, 2002). By applying a high hydrogen pressure, hydrogen production is limited and the reduction of organic products such as VFA becomes thermodynamically more attractive. In this thesis, mixed cultures were fed with VFA at a hydrogen partial pressure of 1.5 bar, which resulted in the formation of the alcohols ethanol, propanol, butanol and hexanol, and the fatty acids *n*-butyrate, caproate and caprylate. Most importantly, conditions were found that mixed cultures successfully converted VFA at a high rate to caproate and caprylate in such a high concentration that the downstream processing could be performed efficiently.

In section 1.2 of the discussion, an update is given on the status of the proof-of-principle experiments. In 1.3, the use of thermodynamics as a tool to investigate ways to steer the mixed culture is discussed. In section 1.4, the possibility of using the process in practice is analyzed; the case of caproate production is further developed, as this product was produced fast and in high quantity and has an advantage in terms of separation.

7.2 Liquid biofuel production by VFA conversion

Biohydrogenation with hydrogen

In chapter two, it was thermodynamically calculated and experimentally demonstrated that mixed culture bacteria can be directed to produce alcohol from volatile fatty acids (VFA) with hydrogen as an electron donor, a mechanism called biohydrogenation. In batch tests, acetic, propionic and butyric acids were reduced by mixed microbial cultures with a headspace of 1.5 bar of hydrogen. Alcohol concentrations were observed to be 3.69 ± 0.25 mM for ethanol, 8.08 ± 0.85 mM for propanol and 3.66 ± 0.05 mM for *n*-butanol. The conversion efficiency based on the electron balance was 55.1 ± 5.6% with acetate as the substrate, 50.3 ± 4.7% with propionate and 46.7 ± 2.2% with *n*-butyrate. Methane was the predominant by-product in each batch experiment, 33.6 ± 9.6% of VFA and hydrogen was converted to methane with acetate as the substrate, 27.1 ± 7.1% with propionate and 36.6 ± 2.2% with *n*-butyrate. In chapter 3, methanogenesis was successfully inhibited and acetate reduction was enhanced after thermal pretreatment and incubation at pH 6. Initially, more ethanol (7.7 ± 3.2 mM) was produced than in the first experiment with a higher efficiency (60.2 ± 8.7%). However, during the experiment ethanol was converted to *n*-butyrate (7.02 ± 0.85 mM) with an efficiency of 76.2 ± 14.0%.

Biohydrogenation with electrons

Chapter 4 studied the possibility of using an electrode instead of hydrogen as an electron donor for biological acetate reduction. Biohydrogenation of acetate was stimulated in the cathodic compartment of a bioelectrochemical system with the same mixed cultures as was used in chapter 2 and 3. Methyl viologen was selected as mediator to accelerate electron transport from the cathode to the bacteria. As soon as methyl viologen was added to the

bioelectrochemical system with an applied cathode potential of -550 mV, ethanol was produced (0.084 g L^{-1}). Hydrogen was co produced at the cathode (0.0035 normalized m^3 hydrogen m^{-2} d^{-1}), making it unclear what the exact electron donor of biohydrogenation was, MV or hydrogen. The advantages of MV addition were that it inhibited *n*-butyrate production and that it lowered the overall potential required for both ethanol and hydrogen production. The current density of 1.33 A m^{-2} at the potential of -550 mV vs NHE was high compared to the current density of a biocathode producing hydrogen (0.2 A m^{-2}) at a similar potential (Jeremiasse et al.). A drawback to this setup was that MV irreversibly reacted at the surface of the electrode. Only 20% of MV was left two days after addition, which inhibited the ethanol production and initiated *n*-butyrate production in the bioelectrochemical system. Since the mediator is highly toxic and continuous addition of MV is not a sustainable way to produce ethanol, further research on this topic should be focused on immobilizing MV at the electrode or on non-mediated ethanol production. It is expected, though, that without MV in the fermentation broth ethanol will most likely be converted to *n*-butyrate or longer chain products.

Chain elongation of acetate with hydrogen or ethanol

The third process to convert VFA to high energy liquid compounds is the coupling of acetate to longer chain fatty acids. The proof–of-principle of chain elongation by mixed cultures was described in chapter 5. It was found that acetate as the main intermediate of anaerobic conversion can be elongated to medium chain fatty acids with 6 or 8 carbon atoms in a simple mixed-culture fermentation. Mixed microbial cultures were able to produce 8.17 g L^{-1} caproate and 0.32 g L^{-1} caprylate under methanogenesis-suppressing conditions in a stable reactor. The highest caproate production rate was 25.6 mM C per day with a product yield of 0.6 mol C per mol C. The production of caprylate in any significant amount has not been previously reported in the literature. Caproate production by mixed cultures was found before in co-cultures of *C. Kluyveri* with ruminal cellulolytic bacteria, but such a high concentration has never been reported before. Kenealy *et al*. (1995) produced 2.6 g *n*-butyrate L^{-1} and 4.6 g caproate L^{-1} from 4.4 g L^{-1} ethanol and 6.0 g L^{-1} cellulose with a yield of 0.647 mol C per mol C.

In this thesis, chain elongation was performed with two electron donors, ethanol and hydrogen, which demonstrated the flexibility of this process. Microbial characterization revealed that the microbial populations were stable and dominated by relatives of *Clostridium kluyveri*. To avoid long lag times and substrate limitations of batch fermentations, it was investigated if MCFA production is possible in a continuous flow operating reactor, as described in chapter 6. Two CSTRs operated 61 and 67 days continuously at an HRT of 22 days. During the operation period, the specific MCFA production activity increased to 2.9 g caproate and 0.09 g caprylate per gram VSS d^{-1}, which was independent of the different start-up method of the CSTR reactors. The final caproate and caprylate concentrations in the fermentation broth were 10.5 g L^{-1} and 0.48 g L^{-1}, respectively. Those concentrations were demonstrated to be high enough to be removed selectively by calcium precipitation and solvent extraction with ethyl hexanoate and petroleum ether.

7.3 Steering mixed culture fermentation

VFA are involved in many anaerobic reactions (Thauer et al., 1977), in metabolism as well as in catabolism. In mixed culture fermentation, the highly diverse population of fermentative bacteria can perform there reactions (Rittmann and McCarty, 2002). As we saw in the chapter 2 and 3, acetate was converted to ethanol, *n*-butyrate and to methane, while a single product was preferred. Since many reactions are possible with VFA and microorganisms are present that can perform these reactions, it is difficult to predict how the fermentation must be steered in order to drive the desired reaction. Thermodynamics can help to understand mixed culture fermentations by knowing the energy limits of reactions, which determines what is possible or impossible under given conditions. Also, it can help in predicting the dominant fermentation under the assumption that the most energy consuming reaction will dominate. Accordingly, the process can be steered by manipulating environmental conditions like pH, temperature or concentration. For example, of the three dominant processes ethanol, *n*-butyrate and methane formation, methanogenesis was energetically the most favorable process and would dominate the fermentation without intervention. The process could be steered toward ethanol and *n*-butyrate production through physical pretreatment with heat to kill the heat-sensitive methanogens and thus enriching for the resistant ethanol and *n*-butyrate

producing organisms. It must be noted, though, that such a sterilization step is energy costly, which will negatively influence the overall energy efficiency of a process.

Comparing biohydrogenation and chain elongation, it shows that it is thermodynamically more favorable to produce *n*-butyrate than ethanol from acetate and hydrogen. Other than the addition of toxic chemicals such as MV, methods to prevent ethanol consumption by *n*-butyrate production were not found. Neither varying the pH nor applying a thermal heat pretreatment inhibited *n*-butyrate production. Production of ethanol by biohydrogenation from acetate will not be realizable with mixed cultures fermentation at applied conditions, because it is thermodynamically more favorable for organisms to produce *n*-butyrate or medium chain fatty acids. Most likely acetate reduction in mixed culture fermentation at high hydrogen pressure will always lead to medium chain fatty acids or longer chain alcohols such as butanol and hexanol (Chapter 5).

7.4 MCFA fermentation as a fuel production technology

Medium chain fatty acid production is a promising technology to produce liquid precursors of fuel or chemicals. Using MCFA fermentation in a biomass conversion process, the production chain will consist of 2 fermentation reactors, acidification and fermentation reactor, followed by a liquid-liquid separation step. The ultimate potential of MCFA fermentation as a viable technology depends on each individual step in the production chain: acidification of the biomass, the MCFA fermentation, and separation of the desired product, but also on the integration of all steps together. The sustainability of the biofuel production process is determined by the feedstock choice for VFA and electron donor production as well as the energy consuming separation step.

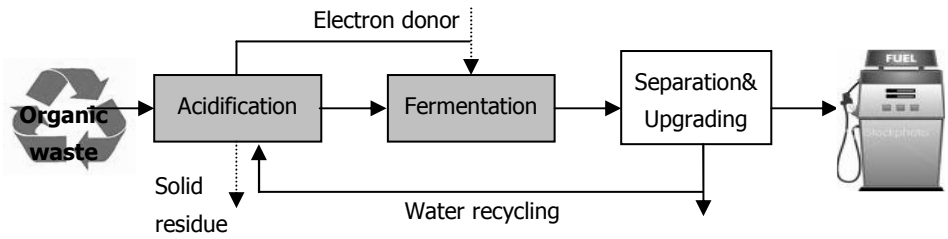

Figure 7.1 Process scheme of integrated MCFA production from organic waste.

7.4.1 Fuel properties of MCFA

MCFA fermentation has a high potential both in terms of the desirable fuel characteristics of MCFA and the good performance of the fermentation with respect to specific MCFA rate, concentration and efficiency. In the conversion of biomass to fuels, the most important change in chemical structure is the removal of oxygen. The ratios of oxygen and carbon or oxygen and hydrogen in a molecule determine some important characteristics of a fuel. For example, the lower the oxygen content is, the higher the heating value. Biomass components such as carbohydrates have a oxygen to carbon ratio of 1:1, while ethanol has a oxygen/carbon ration of 0.5. A comparison of caproate and caprylate based on oxygen/hydrogen (O/H) ratio and oxygen/carbon (O/C) ratio shows that these MCFA have lower O/C ratios than ethanol, but butanol has yet a lower O/H ratio (Table 7.1). Higher than ethanol, the MCFA have a similar energy density to that of butanol. The advantage of MCFA over butanol is the low water solubilities of caproic and caprylic acid, which are, respectively, a factor of 6 and 100 lower than of the solubility of butanol. Therefore, we expect that better and more efficient separation from the fermentation broth is possible with MCFA than with butanol.

Table 7.1 Properties of fermentation products that can be used as liquid fuel.

Biofuel	*Carbon atoms*	*O/H ratio*	*O/C ratio*	*Energy density (MJ kg^{-1})*	*Solubility (g L^{-1})*
Ethanol	2	0.17	0.5	23.1	
Butanol	4	0.1	0.25	29.2	63.4
Caproic acid	6	0.17	0.33	28.1	9.67
Caprylic acid	8	0.13	0.25	30.5	0.68

Regarding the MCFA performance, the highest caproic acid concentration (10.5 g L^{-1}) produced in chapter 6 was high enough to selectively remove acids from the fermentation broth using liquid-liquid extraction. It is expected that the caproic acid concentration can still be increased during fermentation, since the production was thus far substrate limited. The specific caproate production rate could be increased through constant addition of fresh medium in continuous flow operating reactors. The highest specific caproate production rate was 2.9 g caproate g^{-1} VSS d^{-1} in a continuous running

reactor. The further increase in the rate was limited by an operational error, which prevented a full realization of the potential of the MCFA fermentation technology. Further research is needed to study the full capabilities of MCFA production in a more stable reactor run with a higher cell density. For a proof-of-principle study, however, the specific production rate is already quite high compared to the anaerobic conversion rate of optimized acetoclastic methanogenesis, which is 7 g acetate g^{-1} VSS d^{-1} at 35°C (Rittmann and McCarty, 2002).

7.4.2 Electron donor: ethanol and hydrogen

Chain elongation is flexible in electron donor choice, both ethanol and hydrogen can be used (Chapter 5). In the controlled fed-batch and continuous flow reactors where both electron donors were added to the fermentation, ethanol was preferred over hydrogen. The ethanol:acetate consumption ratio of 4:1 was higher than the expected ratio of 2:1 based on equation 5.2. The high ethanol consumption could be explained by an ethanol oxidizing side reaction with the inhibiter 2-bromoethanosulfonic acid as an electron acceptor. This assumption is supported by the fact that little CO_2 was found in the effluent and by the dominance of *Azospira oryzae* in the MFCA producing bioreactor. This nitrogen-fixing beta-proteobacterium can use chlorate or selenate as electron donors (Hunter, 2007), which are comparable to the chemical structure of the methanogenesis inhibiter 2-bromoethanosulfonic acid. Exclusion of the inhibiter is expected to lower the ratio and increase the MFCA product yield. Still, a considerable amount of ethanol will be consumed for the production of MCFA. Depending on the feedstock, ethanol can be prepared sustainably. Ethanol is preferably produced from organic waste via biohydrogenation of acetate, but the most proven ethanol production technology is sugar fermentation by yeasts. With yeast fermentation, ethanol can still be produced sustainably from cellulosic biomass that is sustainably produced, the so-called second generation biofuel production. Hydrogen can be produced more sustainably from waste during acidification or by bioelectrolysis, or from water via electrolysis.

An alternative to the large amount of ethanol needed for MCFA production can be *n*-butyrate. In the experiments described in chapters 5 and 6, a portion of the *n*-butyrate that was formed in the beginning was later converted to caproate. *n*-Butyrate, like acetate, is produced during the

acidification of waste. Thus, perhaps the presence of *n*-butyrate in the influent of the MFCA reactor reduces the amount of ethanol needed for caproate or caprylate production.

7.4.3 Potential of fermentation from low-grade biomass

This thesis studies the possibilities to convert biomass to liquid fuel and or chemicals via VFA. The research focused mainly on proof-of-principle studies on the conversion step of VFA to the liquid fuel. The experiments were performed with synthetic medium containing acetate, and not with effluent from an acidification tank. To give an indication of the potential of biofuel production from real acidified waste, the MCFA production potential was calculated with data from this thesis together with literature data reporting VFA production during solid waste acidification. The potential MCFA production is compared to other anaerobic conversions that can be used to process low grade organic waste to energy rich components such as hydrogen, methane, or liquid biofuel as MCFA. The processes are compared on carbon and energy recovery of the original waste in the final product.

As was discussed in the introduction, the exact acidification products depend on the type of organic waste, and the experimental conditions applied during the acidification. Fang *et al.* (2006) were one of the few researchers who carried out acidification on food waste using mixed cultures at different applied pH, and who reported the exact VFA composition and hydrogen production at each pH. Fang *et al.* acidified food waste rice slurry using mixed cultures (sludge) at pHs between 4 and 7 at 0.5 unit intervals to study hydrogen production potential. The rice was composed of carbohydrate (78.3%), protein (6.6%), lipid (3.2%) and water (11.9%). Acidification of 1 kg of rice food waste at pH 5.5 yielded 3.7 mol acetate, 2.66 mol *n*-butyrate, 0.65 mol ethanol and 9.62 mol of hydrogen. Based on the caproate and *n*-butyrate yield in chapter 6, the conversion efficiency of MCFA production is together 0.75. The efficiency of VFA conversion to methanogenesis is 90%.

If these acidification products would be converted to caproic acid with a conversion yield of 0.75, then 280 g caproate can be produced. The highest energy recovery occurs when the rice slurry is converted to methane. The most carbon is recovered when the rice slurry is converted to caproate. The

Table 7.2 Energy content of conversion of acidification products of 1 kg cooked rice

(Fang et al., 2006)	HHV MJ kg^{-1}	Energy recovery %	Carbon recovery %
0.783 kg carbohydrate	- 12.379[a]	100	100
9.62 mol H_2	- 2.188[b]	17.7	-
11.2 mol CH_4 + 9.62 mol H_2	- 10.430[c]	84	39
2.41 mol caproic acid and 1.12 H_2	- 8.636[d]	69.7	55

[a] assumed that 1 mol of $[C_6(H_2O)_5]_n$ has the same HHV as 1 mol of Glucose

[b] assumed that the headspace has a temperature of 37°C as is described in the article.

least efficient application of organic waste is sole hydrogen production, where only 17% of the total energy in the carbohydrate is recovered and no carbon. In these energy calculations, the separation process is disregarded. This will negatively affect the overall energy balance for caproic acid production, because the gas methane is easier to separate from the fermentation broth.

7.5 Technology comparison

This thesis studies the conversion processes required to utilize organic waste for liquid fuel and or chemical production. In this section, a rough comparison was made with commonly practiced technologies that use organic waste for energy production and with those that use organic waste for fuel. It must be noted that it is difficult to compare MCFA fermentation with current technologies, since the work presented here has yet to be optimized.

MCFA fermentation as competing process for feedstock for energy production

Based on energy recovery, anaerobic digestion is the most energy efficient process: the most energy is recovered from the biomass and the process itself requires low energy input since gas separates naturally from the liquid slurry very easy. Biogas is, however, difficult to transport and has a low economical value. The biogas composition is different from the natural gas and needs energy intensive upgrading before it can be transported in the gas grid. The low energy density of gas makes transport with a truck energy inefficient; it would better to use biogas locally for heat or electricity production.

Costs are mainly determined by the variable cost of feedstock, and fixed costs which include operational cost and maintenance, as determined by the process design. The feedstock costs for anaerobic digestion are the same as those for MCFA production, but the process costs of MCFA production are higher, since a liquid-liquid separation is needed instead of gas-liquid separation. However, considering that the economical value of the product is higher than heat or electricity, the production cost can be higher without influencing the economic viability of MCFA production.

MCFA fermentation as competing process for feedstock for fuel production

The energy input of bio-ethanol production is mainly determined by the feedstock choice and the down-stream process distillation. Using waste biomass saves a considerable amount of energy in comparison to using lignocellulose. This was seen in the study of Granda et al. (2007), where they compared the waste converting MixAlco process with cellulosic ethanol production on energy use to produce ethanol. Here, the ratio between the energy output in ethanol and the energy input during the production process were calculated for different second generation ethanol production processes. It appeared that the MixAlco process, with a ratio of 18.87, was comparable to the cellulosic manufacturing technologies with a ratio between 15-19 depending on the technology (Granda et al., 2007).

We refer to the MixAlco process because it, like MCFA fermentation, uses the same low grade biomass feedstock and mixed cultures to ferment the biomass. The difference between MCFA fermentation and the MixAlco process is that the latter only produces volatile fatty acids with mainly acetic acid. Volatile fatty acids precipitate directly with calcium to calcium carboxylates, which are separated and further converted to ethanol and other alcohols in a thermal hydrogenation process (Holtzapple et al., 1997). The advantage of MCFA production over both MixAlco process and cellulosic ethanol fermentation is the product is a precursor for biodiesel compound.

7.6 Concluding remarks

Chain elongation is a promising technology to convert cheap, low-grade biomass into medium chain fatty acids as precursors for liquid fuels or chemicals. This process converts biomass that is unsuitable as feedstock for sugar fermentations or thermal conversions. This so-called low grade

biomass is abundantly present in many agricultural areas and its use is economically very attractive. The proposed process is straightforward, as it requires two simple and robust mixed culture fermentation steps for acidification and chain elongation. The best application of the processes demonstrated in this thesis should lead to caproate and caprylate production in such a concentration that separation by precipitation or extraction could be performed efficiently. To estimate the potential of the technology, further research is needed to optimize the MCFA production and separation step.

Abstract

The production of renewable fuels and chemicals reduces the dependency on fossil fuels and limits the increase of CO_2 concentration in the atmosphere only if a sustainable feedstock and an energy efficient process are used. The thesis assesses the possibility to use municipal and industrial waste as biomass feedstock to have little of no competition with food production, and to save greenhouse gasses emissions. Waste is a complex substrate with a diverse composition and high water content. It can be homogenized without losing its initial energy value by anaerobic conversion to volatile fatty acids. Using VFA gives the opportunity to process cheap and abundantly present biomass residues to a fuel and chemical instead of sugar containing crops or vegetable oil. This thesis describes the feasibility to convert VFA to compounds with a higher energy content using mixed culture fermentations by eliminating of oxygen and/or increasing the carbon and hydrogen content. At high hydrogen pressure, protons and electrons release via the reduction of organic products such as VFA becomes thermodynamically more attractive. Three VFA reduction reactions were studied: hydrogenation to an alcohol with 1) hydrogen and 2) an electrode as electron donor, and 3) by chain elongation with hydrogen and ethanol.

Based on concentration, production rate and efficiency, elongation of acetate with hydrogen and/or ethanol was the best technique to convert VFA into a fuel. In a continuous flow CSTR, 10.5 g L^{-1} caproic acid and 0.48 g L^{-1} caprylic acid were produced with ethanol and/or hydrogen at a specific MCFA production activity of 2.9 g caproate and 0.09 g caprylate per gram VSS d^{-1}. The products were selectively removed by calcium precipitation and solvent extraction with ethyl hexanoate and petroleum ether. Microbial characterization revealed that the microbial populations were stable and dominated by relatives of *Clostridium kluyveri*.

VFA could also be reduced to alcohols. Acetic, propionic and butyric acids were biohydrogenated with hydrogen and acetic acid also with an electrode. Observed alcohol concentrations were 0.62 g L^{-1} ethanol, 0.49 g L^{-1} propanol and 0.27 g L^{-1} *n*-butanol. Methanogenesis was successfully inhibited after thermal pre-treatment incubated at pH 6, while acetate reduction was enhanced. In the second study, ethanol (0.084 g L^{-1}) was produced at the cathodic compartment of a bioelectrochemical system, in which the electron transport was mediated by methyl viologen. The ethanol production activity at the cathode was only of very short term, since the mediator irreversibly reacted at the surface of the cathode.

Of the two VFA conversion processes, biohydrogenation and chain elongation, chain elongation was a more dominant process that consumes ethanol with acetate to medium chain fatty acids. With this technology, wet organic waste can be converted to biofuels carbon and energy efficient. The technology is promising due to the good fuel and separation properties of medium chain fatty acids, and the possibility to produce them at high concentrations and specific production rates comparable to other anaerobic conversions.

Nederlandse samenvatting

De productie van duurzame biobrandstoffen en chemicaliën vermindert de afhankelijkheid van fossiele brandstoffen en beperkt de toename van de CO_2 concentratie in de atmosfeer mits duurzame grondstoffen en een energie-efficiënt proces hiervoor worden gebruikt. Het proefschrift beoordeelt de mogelijkheid om stedelijk en industrieel organisch afval te gebruiken als grondstof voor de productie van duurzame biobrandstoffen om zo weinig of geen concurrentie aan te gaan met voedselproductie, en om de uitstoot van broeikasgassen te verminderen ten opzichte van eerste generatie biobrandstoffen. Organisch afval is een complexe grondstof met een gevarieerde samenstelling en een hoog vochtgehalte. Het afval kan zonder verlies van haar oorspronkelijke energetische waarde gehomogeniseerd worden door anaërobe omzetting naar vluchtige vetzuren. Door het gebruik van deze vluchtige vetzuren voor brandstof productie kunnen goedkope en overvloedig aanwezige organische afvalstromen gebruikt worden in plaats van suikerhoudende gewassen of plantaardige oliën. Dit proefschrift beschrijft drie haalbaarheidstudies om vluchtige vetzuren om te zetten naar voorlopers van biobrandstoffen of chemicaliën met een hogere energetische dichtheid. Onder hoge waterstofspanning kunnen organismen met hun protonen en elektronen, die vrijkomen tijdens het metabolisme, vluchtige vetzuren omzetten naar gereduceerde verbindingen. Drie reductiemechanismen van vluchtige vetzuren werden bestudeerd: biohydrogenering tot een alcohol met 1) waterstof en 2) een elektrode als elektronen donor, en 3) door de microbiële ketenverlenging met waterstof en ethanol als elektronen donor.

Op basis van concentratie, productiesnelheid en omzettingsefficiëntie, is ketenverlenging met waterstof en/ of ethanol de beste techniek om azijnzuur om te zetten naar een brandstof. Een continue draaiende CSTR met azijnzuur als substraat, en ethanol en/of waterstof als elektronen donor, produceerde 10.5 g l^{-1} capronzuur en 0.48 g l^{-1} caprylzuur met een specifieke productie activiteit van 2.9 g caproaat en 0.09 g caprylate per gram VSS d^{-1}. De

producten konden selectief verwijderd worden door precipitatie met calcium en door vloeistof-vloeistof extractie met ethylhexanoaat en petroleumether. Uit karakterisering van de microbiële populatie in de CSTR bleek dat de microbiële populaties stabiel waren en werden gedomineerd bacteriën die gerelateerd zijn aan *Clostridium kluyveri*.

Een ander mechanisme om vluchtige vetzuren om te zetten naar biobrandstoffen is biohydrogenatie naar alcoholen. Azijnzuur, propionzuur en boterzuur werden met waterstof omgezet naar 0.62 g l^{-1} ethanol, 0.49 g l^{-1} propanol en 0.27 g l^{-1} *n*-butanol. Methaanvorming verminderde de biohydrogenatie efficiency, maar kon selectief geremd worden na thermische voorbehandeling en incubatie bij lage pH. In het tweede biohydrogenerings studie werd azijnzuur aan de kathode van een bioelectrochemisch systeem omgezet naar ethanol (0.084 g l^{-1}). In het systeem werd het elektron transport versneld door een mediator methyl viologen. Door een instabiele mediator echter hield de ethanol productie aan de kathode niet stand Concluderend, hoewel biohydrogenation van azijnzuur met waterstof mogelijk was, domineerde microbiële ketenverlening in de fermentatie door ethanol en azijnzuur om te zetten naar de middellange vetzuren ketens als capron- en caprylzuur. Door de goede brandstof eigenschappen en scheidings-mogelijkheden van deze middellange vetzuren, en de mogelijkheid om deze verbindingen koolstof- en energieefficiënt tot hoge concentraties en snelheden te produceren, biedt deze technologie een nieuwe mogelijkheid om natte organische afvalstromen te verwerken tot biobrandstoffen.

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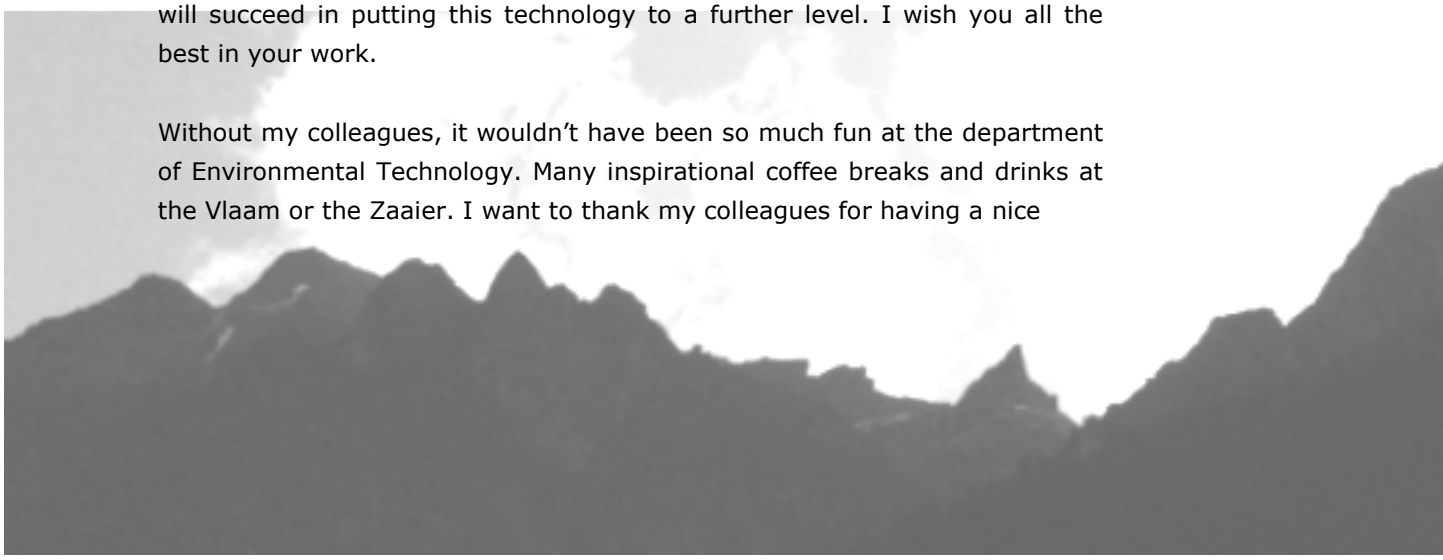
Without my colleagues, it wouldn’t have been so much fun at the department of Environmental Technology. Many inspirational coffee breaks and drinks at the Vlaam or the Zaaier. I want to thank my colleagues for having a nice



time, especially Pim, David, my twin sister Darja, Christel, Marjolein, Paula, Roel, Martijn Smit, Cees, Jana, Jan, Fernando, Sara, Tania, Claudia, Lina, Titia, Joost van Buren, Marjo, Hardy, Mirjam, my collegues of Wetsus, especially Jan en René in the bio-energy group. The moments of sharing our experiences were pleasant. Annemiek and Alex, my paranimphs, I am very glad that you both started as PhD's in the same year at Environmental technology as I did. Annemiek, you have a warm personality. I really appreciated our conversations, thank you for sharing experiences. Alex, it was really pleasant working at your site in the lab. Thank you for the good laugh, which brought things in perspective. It was very special to have worked with you both and hopefully this friendship will continue. Thank you Niels for joining me in the next adventure the start of the company Waste2Chemical. Thinking of how to commercialize the chain elongation process, and the long road that has to continued to realize that, were of useful help during the writing of the last discussion chapter.

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Curriculum vitae

Kirsten Steinbusch was born in 27 Februari 1980 in Weert, the Netherlands. She grew up in the south of Limburg and graduated secondary school in Grotius College in Heerlen. She already knew in early stage that she would study after college "Milieuhygiëne" (Environmental Studies) in Wageningen, where she started in 1998. During her study, she first focused on aquatic chemistry, but after 2 months she realized that analyzing problems was not her true motivation, solving problems was more her style. She then specialized in Environmental Technology where she finished both her Major and Minor thesis. After graduation in 2004, she was offered to join the bioenergy group at the sub-department of Environmental Technology of WUR as junior researcher. In 2005, she got the opportunity to graduate on the same topic with the B-basic project.

End of 2009, she continued working in this group as Post-doc on the EU Plantpower project. Next to that, she is writing a business plan for the spin-off company "Waste2Chemical", to valorize the patented technology discovered during her PhD.

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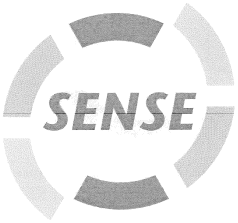

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SENSE PhD courses:

- **Environmental Research in Context**
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- **Sustainable Bio-energy and Innovation, WUR**

Other Phd and MSc courses:

- **Thermodynamic in biochemical engineering, TU Delft and EPFL**
- **Bioreactor design and operation, WUR**
- **Wisconsin entrepreneurial boot camp 2008 and Dutch block, University of Wisconsin and WUR**
- **EU Intensive Program: renewable biomaterials, University of Ghent**
- **Biological processes in Environmental Technology, WUR**
- **Techniques for writing and presenting scientific papers**
- **The art of writing**
- **Project- and time management**

Management Skills Training:

- **Organization of the PhD writing week**
- **PhD representative in the education committee of the sub-department of Environmental Technology, WUR**
- **Co-organization of the PhD course: Sustainable bio-energy and innovation**
- **Writing two grant proposals (SenterNovem NEO T02010)**
- **Inventing and writing of patent application on fatty acids production (EP07076132.5)**

Oral Presentations:

- **"Alcohol production from wastewater", Sense summer symposium Kyoto and Beyond, 23 June 2005, Ede, The Netherlands**
- **"Alcohol production from wastewater - Alternative for methane production?" Workshop on Mixed Culture Biotechnology for production of chemicals and energy, 12-13 June 2006, Delft, The Netherlands**
- **"Selective inhibition of methanogens for increasing ethanol production from acetic acid by mixed cultures", International Orbit conference, 13 - 15 October 2008, Wageningen, The Netherlands**

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SENSE Coordinator PhD Education and Research

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