Masteroppgave

NTNU
Norges teknisk-naturvitenskapelige universitet
Fakultet for naturvitenskap
Institutt for bioteknologi og matvitenskap

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Effects of a Hyperthermophilic Pre-Treatment applying *Thermotoga Lepl 10* in Biological Waste for Anaerobic Digestion

Masteroppgave i Biotechnology (5 years)
Veileder: Olav Vadstein, Ketil Stoknes
Mai 2019

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Vilde Holen

May 2019

Abstract

Anaerobic digestion (AD) of biological waste has become a widely used technology in the production of the renewable energy source, biogas. Introducing a pre-treatment to the waste is common to hygienise and hydrolyse to increase the efficiency of AD. A pre-treatment technology has been developed by Hyperthermics ™ AS, utilising the hydrolysing and H_2-producing properties of the hyperthermophilic bacterium *Thermotoga Lepl 10*. In laboratory-scale, *Thermotoga* spp. is well studied in fermentation of complex carbohydrates, but little is known on full-scale industrial operation. In this thesis, a study was performed on the first Hyperthermics™ pilot-plant, installed and connected to the biogas plant at Lindum AS in Drammen. Biowaste was sampled before and after the pre-treatment and used as feedstock in lab-scale AD to study the effect on methane gas (CH_4) production and digestate. Samples were also taken from the pilot-plant to analyse the fermentation products, applying spectrophotometric analyses (Hach Lange) and High-performance liquid chromatography (HPLC). In addition, quantitative real-time PCR (qPCR) was conducted on samples to study the amount of *T. Lepl 10* compared to the total amount of bacteria. The hyperthermophilic pre-treatment did not show any improvement in the production of CH_4, and no *Thermotoga* spp. could be detected. Still, a slightly higher diversity in the fermentation products was observed compared to waste that was only hygienised. This project has emphasised some of the challenges of up-scaling to industrial production. Also, it is indicating a need for further optimising of the process in order to improve growth conditions for the bacterium.

Sammendrag

Anaerob nedbryting av biologisk avfall har blitt en utbredt teknologi for produksjon av den fornybare energikilden biogass. Forbehandling av avfallet er vanlig for å utføre lovpålagt hygienisering, og å hydrolysere for å oppnå økt effektivitet av fermenteringen. Hyperthermics™ AS har utviklet en forbehandlingsteknologi som utnytter de hypertermofile og H_2-produserende egenskapene til bakterien *Thermotoga Lepl 10*. I laboratorieskala er arter av *Thermotoga* nøye studert i fermentering av komplekse karbohydrater, men lite er kjent om prosessene i fullskala. I dette prosjektet ble det første pilotanlegget til Hyperthermics™ studert, og pilotanlegget er installert og koblet til biogassanlegget til Lindum AS i Drammen. Prøver av biologisk avfall ble tatt før og etter forbehandlingen og testet som substrat i lab-skala biogassproduksjon, for å studere effekten på metanproduksjon (CH_4) og biorest. I tillegg ble det tatt prøver fra pilotanlegget for å studere ulike fermenteringsprodukter. Dette ble gjort ved hjelp av spektrofotometriske analyser (Hach Lange) og høypresisjonsvæskekromatografi (HPLC). I tillegg ble kvantitativ real-time PCR (qPCR) gjennomført i et forsøk på å kvantifisere *T. Lepl 10* og sammenligne med totalt antall bakterier i prøvene. Den hyperthermofile forbehandlingen viste ingen effekt på produksjon av CH_4 eller biorest. Forbehandlingen viste noe større diversitet i fermenteringsprodukter sammenlignet med biologisk avfall som kun var hygienisert. Dette prosjektet har understreket mange av utfordringene ved å oppskalere fra lab-skala til industriell produksjon. I tillegg viser det antydning til at videre optimalisering av prosessen er nødvendig for å forbedre vekstforholdene til *T. Lepl 10*.

Abbreviations

AD Anaerobic Digestion

AMPTS Automatic Methane Potential Test System

BT-nonR Buffer tank content, not regulated

BT Buffer Tank

BTpH pH regulated and hygienized buffer tank content

CSTR Continuous Stirred-Tank Reactor

HPLC High Performance Liquid Chromatography

HRT Hydraulic Retention Time

HT Hyperthermophile reactor

NTC Non-Template Control

OLR Organic Loading Rate

PCR Polymerase Chain Reaction

RI Refractive Index

RT Anaerobic Digester

THP Thermal Hydrolysis Process

UV/Vis Ultraviolet/Visible light

V3 region Variable region 3

bp Nucleotide Base Pairs

dsDNA Double Stranded DNA

pH_2 Hydrogen partial pressure

qPCR Real-Time PCR

rt Broad-range real-time PCR primers

spp Species

ssDNA Single Strand DNA

Contents

Chapter 1

Introduction

Methane gas (CH_4), when released into the atmosphere, has a greenhouse gas effect 25 times higher than carbon dioxide (CO_2) (Forster Piers, 2007). However, if burned, methane can be used as a source of fuel, heat and electricity (Jørgensen, 2009). In October 2018, the intergovernmental panel on climate change (IPCC) reported that to reach the 1.5 °C target, there is a need of 40-50 % reduction in greenhouse gas emissions within 2030 (Miljødirektoratet, 2018). The climate challenge has become a fact, and solutions for replacing fossil fuels with more sustainable alternatives are needed now more than ever.

One of the most important sources of methane release into the atmosphere is the anaerobic degradation of biological matter. Therefore, the European Union (EU) have implemented regulations for preventing the landfilling of biodegradable wastes such as sewage sludge and food waste (FW) (European Comission, 2016). The natural production of methane has been known since the 17th century, and the utilisation of methane as an energy source is a 100-year-old technology (Jørgensen, 2009). However, technological development stagnated in the '50s when fossil fuels became cheap. The EU's legislation about biodegradable waste has in the 21st century lead to a major increase in research on this field (European Comission, 2016). As a result of this, anaerobic digestion has become a widely used treatment technology, and biogas production has become a relevant research field.

1.1 Production of Biogas

The microbial fermentation of biological waste is called anaerobic digestion (AD), and the gas produced can be defined as biogas. This section gives an introduction to the whole process and limitations and challenges in industrial optimisation of biogas production.

1.1.1 Anaerobic digestion

AD is the anaerobic process where some biological matter is degraded into biogas and a liquid/solid fraction called digestate (Gerardi, 2003). The degradation steps presented in Figure 1.1, shows the main stages; hydrolysis and acidogenesis, acetogenesis and methanogenesis. Each step is dependent on different groups of microorganisms (Østgaard, 2005). The final product, biogas, is produced in the methanogenesis step and consists mainly of methane (CH_4) and carbon dioxide (CO_2) (Gerardi, 2003).

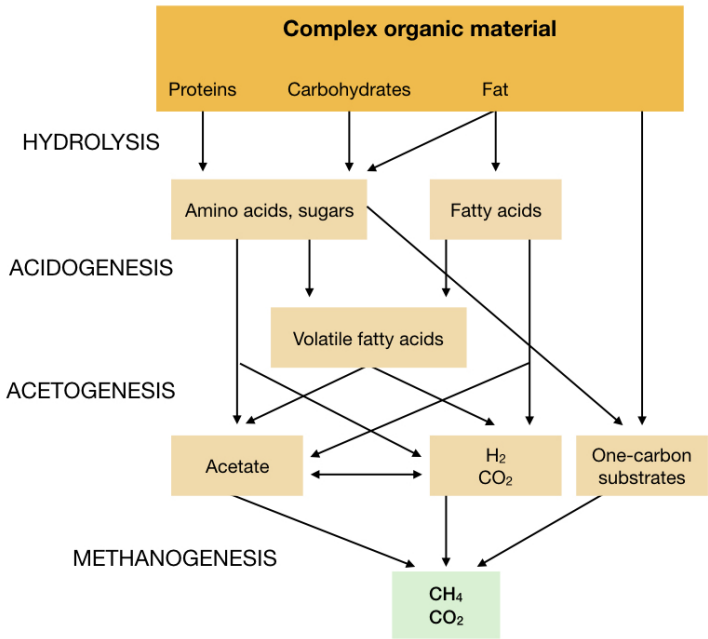


Figure 1.1: An overview of the anaerobic digestion process. Complex polymers are hydrolysed and acidified to volatile fatty acids (VFA) and further degraded to methane through acetogenesis and methanogenesis. Adapted from (Nordgård, 2017) and (Østgaard, 2005)

In hydrolysis, polymers such as long-chained and complex carbohydrates, proteins and lipids are hydrolysed into smaller soluble compounds by extracellular enzymes (Equation 1.1 and 1.2) (Gerardi, 2003). In acidogenesis, products of hydrolysis are further converted to volatile fatty acids (VFA) as exemplified with glucose degradation in Equation 1.3, 1.4 and 1.5 (Østgaard, 2005). VFA are produced from fermentation of sugars, amino acids and more lipids, and are typically butanoic acid ($CH_3CH_2CH_2COOH$), propionic acid (CH_3CH_3COOH) and acetic acid (CH_3COOH) (Østgaard, 2005).

$$\text{cellulose} + H_2O \longrightarrow \text{soluble sugars} \tag{1.1}$$

$$\text{proteins} + H_2O - > \text{soluble amino acids} \tag{1.2}$$

$$C_6H_{12}O_6 + 2\,H_2O \longrightarrow 2\,CH_3COOH + 4\,H_2 \tag{1.3}$$

$$C_6H_{12}O_6 + 2\,H_2 \longrightarrow 2\,CH_3CH_2COOH + 2\,H_2O \tag{1.4}$$

$$C_6H_{12}O_6 \longrightarrow 2\,CH_3CH_2CH_2COOH + 2\,CO_2 + {}_2H_2 \tag{1.5}$$

In uncharged form, VFA can diffuse through the bacterial cell membrane leading to the second stage, the acetogenesis (Gerardi, 2003). Here, acetogenic bacteria produce acetate and molecular hydrogen (H_2) by oxidation of VFA, as exemplified with propionic acid and butyric acid in Equation 1.6 and 1.7 (Gerardi, 2003; Østgaard, 2005).

$$CH_3CH_2COOH + 2\,H_2O \longrightarrow 2\,CH_3COOH + 2\,CO_2 + 3\,H_2 \tag{1.6}$$

$$CH_3CH_2CH_2COOH + 2\,H_2O \longrightarrow 2\,CH_3COOH + 2\,H_2 \tag{1.7}$$

One possible limitation of the acetogenic microorganisms is the inhibition of acetogens if the partial pressure of H_2 (pH_2) becomes too high (Gerardi, 2003). Acetogenesis alone is not thermodynamically favourable, that is, the free energy change ($\Delta G°$) is positive (Khanal, 2011). Therefore, the acetogens are dependent on their close syntrophy with the methanogens. The *hydrogenotrophic* methanogens consume H_2 and produce CH_4 by reduction of CO_2, according to Equation 1.8 (Gerardi, 2003; Østgaard, 2005). In this

way, methanogens are keeping the pH_2 low and favourable for the acetogens. The two other main types of methanogenic pathways are called *aceticlastic* and *metylotrophic* methanogenesis (Nordgård, 2017). The aceticlastic methanogens grow on acetate and release methane and CO_2 according to Equation 1.9 (Gerardi, 2003). Lastly, compounds containing a methyl group (-CH_3), serve as an energy source for the methylotrophic methanogens, and methane is released in the breakdown (Nordgård, 2017).

$$4\,H_2 + CO_2 \longrightarrow CH_4 + 2\,H_2O \tag{1.8}$$

$$CH_3COOH \longrightarrow CH_4 + CO_2 \tag{1.9}$$

1.1.2 Industrialised Anaerobic Digestion

Performance of AD reactors can be affected by different factors (Babaee and Shayegan, 2011). This subsection is dedicated to presenting some of the most important aspects known.

Temperature of Anaerobic Digestion

Temperatures of AD processes are normally mesophilic (30-40 °C) or thermophilic (50-60 °C), both having advantages and disadvantages (Kim, Ahn and Speece, 2002). Compared to mesophilic AD, thermophilic AD usually leads to increased hydrolysis and solubilization rates of organic compounds, as well as reduced amounts of pathogens. At the same time, thermophilic conditions create systems of more narrow selectivity and a lower microbial diversity than mesophilic (Zamanzadeh et al., 2017). Microbial communities with lower diversity, are more prone to stress if operational conditions change. Mesophilic AD has the advantage of being less selective and can be more stable due to higher microbial diversity in the AD microbial community.

Organic Loading Rate

One important aspect in AD reactor stability is the organic loading rate (OLR), which indicates the amount of organic material available for digestion and biogas production (Babaee and Shayegan, 2011). It can be defined in terms of kg Volatile Solids (VS) per day (d) in relation to the m^3 of working volume (kg VS/m^3·d).

As mentioned, VFA are an essential part of the AD process, but can also lead to challenges with stability (Wijekoon, Visvanathan and Abeynayaka, 2011). An increase of OLR will also introduce higher production of VFA, which can result in high acidification and unfavourable pH in the reactor. Besides, an increased OLR can affect the types of VFA produced in the process, shifting from acetate towards higher butyrate concentrations, which is less favourable in methanogenesis. This will be explained in further detail in Section 1.1.3.

Substrate type

Biological waste is a heterogeneous group with large variations in composition and degradability (Curry and Pillay, 2012). Different types of biological waste have a natural variation in degradable content. As an example, food waste can have an OLR of 7-10 kgVS/m^3, in contrast to, e.g. wastewater that commonly has an OLR of 1-5 kgVS/m^3. Therefore, it is important to take the volumetric loading rate of the AD system into consideration (Wijekoon, Visvanathan and Abeynayaka, 2011).

It is possible to estimate the biogas potential of a substrate from the content of carbohydrates, proteins and lipids (Curry and Pillay, 2012). Carbohydrates (as $C_6H_{10}O_5$), proteins (as $C_5H_7NO_2$) and lipids (as $C_{57}H_{104}O_6$) have a biogas potential of 415 Nm^3/tVS, 496 Nm^3/tVS and 1014 Nm^3/tVS, respectively. Because of this, lipid-rich waste introduce substrates with high biogas potential to the system.

Substrate pre-treatment

Before introducing the waste to AD, adding one or more pre-treatments is common (Kim et al., 2003). Various pre-treatment options are available, and can for instance be thermal, alkaline or mechanical, or a combination. The pre-treatment is normally introduced to hygienise according to statutory requirements, and to reduce the rate-limiting effect of substrate hydrolysis (Avfall Norge and Norsk Vann BA, 2009; Kim et al., 2003).

1.1.3 Challenges and Limitations in Anaerobic Digestion

Keeping optimum reactor conditions is a challenge in AD. The digester contains a microbial community with microbes with variations in optimum conditions like pH, ammonia concentrations and temperature (Appels et al., 2008). Changes in for instance temperature can have a substantial effect on other variables and may cause instability in the

total system. This vulnerability makes regulation and control of industrialised AD a complicated matter.

Rate-Limiting Effect of Methanogenesis

In most cases, the methanogenesis is the rate-limiting step of AD (Gerardi, 2003). Methanogens are in general slow growing and sensitive to changes in their environment. The slow growth rate of methanogens is due to the low energy obtained from the consumption of acetate. As a consequence, this leads to a microbial population in need of optimal growth conditions to keep it stable and CH_4 production as efficient as possible. The aceticlastic methanogens are the least energy efficient but are known to be responsible for the largest fraction of produced CH_4. In fact, hydrogenotrophic methanogens get higher energy yields from methane production but are limited in growth due to a low pH_2 in the anaerobic digesters.

Acetate is the most favourable VFA for methanogenesis, but the production of actetate is another rate-limiting step in AD (Gerardi, 2003; Wijekoon, Visvanathan and Abeynayaka, 2011). Also, the hydrolysis stage of AD can become the rate-limiting step if the substrate is of complex matter (Appels et al., 2008).

Accumulation of Volatile fatty acids

As already described, VFA are a central part of the AD process. However, if VFA accumulate up to relatively high concentrations, inhibition of the AD microbial community will most likely happen (Appels et al., 2008). VFA accumulation usually happens due to instability in operational conditions such as temperature, organic loading rate (OLR) or different toxic compounds. The increased concentrations of VFA leads to a lowered pH, and unfavourable growth conditions, especially for the slow-growing methanogens, because their enzymatic activity stagnates when pH is 6.2 (Gerardi, 2003). When the enzymatic activity in methanogens stops, the consumption-rate of VFA also slows down, and the pH decreases even further, leading to unfavourable growth-conditions also for the hydrolysing and acetogenic microorganisms (Appels et al., 2008; Gerardi, 2003).

The effect of VFA accumulation on the microbial community is dependent on the buffer capacity of the AD system (Palacios-Ruiz et al., 2008). Therefore, monitoring total alkalinity is essential, because this indicates the buffering capacity of the process. A high buffering capacity, for instance, due to the presence of bicarbonates, can counteract changes in pH caused by high VFA concentrations.

1.2 *Thermotoga* spp. in Bioenergy Production

Bacteria from the genus *Thermotoga* are hyperthermophiles, with certain species being able to grow at up to 90 °C (Reichelt, Baumann and Baumann, 1976). They are dissimilative iron reducing bacteria and play an important role in degrading organic matter under anaerobic conditions (Madigan et al., 2015). The *toga* part of the name comes from the outer membrane enclosing the cytoplasm as shown in Figure 1.2 (Frock, Notey and Kelly, 2010).

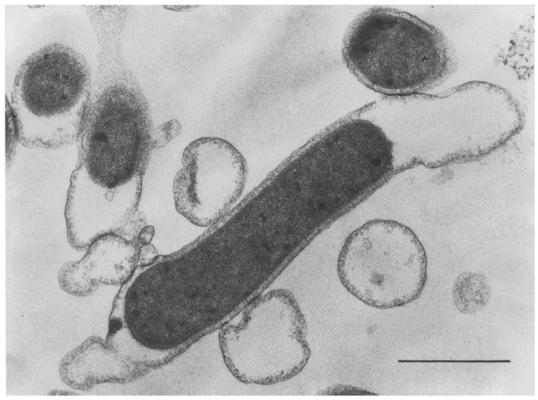


Figure 1.2: Thin section of *Thermotoga maritima* showing the characteristic toga-like outer membrane (Huber et al., 1986).

Species of the *Thermotoga* genus can ferment both simple and complex carbohydrates of different molecular weights (Frock, Notey and Kelly, 2010). The fermentation products are typically short chained organic compounds like acetate, ethanol, lactate, butyrate and butanol, and in addition, CO_2 and H_2 are produced in the fermentation.

One of the interesting biotechnological aspects of the genus *Thermotoga* is the ability to produce H_2 with high yields (Frock, Notey and Kelly, 2010). Among thermophilic bacteria, *Thermotoga neapolitana* and the closely related *Thermotoga maritima* have the highest theoretical H_2 yield in the fermentation of sugar-based substrates (Pradhan et al., 2015). The species have a theoretical maximum yield of 4 mol H_2 per mol glucose consumed, as shown in Equation 1.10. As H_2 is not bound to any carbon, it is an environmentally friendly energy source. Also, *Thermotoga* species (spp.) ability to grow on a large variety of organic substrates, could make them good candidates for the treatment of organic waste in the production of biohydrogen.

$$C_6H_{12}O_6 + 4\,ADP + 4\,Pi \longrightarrow 2\,CH_3COOH + 2\,CO_2 + 4\,H_2 + 4\,ATP + 2\,H_2O \quad (1.10)$$

The species of *Thermotoga* can degrade different kinds of carbon-containing polymers present in the biological waste and are therefore also interesting in an AD-aspect. (Frock, Notey and Kelly, 2010; Gerardi, 2003). It is known that *T. neapolitana* degrades organic substrates through glycolysis, producing pyruvate as shown in Figure 1.3. The highest observed ATP-yield is when pyruvate is further fermented into acetate and H_2 (Pradhan et al., 2015). However, if the pH_2 becomes too high (> 20 kPa), it inhibits the fermentative H_2 production and leads to the conversion of pyruvate into lactate.

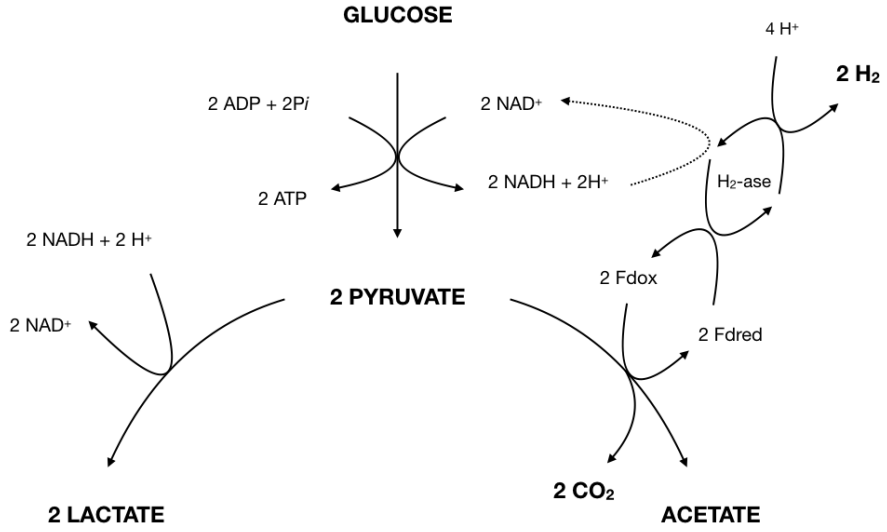


Figure 1.3: Simplified reaction pathway of *Thermotoga neapolitana* production of hydrogen (H_2), carbon dioxide (CO_2), acetate and lactate. Adapted from (Dipasquale, D'Ippolito and Fontana, 2014).

Thermotoga spp.'s most important fermentation products, acetate, H_2 and CO_2 can be directly consumed by aceticlastic and hydrogenotrophic methanogens (Gerardi, 2003). A study from Johnson et al., 2005, used a co-culture with *T. maritima* and a hyperthermophilic and hydrogenotrophic methanogen, *Methanococcus jannaschii*, and

compared it to a pure-culture of *T. maritima*. The co-culture showed a five-fold increase in population density of *T. maritima* (Johnson et al., 2005). Also, hydrogen transfer between hyperthermophiles and methanogens is interesting because it has been shown that injection of H_2 to AD can increase methane production to a large extent (Bassani et al., 2015).

Thermotoga spp.'s ability to ferment a variety of carbohydrates and produce H_2 is well established in laboratory scale (Pradhan et al., 2015). However, the suitability in full-scale industrial production still needs further investigation. The high temperatures needed for optimal growth of *Thermotoga* spp. could also increase the rate of hydrolysis and help killing pathogens (Kim, Ahn and Speece, 2002). The pilot plant technology the experimental procedure of this thesis is based on is the first of its kind (Hyperthermics AS, 2019). It is also the first full-scale trial of a *Thermotoga* species in pre-treatment of biological waste.

1.3 Objectives and approach

The main objective of this thesis was to study the effects of a hyperthermophilic treatment of biological waste, to study the applicability of the technology as a pre-treatment alternative in biogas production. This lead to the following secondary objectives:

1. Study the Hyperthermophilic pre-treatment and compare it to un-treated biological waste. This was done in terms of
    - Effect on anaerobic digestion
    - Fermentation products
2. Study the presence of the hyperthermophilic *Thermotoga Lepl 10* in Hyperthermics™ AS full-scale pilot-plant

Approach

This thesis consisted of two main experiments, where the first was applying Continous Stirred-Tank Reactors (CSTR) in anaerobic digestion. The CSTRs were first inoculated with biomass from a large-scale anaerobic digester. Food waste, both non-treated and treated in a hyperthermophilic reactor, was fed into separate reactors to study potential differences in methane yield. The hyperthermophilic reactor had been inoculated with *Thermotoga Lepl 10* a while before the experiment started. In the second experiment, a mixture of sludge and food waste was studied. The fermentation profile, using HPLC and measuring total concentration of organic acids, of the Hyperthermophilic treatment was compared to the one of un-treated material. In addition, qPCR was used as a part of both experiments to study the quantiative occurance of *T. Lepl 10*.

Chapter 2

Materials and Methods

2.1 Process Description of Biogas Plant at Lindum

Biogas Plant Description

The methods of this thesis are applied to material collected from the full-scale biogas plant at Lindum AS in Drammen illustrated in Figure 2.1. Here, suitable types of organic waste are delivered to the plant. First, unwanted residuals such as plastic bags and metals are removed, before mixing the waste types, and water is added to achieve a dry matter (DM) content of approximately 13 %. After this, the "Thermal Hydrolysis Process" (THP, Cambi) pretreats the material for 20 minutes. In THP, the material is hydrolysed and sterilised with high temperature and pressure (133 °C, 6 bar).

After THP, the hygienised material is continuously pumped in to one of two anaerobic digesters (2000 m^3, 1750 m^3 working volume, 43 °C), at a daily OLR of 3,7 kg VS/m^3 working volume. The biogas is produced in the anaerobic digesters as explained in 1.1.1. The biogas is further purified to a methane content of 98 %, which at Lindum is used as fuel.

Hyperthermophilic Pilot-Plant

Hyperthermics™ AS has developed a patented pre-treatment technology based on utilising the hyperthermophilic properties of the bacterium *Thermotoga Lepl 10* in a reactor with hyperthermophilic conditions (80 °C). *T. Lepl 10* was isolated from a hot

spring in Lesbos, Greece, and single cell cultures were made using optical tweezers. Based on 16S rRNA analysis conducted by Leonardo Torres at the University of Regensburg, The *T. Lepl 10* has a 99 % and 98 % genetic similarity with *Thermotoga petrophila* and *T. maritima*, respectively.

The first Hyperthermics™ pilot plant is installed at Lindum (2.1) to investigate it as a pre-treatment option for biological waste. The pilot plant technology can potentially be an alternative to the already existing THP pre-treatment.

Biological waste is pumped into the buffer tank (BT, 100 m^3) where it is preheated to 50-60 °C. Leading on, the content in BT is semi-continuously pumped into the hyperthermophilic reactor (HT, 6 m^3), already inoculated with *T. Lepl10*. Ideally, the temperature and pH in HT should be held at the optimum of *T. Lepl10*, which is 80 °C and a pH of 7. To maintain the pH at the desired level, automatic addition of sodium hydroxide (NaOH, 32 %) adjusts the pH. In HT, the material is hydrolysed and acidified by the bacterium and high temperature. To meet statutory criteria for sterilisation, the treated content is lead to one of three holding tanks keeping the material at 70 °C for at least one hour. HT and the holding has an outlet at the top, where the gas produced exits and is lead to the anaerobic digesters 1 m^3 below the liquid surface.

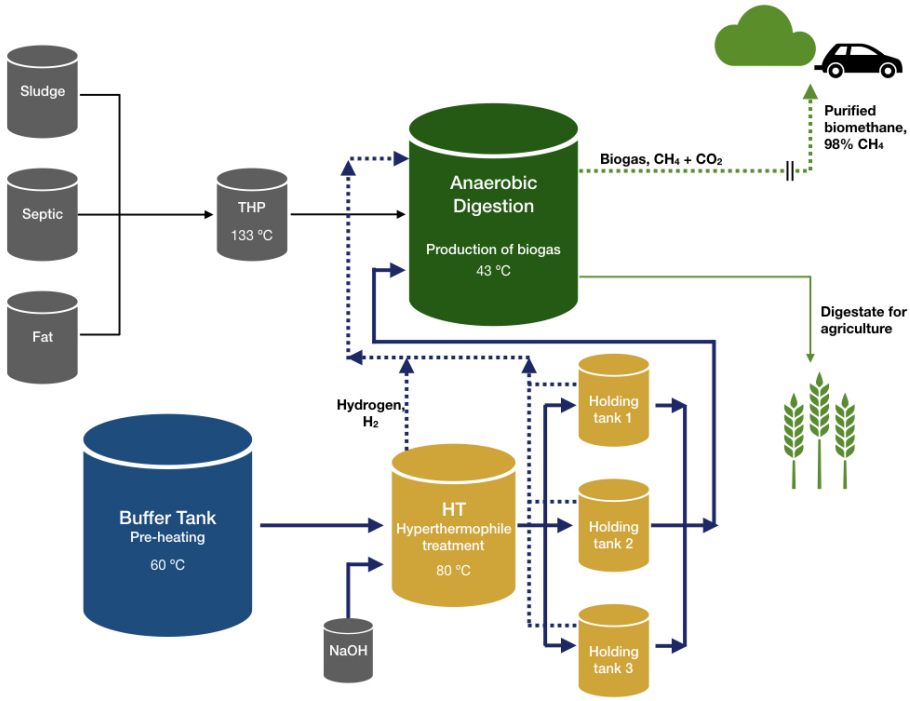


Figure 2.1: Simplified diagram of the biogas plant at Lindum, showing how incoming waste (fat, sludge and septic) is hygienised in the Thermal Hydrolysis Process (THP) before it enters anaerobic digestion (AD). The biogas produced AD is further upgraded to pure methane (CH_4). The blue bold arrows shows the connection of the Hyperthermophile pre-treatment plant to the main biogas plant. Incoming waste is fed into the buffer tank and further transferred to the hyperthermophilic reactor (HT). Produced gas, ideally hydrogen (H_2), is led to the AD reactor below the liquid surface.

2.1.1 Waste Composition in the Pilot-Plant

The AD plant and the Hyperthermics™ AS pilot-plant is fed with biological waste from different sources. It can consist of, for instance, alcoholic beverages, septic and household food waste (FW). All the raw materials used in this project were collected from the full-scale plant.

in the first main experimental part (Section 2.2), FW was the only waste type treated in the pilot-plant. When the second part (Section 2.3) started up, only sludge was treated. Also during the second experiment, there was a shift from treating pure sludge to a mixture of sludge and FW.

2.2 Monitoring biogas production using Continous Stirred-Tank Reactors

This Section is the first main experimental part of the thesis. FW was collected from the hyperthermophilic pre-treatment plant, to be used as feedstock in small-scale AD. The collection of feedstock was on the 30.08.2018, and the reactor had been inoculated with *Thermotoga Lepl 10* at the 11.06.2018. The collected FW was fed into inoculated continuous stirred-tank reactors (CSTRs) to study the effect on AD.

2.2.1 Inoculum collection

The inoculum was retrieved from the anaerobic digesters at Lindum in plastic buckets (20 L) and degassed for 48 hours. After degassing, the inoculum was added to all 12 reactors in the AMPTS system, to a working volume of 1750 mL. At the first feeding with the substrate, the output digestate from each reactor was collected and analysed as inoculum samples. In each sample, the total solids (TS), volatile solids (VS), pH, volatile fatty acids (VFA), total alkalinity (TA), total and soluble chemical oxygen demand (TCOD, SCOD) and ammonium-nitrogen (NH_4-N) was determined. In addition, high-performance liquid chromatography was applied to half of the samples, and quantitative real-time PCR (qPCR) was applied to the second half. Section 2.4 includes detailed procedure descriptions of the used analytical methods.

2.2.2 Collection and pre-treatment of food waste

The FW used as substrates in the feeding of the CSTRs were collected from BT and HT in the full-scale plant (Figure 2.1). FW from BT was poured into plastic boxes (1 L) with lids. 15 L of content from BT was manually hygienised at 80 °C. 8 L of FW from HT was collected and added to sealed plastic boxes (1 L). For both substrate types, enough material for feeding of 24 days was kept at 2 °C for the whole experimental period.

To adjust for the pH difference between material from BT and HT, half of the collected FW from BT was pH adjusted BT-content (BTpH), and added as a third treatment. Table 2.1. The BTpH feedstock was regulated continuously throughout the experimental period, with NaOH (32 %) to approximately the same pH as HT (6.0-6.3). The collection and pre-treatment procedure is illustrated in Figure 2.2.

Different analytical methods were applied in the characterisation of the feedstocks, and description of the methods applied can be found in Section 2.4. The FW substrate was characterised every second to third day by TS and VS, and SCOD was determined once

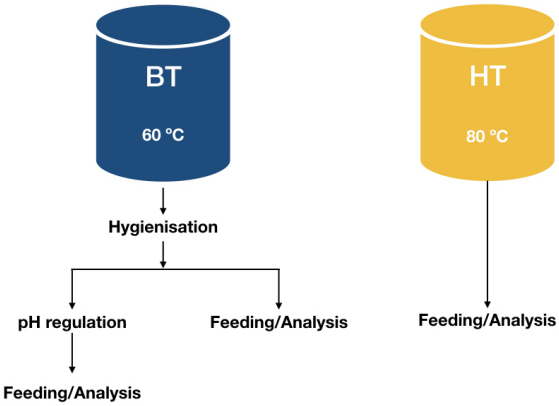


Figure 2.2: FW from a hyperthermophilic pilot-plant was collected to be used as substrate in Anaerobic digestion (AD) in laboratory scale. The FW was pre-treated in a hyperthermophilic reactor (HT) or the buffer tank (BT), and half of the collected BT content was also pH adjusted.

a week (day 1, day 10, day 19). Also, TCOD was analysed on day 1 and day 19. HPLC and qPCR were applied to one sample from HT and one sample of unhygienised BT content.

Table 2.1: Pre-treatments of the food waste (FW) used in a continuous stirred tank reactor (CSTR) experiment. FW was collected from the buffer tank (BT) or hyperthermophilic reactor (HT) and "Hygienisation" describes the type of pre-treatment used to sterilize the content. For HT, pH was adjusted (NaOH, 32 %) automatically in the plant, and half of the collected BT-substrate was manually pH-regulated in the lab.

Treatment name	Hygienisation	pH regulation
BT	80 °C for 1 hour	-
BTpH	80 °C for 1 hour	In lab
HT	In plant	In plant

2.2.3 Automatic Methane Potential Test System

The AD was operated using the Automatic Methane Potential System (AMPTS), a system specifically made to run and measure real-time methane production in small-scale. The specific system used was AMPTS II in combination with reactors made for continuous operation (Bioprocess Control). The system consisted of lab scale CSTRs (described in detail below), water bath for controlled temperature, a CO_2 absorbing Unit, and the gas volume measuring device (Unit C) which is responsible for detecting the methane gas flow.

In this thesis, 12 glass reactors (2000 mL each) designed for continuous feeding as illustrated in Figure 2.3 were used. The reactors had one glass tube for feeding (1) and one for digestate collection (2). Both tubes were equipped with valves for opening/closing (4). Each reactor was provided with a mechanical mixing arm run by a connected motor (3), set to 40 rpm with 30 seconds off-time between 1 minute intervals of mixing. Two gas ports were available at the top of the reactor, to which Tygon® tubes were connected. One was used for flushing the system with N_2 (6), and the other was used for leading produced biogas to CO_2 absorbtion Unit (7). Both Tygon® were equipped with plastic tubing clamps (5) as valves.

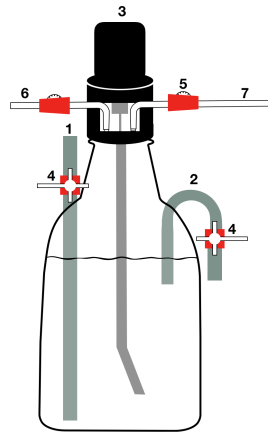


Figure 2.3: Illustration of the reactor Automatic Methane Potential Test System (AMPTS) used in laboratory-scale anaerobic digestion (AD). (1) Feeding tube, (2) Outlet tube, (3) Stirring system, (4) Feed/Outlet valve, (5) Gas valve, (6) Flushing tube, (7) Biogas conduction tube.

The water baths were set to 43 °C to match the temperature of the full-scale AD. Also, the CO_2-absorbing Unit contained an indicator solution with NaOH (3M) to remove CO_2 and H_2S from the biogas. Thymolphthalein (0.4%, 5mL per 1L NaOH) was added as the pH indicator, which changes from blue to colourless when acid binding capacity of the

solution becomes low (BioprocessControl, 2016). When the solution turned colourless, it was replaced with new indicator solution. The complete experimental setup with AMPTS is shown in Figure 2.4.

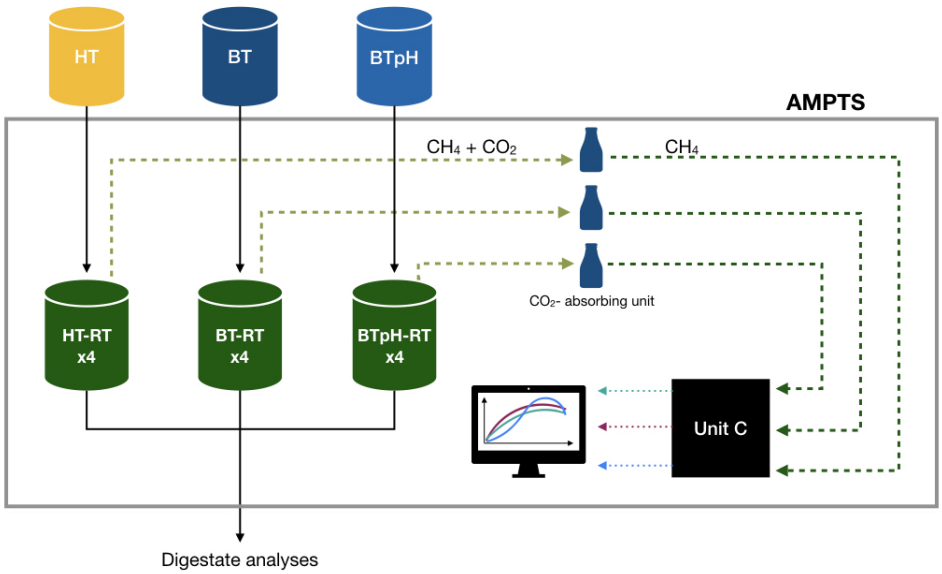


Figure 2.4: Illustration of the experimental setup and flow diagram using Automatic Methane Potential System (AMPTS) in the monitoring of lab-scale biogas production. Food waste from the hyperthermophilic reactor (HT), buffer tank content (BT) and pH-regulated BT (BTpH) were used as substrates with four replicate reactions each. At the time of feeding, digestate was collected from the reactors, which was analysed further. In the AMPTS, the substrate was fed into a reactor (RT) where biogas (CH_4 + CO_2) was produced. CO_2 was removed in the NaOH solution of the CO_2-removal unit. The volume of pure CH_4 was detected for each reactor in Unit C and registered as a computerized signal.

2.2.4 Feeding and digestate collection

Before starting up, the inoculum was added to all the CSTRs, and the whole system was flushed with nitrogen gas (N_2) to remove oxygen. After making sure the system was closed, feeding with FW substrate started. The daily feeding of the reactors in the AMPTS-system was approximated to and OLR 3.7 kg VS/m^3. OLR calculations were done based on an estimated TS content of 10 % and VS content of 8.4 %. The exact OLR was calculated based on the VS analyses several times during the experiment.

Three different waste treatments (HT, BT and BTpH) and 12 reactors, gave a reactor (RT)

setup with four replicates as shown in Figure 2.5.

Figure 2.5: Reactor (RT) setup in monitoring of lab-scale biogas production. Reactors were fed with substrate from the hyperthermophilic reactor (HT), buffer tank (BT) and pH-regulated BT (BTpH). The unedited image was retrieved from Bioprocess Control.

Feeding of substrate to each reactor was done by using a syringe (100 mL). The AMPTS reactors are closed systems, meaning that during feeding of the substrate into the RTs, digestate came out due to increased pressure. The digestate from each RT was collected and then analysed using the analytical methods described in detail in Section 2.4. Every second to third day, the content of TS, VS, pH, VFA and TA was determined in the digestate. Also, NH_4-N, SCOD and TCOD were determined on day 1, 3, 5, 13 and 24. HPLC and qPCR were applied on digestate from day 24. HPLC was used on samples from HT-RT2, HT-RT3, BT-RT2, BT-RT3, BTpH-RT2, BTpH-RT3. qPCR was applied to the remaining half of the RT samples.

2.3 Reactor Study of the Hyperthermophilic Pilot-Plant

This section is based on the need for a closer study of the processes happening in the Hyperthermophilic pre-treatment. Collected material from HT was compared to samples retrieved from BT. Reinoculation of HT happened 06.10.2018, and sample collection started on 21.11.2018.

2.3.1 Collection of Waste Samples

Every third to fourth day over 24 days, a sample from BT and HT was collected. The OLR of HT was set to 0.5 m^3h^{-1}, and the HRT was 4-8 hours in continuous operation, depending on the volume in HT (2-4 m^3) at the time of sampling. Ideally, to sample material of the same origin, the time interval between sampling of BT and HT should be equal to the HRT. As shown in Table 2.2, technical issues at the pilot plant lead to the need for keeping both reactors (BT and HT) in circulatory mode.

Also, the composition of biological waste was different in the second experimental part. In the beginning, pure sludge was treated. Sludge naturally has a higher pH and a relatively high buffer capacity and was therefore demanded lower amounts of NaOH (32 %) to get an optimum pH for *T. Lepl 10*. At day 13, a mixture of FW and sludge was used in the pilot-plant (Table 2.2).

2.3.2 Sample Pre-Treatment

From each sampling day, two kinds of samples were prepared from BT. One was hygienised at 80 °C and pH adjusted with NaOH to the approximate pH of the HT sample. The other sample type was not pre-treated before analyses, as shown in the sampling procedure of Figure 2.6.

In all samples, TS, VS, VFA, TA, NH_4-N, TCOD and SCOD was determined. In addition, samples were frozen down with liquid nitrogen and later analysed with HPLC and qPCR.

Table 2.2: Description of the operational status and waste composition of the hyperthermophilic pilot plant during a sampling period of 24 days. Samples were content from the buffer tank (BT) and the hyperthermophilic reactor (HT), and the reactors contained sludge (S) or a mixture of sludge and food waste (S/FW). The composition of HT was estimated based on the operational mode (Cont./Circ.).

Day	Op. mode	BT	HT	Comment
1	Circ.	S	S	One day since last addition of waste to HT. 24 m^3 sludge added to BT before sampling
2	Cont.	S	S	6.3 m^3 fresh waste added to BT. Continuous operation since evening of day 1
6	Circ.	S	S	Continuous operation until day 3. Stopped due to safety reasons
9	Circ.	S	S	Day 7 was the last day material was added to HT
13	Cont.	S/FW	S	FW (24 m^3) was added to BT this day
16	Circ.	S/FW	S/FW	HT had been running continuously until day 15. Waste composition: 50/50 FW/Sludge
21	Circ.	S/FW	S/FW	HT in circulatory mode since day 15
23/24	Cont.	S/FW	S/Fw	Cont. operation from afternoon day 23. Day 23: sampling from BT, day 24: sampling from HT

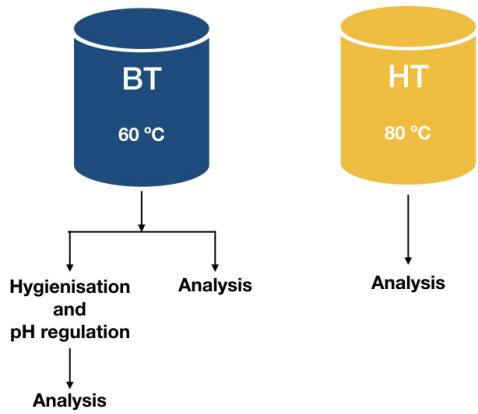


Figure 2.6: Description of sample collection and pre-treatment procedure for biological waste collected from the buffer tank (BT) and hyperthermophilic reactor (HT). Samples were collected to apply different analyses.

2.4 Analytical Methods

2.4.1 Total Solids and Volatile Solids

The measurement of total solids (TS) was used to find the amount of dry matter (DM) in a sample. The procedure was done by adding some fresh liquid sample to a weighted aluminium cup, and leave it at 100 °C for 24 hours. TS was calculated by finding the fraction between the fresh and dry sample as shown in Equation 2.1.

Volatile solids (VS) was performed by removing all available carbon in the dried sample by burning it at 550 °C for 2 hours. This was followed by cooling down to room temperature in a desiccator. As shown in Equation 2.2, VS of the fresh sample was calculated by first finding the VS fraction in the dried sample, and from this find the VS content of the fresh sample. All analyses of TS and VS analyses were done in duplicate or triplicate.

$$\text{Total Solids} = \left(\frac{\text{Dried sample [g]}}{\text{Fresh sample [g]}}\right) \tag{2.1}$$

$$\text{Volatile Solids} = \left(\frac{\text{Burned sample [g]}}{\text{Dried sample sample [g]}}\right) \times \left(\frac{\text{Dried sample [g]}}{\text{Fresh sample [g]}}\right) \tag{2.2}$$

2.4.2 Spectrophotometric methods

Spectrophotometric analyses were conducted using LCK Cuvette Test System (Hach) compatible with DR2700™Portable Spectrophotometer (Hach). Analyses run on the spectrophotometer were VFA, total alkalinity (TA), ammonium-nitrogen (NH_4-N), total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD). In some cases, the samples had to be diluted to be within the measuring ranges of the specific cuvette tests, which was accounted for in the calculations. Before the spectrophotometric analyses, fresh samples (3,5 mL each) were centrifuged at 11000 rpm for 20 minutes.

Volatile Fatty Acids

VFA was measured using LCK 365 Organic Acids (range 50 - 2500 mg/L). The method makes fatty acid esters by creating an acidic environment (HACH, 2015). Addition of Iron(III) salts reduces the fatty acid esters, resulting in a red complex which can be detected by the spectrophotometer. The method was conducted according to the producer's protocol as shown in Appendix D.

Total Alkalinity

For measuring total alkalinity (TA), LCK 362 (0.5 - 8.0 mmol/L $K_{S4.3}$, 25-400 mg/L $CaCO_3$) was applied. The method includes the use of vials pre-made with a reagent reacting with buffering substances in the sample, which decreases the pH of the sample (Hach, 2004). An indicator solution was also added, which indicates a pH between 4.3 and 4.9 that can be measured by the spectrophotometer (HACH, 2011). The method was conducted according to the producer's protocol shown in Appendix E.

Ammonium-Nitrogen, NH_4-N

The LCK 302 and 303 (range 47-130 mg/L and 2-47 mg/L) were applied to find the NH_4-N values. In this method, pH is increased to make ammonium ions react with hypochlorite ions (ClO^-) and salicylate ions which happens at pH 12,6 (HACH, 2013). The indicator, indophenol blue, is formed using nitroprusside as a catalyst. The method was conducted according to the producer's protocol shown in Appendix G.

Total and soluble chemical oxygen demand

Measurement of TCOD and SCOD was based on the same approach using LCK 914. In this analysis, COD is defined as mg O_2 consumed per litre of the sample under the certain reaction conditions applied (HACH, 2014). The vial is pre-made with the COD reagent, containing sulfuric acid (H_2SO_4), potassium dichromate ($K_2Cr_2O_7$, silver sulfate (Ag_2SO_4) and mercury (II) sulfate ($MgSO_4$). The silver in $AgSO_4$ works as a catalyst, and mercury is added to prevent interference from chloride. The dichromate ion ($Cr_2O_7^{2-}$) is a strong oxidising agent which is reduced to chromic ions (Cr^{3+}) in reaction with available organic compounds in the sample. Cr^{3+} is a green compound, and the amounts produced can be detected by the spectrophotometer.

The procedure was conducted according to the producer's protocol in Appendix F.

2.4.3 Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) is a PCR method where the quantification of PCR product is monitored continuously throughout the DNA amplification (Malinen et al., 2003). The method is commonly applied with the fluorescent dye SYBR®Green I, which emits a fluorescent signal when bound to dsDNA, and is otherwise non-fluorescent. The fluorescent signal is registered when it reaches a certain fluorescent threshold. The threshold is reached in the early exponential phase of the amplification, and is called the quantification cycle (Cq) value or threshold cycle (Ct) (Amani, Harris and Fitzgerald, 2017).

qPCR was used to quantify both *Thermotoga* spp. and the total number of bacteria in selected samples. First, regular PCR was used to amplify targeted sequences in the pure-cultures. The PCR amplicons were used later to make standard curves in the qPCR. DNA from pure-cultures of *Thermotoga Lepl 10* was extracted using DNeasy® PowerSoil® Kit(Qiagen) according to the protocol in Appendix A.

PCR was performed with the primer-pairs listed in Table 2.3. The primer-pair *Thermotoga FWD* and *Thermotoga REV* were used in reactions with template DNA from *T. Lepl 10*, to amplify a 100 bp amplicon of 16S rDNA (Okonkwo et al., 2018). DNA from a pure *Vibrio*-strain was used as template with the broad-range RT-996F and RT1089R primer-pair, targeting a bacterial 16S rDNA sequence of 123 bp. In addition, broad-coverage bacterial primers, EUB8f and 518R, was applied on *T. Lepl 10* DNA to amplify the variable region V1-V3. This PCR product was sequenced by Sanger Sequencing.

For each primer pair, a reaction mixture was made. Each PCR reaction was conducted with forward and reverse primers (0.3 µM each), DNA-free water, dNTP (Thermo Fisher Scientific, 0.25 mM each), Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific, 0.02 U/µL), 5x Phusion HF Buffer (Thermo Fisher Scientific). Lastly, isolated template DNA (1 µL) was added to the reaction mixture. In addition, for each primer pair, a non-template control (NTC) was added.

PCR reactions were performed in a total volumes of 25 µL on a T100™Thermal Cycler (BioRad) for 38 cycles. For the qPCR-primers (Table 2.3), the conditions were denaturation at 98 °C for 15 seconds (s), annealing at 65 °C for 20 s and elongation at 72 °C for 20s. The annealing temperature was 55 °C for the V1-V3 primer-pair, otherwise the conditions were identical.

The PCR products were inspected by gel-electrophoresis (1.5% agarose), to see if they were of expected size. All PCR products were purified with QIAquick PCR purification kit (Qiagen) according to the protocol in Appendix B. Concentration of the purified DNA was

Table 2.3: PCR primers used to target DNA of *Thermotoga Lepl 10*. EUB8F and 518R targeted the V1-V3 region of bacterial 16S rRNA gene, and the PCR product was purified and was sequenced by Sanger sequencing. The two other primer-pairs were used to make amplicons to be used in quantitative real-time PCR (qPCR). Both primer-pairs targets 16S rDNA, one is a broad-range (RT-996F, RT-1089) and one is specific, targeting DNA from *Thermotoga* spp.

Primer name	Sequence (5´-3´)	Application
EUB8F	5´-AGA GTT TGA TCM CTC AG - 3´	Sanger sequencing
518R	5´-CGA ACG CGM RGA ACC TTA CCT - 3´	Sanger sequencing
RT-966F	5´-CGA ACG CGM RGA ACC TTA CCT A - 3´	qPCR (broad-range)
RT-1089R	5´- CSG GAC TTA ACC SAA CAT YTC - A 3´	qPCR (broad-range)
Thermotoga FWD	5´- TAC CCC ATA CGC TCC ATC AA - 3´	qPCR (specific)
Thermotoga REV	5´- CCG TTA CCC CAC CAA CTA - 3´	qPCR (specific)

measured using iQuant™ HS dsDNA quantitation assay with Qubit® 3.0 Fluorometer as explained in Appendix C.

The qPCR reaction applied the SYBR® Green PCR Master Mix (Thermo Fisher) which is specifically made for qPCR analyses in the Applied Biosystems Real-Time PCR Rapid Assay. It contained 2x SYBR® Green I as the dye, AmpliTaq Gold® as DNA polymerase, dNTP with dUTP, passive reference (ROX) and buffer components. The final qPCR reactions consisted of 1xSYBR ® Green PCR Master Mix and 0.5 µM of each primer. To each reaction, 5 µL of DNA template was added, and the total reaction volume was 25 µL. Reactions mixes were distributed in a 96-well reaction plate, and each reaction was performed in triplicates.

One run was performed with the *Thermotoga FWD* and *Thermotoga REV* qPCR primer-pair, to quantify *Thermotoga* spp. in samples. Also, broad-range primers, RT-966F and RT-1089R were applied on the same samples to quantify the total amount of bacteria. The broad-range assay was meant as a normalisation when determining the amount of *Thermotoga* spp. in the analysed samples.

The qPCRs were conducted and data obtained from the QuantStudio 5 Real- Time PCR System (Thermo Fisher Scientific). Samples were run in triplicates with *Relative standard curve* as experiment type. Reactions with primer-pair for quantification of *Thermotoga* spp. were run for 40 cycles with 15 seconds (s) at 95 °C (denaturation), 60 s at 60 °C (annealing and elongation) and 15 s at 95 °C (denaturation). The broad-range primer-pair (RT-996F, RT-1089) were used in reactions having 40 cycles with 15s at 95 °C (denaturation), 20 s at 60 °C (annealing), 20 s at 72 °C (elongation).

A standard curve was made for each of the amplification targets, using the RT-996F and RT-1089 primer-pair, and the specific *Thermotoga FWD* and *Thermotoga REV* primer-pair. The purified PCR product of the *T. Lepl 10* and *Vibrio* pure-cultures of known concentrations, were applied as templates in the standard solutions. Firstly, the DNA was diluted to 1.0 ng/µL and a dilution series diluting 1:5 down to 6.4×10^{-5} ng/µL was made.

Determination of Copy Number and Amplification Efficiency

The standard curves were obtained by plotting the log copy number (CN) against the registered Ct-value and doing a linear regression. The Ct-value was obtained from the qPCR assays. The CN was calculated according to Equation 2.3, and represents the number of copies of the certain DNA fragment of known length (Okonkwo et al., 2018). The slope from the regression line was used in Equation 2.4 to estimate the amplification efficiency.

$$\mathrm{CN}\left[\frac{\text{molecules}}{\mu L}\right] = \frac{\text{DNA concentration}[ng/\mu L] \times 6.022 \times 10^{23}[\text{molecules/mol}]}{\text{Genome size [bp]} \times 660[\text{g/mol}]} \tag{2.3}$$

Where genome size refers to the amplicon length and bp is the number of base pairs. 660 g/mol is the average molecular weight for one base-pair in dsDNA (Ritalahti et al., 2006).

$$\text{Amplification Efficiency} = \left(10^{\frac{-1}{\text{slope}}} - 1\right) \times 100\% \tag{2.4}$$

2.4.4 High Performance Liquid Chromatography

Liquid Chromatography (LC) is a technique from the early 1900s and is a type of chromatography where compounds in a sample are physically separated based on one or more physicochemical properties. LC applies a liquid mobile phase, typically a pure solvent with low viscosity, and a solid stationary phase (Poole, 2003). High-performance liquid chromatography (HPLC) is an identification and quantification method applying high pressure and robust column to separate, identify and quantify specific compounds (Nelson and Cox, 2013).

Figure 2.7 shows how the HPLC system is built up. The solvent delivery system involves

a pump applying high pressure, leading to a certain flow-rate when transferring the mobile phase from the reservoir through the system (Waters Cooperation, 2015). The sample of interest is injected at the sample inlet and is brought to the column by the mobile phase. The separation method and order of elution is dependent on the type of HPLC column (Poole, 2003). The system also includes a detector with the ability to detect analytes at low concentrations, which sends electrical signals to a computer that generates the chromatogram.

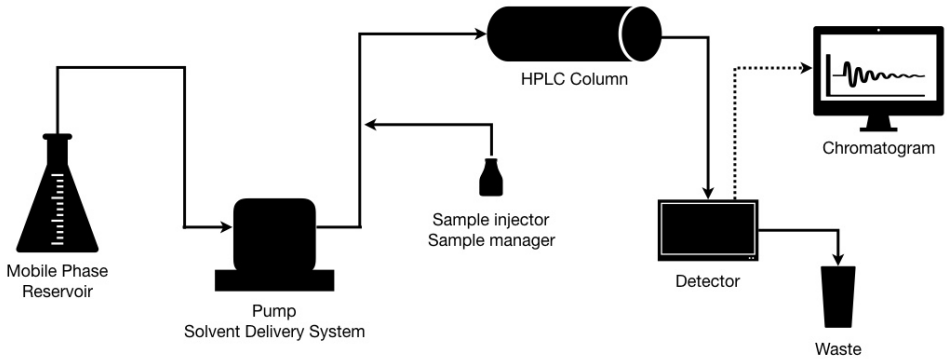


Figure 2.7: Simplified diagram of the High-Performance Liquid Chromatography (HPLC), showing the reservoir keeping the mobile phase, the high-pressure pump, area for sample injection, HPLC column, and the detector sending signals that leads to a chromatogram of the analysed sample.

The column of HPLC is constructed to handle high pressure from the solvent delivery system (Nelson and Cox, 2013). The separation can lead to an identification and quantification of the compounds when compared to standards of known concentrations and retention times.

In this thesis, HPLC was used to determine the profile of a limited selection of VFA and sugars as presented in Table 2.4. The specific HPLC instrumentation applied was Alliance e2695 Separations Module (Waters) with 2489 UV/Visible Detector (Waters) and 2414 Refractive Index Detector (Waters). The column used for detection was an Agilent Hi-Plex H (Agilent), which is an ion-exchange ligand-exchange column. This means the compounds in the samples were retained based on the degree of electrostatic interactions (ion-exchange) and metal-ion-mediated complex formation (ligand-exchange) to the stationary phase (Davankov and Semechkin, 1977; Kopaciewicz et al., 1983).

Standard preparation

A standard solution mix was prepared with analyte concentrations as shown in Table 2.4. From this, a 1:4 (solution:water) dilution series was made. Table 2.4 also shows the retention times and applied detector type for each compound in the standard solution.

Table 2.4: Original concentrations of compounds in the standard solution mix used in High performance liquid chromatography (HPLC) analysis. The two different detectors applied were refractive index (RI) and UV/Visible (UV/Vis)

Compound	Retention Time [min]	Concentration [g/L]	Detector type
Glucose	12.04	5.00	RI
Fructose	13.13	5.00	RI
Maltose	10.017	5.00	RI
Glycerol	17.98	2.50	RI
Succinic acid	16.1	0.50	UV/Vis
Citric acid	10.66	0.50	UV/Vis
Lactic acid	17.2	2.00	UV/Vis
Ethanol	27.88	10.00	RI
Acetic acid	20.56	0.50	UV/Vis
Propionic acid	24.58	0.50	UV/Vis
Butyric acid	29.88	0.50	UV/Vis

All samples analysed with HPLC were thawed, centrifuged (10.000 rpm, 5 min) and filtrated. After this, 0.2-1 mL of each sample was transferred to vials (2 mL) and vortexed briefly to remove air from the bottom.

All standard solutions and samples were analysed at a flow rate of 0.6 ml/min, column temperature at 45 °C, sample temperature at 5 °C and wavelength (UV/Vis detector) at 210 nm. The mobile phase was a 0.05 M solution of H_2SO_4 and the running time for each sample was set to 40 minutes. Automatically, RI detected sugars and alcohols, and UV/Vis detected organic acids.

Chapter 3

Results

3.1 Anaerobic Digestion Using Continuous Stirred-Tank Reactors

3.1.1 Inoculum characteristics

The inoculum was collected from the biogas plant at Lindum and analysed as described in Section 2.2.1. In samples from digesters fed with pH-regulated BT-content (BTpH-RT), the VFA concentration was higher compared to HT-RT and BT-RT. Otherwise, little variation was seen between the three reactor types as presented in Table 3.1.

The fermentation products of the inoculum samples were also analysed, and the detected concentrations are presented in Table 3.2. The peak diagrams of all HPLC analyses are shown in Appendix **??**. Variation was observed between reactor types and within the duplicate samples. Ethanol was detected in all samples, but was the only identified compound in HT-RT2 and BTpH-RT2. In HT-RT3, BT-RT3 and BTpH-RT3, butyric acid was detected in concentrations from 5.64 to 6.01 g/L, and propionic acid was detected in concentrations at 0.3 g/L. Acetic acid and lactic acid was only detected in BT-RT2.

3.1.2 Substrate characteristics

The food waste (FW) substrates were collected and pre-treated as described in Section 2.2.2. The pH of the HT substrate was measured to an average of 6.2. To make the BTpH, NaOH (32%) had to be added to BT content to achieve a pH similar to HT. Per 1 L of

Table 3.1: Characterisation of inoculum used for anaerobic digestion in continous stirred-tank reactor with standard deviation (SD). Analytical made were total solids (TS), volatile solids (VS), pH, volatile fatty acids (VFA), total alkalinity (TA), ammonium-nitrogen NH_4-N and total and soluble chemical oxygen demand (TCOD and SCOD).

Reactor type	HT-RT (SD)	BT-RT (SD)	BTpH-RT (SD)
TS [%]	4.0 (0.6)	4.4 (0.4)	4.4 (0.7)
VS [%]	1.9 (0.3)	2.2 (0.2)	2.1 (0.3)
pH	8.2 (0.08)	8.2 (0.02)	8.2 (0.08)
VFA [g/L]	0.44 (0.03)	0.44 (0.03)	0.54 (0.09)
TA [g/L]	3.7 (0.03)	3.8 (0.09)	3.7 (0.06)
NH_4-N [g/L]	1.63 (0.03)	1.6 (0.03)	1.48 (0.09)
TCOD [g/L]	23.9 (2.0)	27.3 (4.0)	24.5 (4.9)
SCOD [g/L]	3.4 (0.1)	3.4 (0.1)	3.4 (0.1)

Table 3.2: Inoculum from an anaerobic digester was analysed to find the concentration of specific fermentation products. The inoculum was used in lab-scale anaerobic digestion, where three different substrates (HT, BT and BTpH), with four replicate reactions each (RT1, RT2, RT3, RT4) each.

Compound	Acetic acid [g/L]	Butyric acid [g/L]	Propionic acid [g/L]	Lactic acid [g/L]	Ethanol [g/L]
HT-RT2	-	-	-	-	5.33
HT-RT3	-	5.69	0.30	-	4.75
BT-RT2	0.13	-	-	0.15	7.02
BT-RT3	-	5.64	0.31	-	4.82
BTpH-RT2	-	-	-	-	4.09
BTpH-RT3	-	6.01	0.30	-	6.43

BT, 60 ± 2 mL of NaOH had to be added to obtain a suitable pH. Characterisation of the substrates was conducted throughout the experimental period, and the results are presented in Table 3.3. The average pH of BT was 4.0, compared to 6.2 in both HT and BTpH. The highest VS and TCOD content was in BT at 12.63 % and 268.63, while the highest concentration of SCOD was detected in HT (88.56 g/L).

In addition, the composition of specific VFA, sugars and alcohols in the three different substrate types were analysed. Table 3.4 shows the detected concentrations from HT and BT (un-hygienised). Generally, HT showed higher concentrations and variation in the fermentation products. In HT, the compounds of highest concentrations were lactic acid, glycerol and butyric acid, detected at 23.6, 17.8 and 14.8 g/L, respectively. In BT, only butyric acid, ethanol and propionic acid were detected.

Table 3.3: Food waste (FW) characteristics of the feedstock used in lab-scale anaerobic digestion with standard deviation (SD). The FW was collected from a pre-treatment plant with a buffer tank (BT) and a hyperthermophilic reactor (HT). BTpH is BT feedstock regulated with NaOH (32%). Analytical made were total solids (TS), volatile solids (VS), pH, volatile fatty acids (VFA), total alkalinity (TA) and total and soluble chemical oxygen demand (TCOD and SCOD)

Pre-treatment	HT (SD)	BT (SD)	BTpH (SD)
TS [%]	13.12 (0.67)	12.63 (1.57)	13.05 (1.54)
VS [%]	9.83 (0.99)	11.25 (1.47)	9.96 (1.56)
pH	6.2 (0.05)	4.0 (0.02)	6.2 (0.09)
TCOD [g/L]	205.6 (21.0)	268.6 (29.1)	188.8 (30.4)
SCOD [g/L]	88.6 (6.3)	76.2 (3.1)	86.4 (2.5)
VFA [g/L]	6.0 (1.3)	6.2 (0.6)	6.4 (0.1)
TA [g/L]	4.5 (0.2)	N/A	4.0 (0.02)

Table 3.4: Concentration of seven fermentation products in food waste (FW) used as feedstock in lab-scale anaerobic digestion. The FW was collected from a pre-treatment plant with a hyperthermophilic reactor (HT) and buffer tank (BT).

Compound	HT [g/L]	BT [g/L]
Glycerol	17.8	-
Succinic acid	2.2	-
Lactic acid	23.6	-
Ethanol	4.7	1.2
Acetic acid	8.0	-
Propionic acid	2.8	0.3
Butyric acid	14.8	2.2

3.1.3 Organic Loading Rate

The average VS content in the substrates variated from 9.83 - 11.25 % (Table 3.3) for each treatment, which gave a higher OLR (gVS/m^3) than estimated. The actual OLR of each feeding was calculated regularly during the experimental period, and is presented in Table 3.5. In the beginning of the CSTR operation, the hourly CH_4 production in all reactor types went from about 200-150 mL of CH_4 right after feeding, down to approximately 5 mL of CH_4 before the next feeding. Therefore, the feeding volume was increased to 90 mL on day 3 in an attempt to get a more stable gas production. After this, an increase in VFA concentration was observed, while the pH decreased. The feeding was therefore reduced to 50 mL.

Table 3.5: Daily feeding of each continuous stirred tank reactor, both in terms of volume and the organic loading rate (OLR). OLR in grams of volatile solids per day (g VS/d) was calculated in the hyperthermophilic reactor (HT), hygenized buffer tank content (BT) and pH regulated BT (BT-pH)

Day	Volume [mL]	OLR HT [g VS/d]	OLR BT [g VS/d]	OLR BT-pH [g VS/d]
0-2	75	7.99	8.29	9.13
3-4	90	7.28	8.80	6.96
5-7	90	9.17	12.17	7.18
8-9	50	5.03	5.88	5.23
10-11	50	4.46	6.15	4.51
12-14	50	5.14	5.59	5.09
15-16	75	6.68	8.14	7.99
17-18	75	6.45	6.77	6.75
19-21	75	7.62	8.03	8.09
22-24	75	7.93	9.28	8.43

3.1.4 Monitored biogas production using AMPTS

Methane production from HT, BT and BTpH was monitored using the CSTR system AMPTS (Bioprocess Control). The average daily gas production from each of the three treatments is shown in Figure 3.1 The gas production was calculated in terms of the actual OLR (Section 3.1.3) of each reactor.

In the first two days, a similar increase in methane production was observed in all treatments. After this, HT-RT and BT-RT showed a similar CH_4 production rate. HT-RT showed the lowest production overall, with an accumulated methane production of 16.97 L CH_4/gVS compared to 18.26 L CH_4/gVS and 22.24 L CH_4/gVS for BT-RT and BTpH-RT, respectively.

A sudden decrease in gas production was observed between day 11 and 14, which was due to a down-regulation in substrate volume from 90 to 50 mL (Section 3.1.3). Beween day 15 and 23, a more stable methane production was observed.

3.1.5 VFA and Total Alkalinity of Digestate

Concentration of volatile fatty acids (VFA) was monitored using spectrophotometric analyses on the digestate during the operation of the CSTRs. As a function of time, VFA concentrations were compared to measurements of total alkalinity (TA), as seen in Figure 3.2. A similar increase in VFA concentration was observed for all CSTRs until day 5. At day 8, the VFA concentration of BT-RT increased to 5.5 g/L, while it was 3.7 g/l in HT-RT and 4.8 g/L in BTpH-RT. As also observed in BT-RT, the gap between VFA

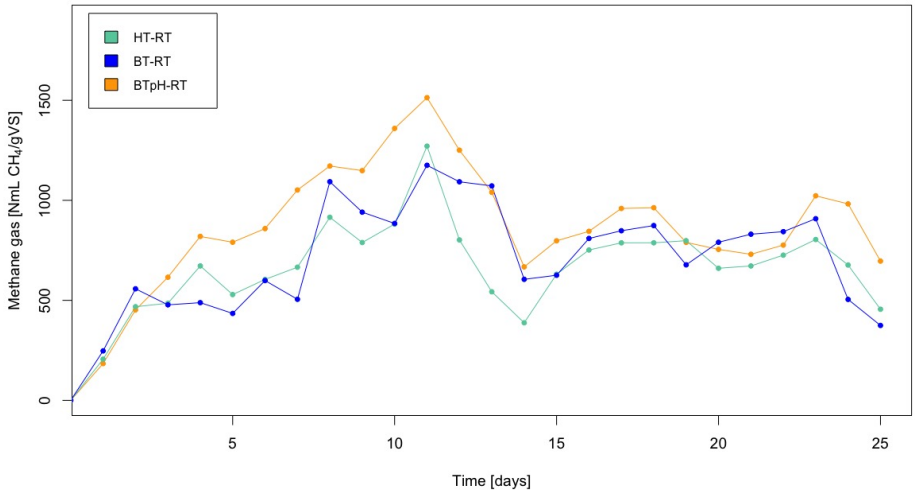


Figure 3.1: A comparison of methane CH_4 gas produced from food waste used in anaerobic digestion. The feedstock was collected from a pre-treatment plant with a buffer tank (BT) and a hyperthermophilic reactor (HT). In addition, BT regulated with NaOH (BTpH) was used as a third waste treatment. The CH_4 production was monitored for 24 days in lab-scale anaerobic digesters (RT).

and TA concentration also increased after day 5, and was stabilised at approximately day 14.

Also measured at day 8, the average pH in BT-RT had gradually decreased from 8.2 (day 1) to 7.4, while it had decreased from a pH of 8.2 to 7.9 in both HT-RT and BTpH-RT. After day 8, the OLR was reduced (Section 3.1.3), and after this, all VFA concentrations decreased. In HT-RT and BTpH-RT, the TA concentration increased during the whole experiment from 3.69 g/L and 3.67 g/L to 14.79 g/L and 13.80 g/L, respectively. The TA concentration in BT was lower and more stable, starting at 3.81 g/L and increasing to 6.64 g/L.

The content of volatile solids (VS) also showed an overall increase over time as seen in Figure 3.3. Starting at the inoculum concentrations of 1.9-2.2 % (Table 3.1.1) to 3.8 %, 3.6 % and 3.2 % for HT-RT, BT-RT and BTpH-RT, respectively.

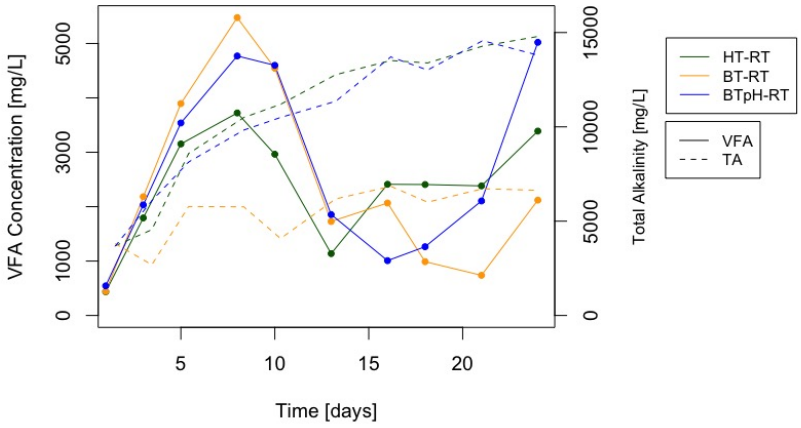


Figure 3.2: Concentration of volatile fatty acids (VFA) and total alkalinity (TA) in digestate from continuous stirred-tank reactors (CSTR). Hyperthermophilic reactor content (HT), hygienised buffer tank content (BT) and pH-regulated BT (BTpH) were applied as pre-treatments of the foodwaste used as feedstock.

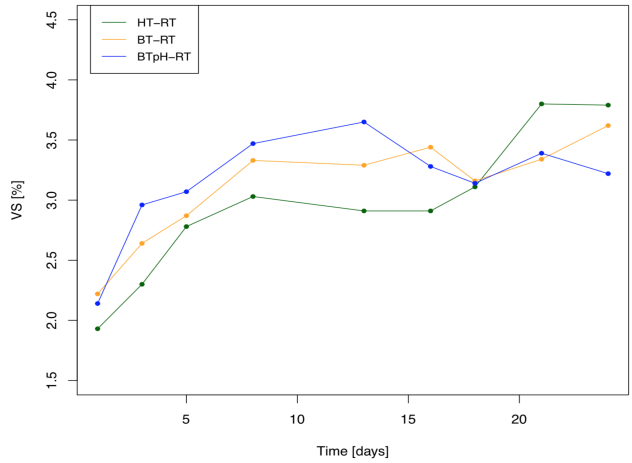


Figure 3.3: Content of volatile solids (VS) in digestate from anaerobic digestion operated in continuous stirred-tank reactors. The feedstock was food waste (FW) pretreated in a pre-treatment blant with a buffer tank (BT) and hyperthermophilic reactor (HT). In addition pH regulated BT content was also added as feedstock.

3.1.6 Fermentation Profile of Digestate

To study some central fermentation products of the digestate from day 24, HPLC was applied on two out out of the four replicate samples. As seen in Figure 3.4, samples from digesters fed with HT (HT-RT) showed propionic acid as the only VFA in the samples. The concentration was 0.31 g/L in both, which was lower than the total concentration of VFA at this point (2.81 g/L and 4.80 g/L). Ethanol was measured to 5.13 g/L, and was the only other compound detected in samples from HT-RT. Two broad un-identified peaks were also present in the chromatograms for HT-RT (Appendix H.1).

The HPLC analysis also showed different profiles in VFA composition in BT-RT2 and BT-RT3 (Figure 3.4). In BT-RT3, butyrate was detected at high concentrations (17.86 g/L), while in BT-RT2 it was not detected at all. Concentration of glycerol, lactic acid and acetic acid was also higher in BT-RT3.

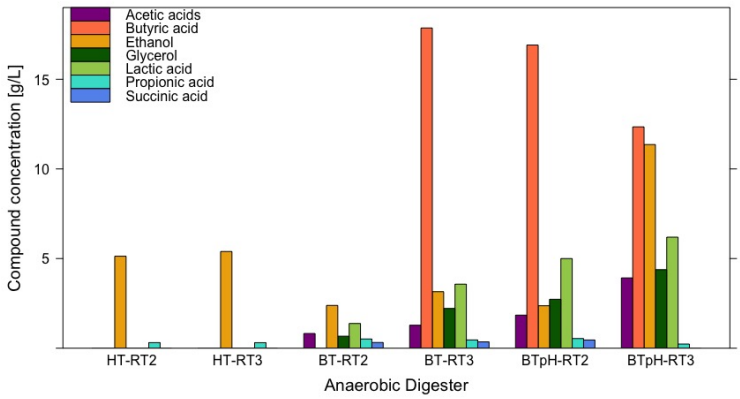


Figure 3.4: Concentration of fermentation products of 6 reactors (RT) in lab-scale anaerobic digestion (AD) after operation of 24 days. Food waste (FW) collected from the buffer tank (BT) and a hyperthermophilic reactor (HT) of a pre-treatment plant was used as feedstock. Content from BT was also pH-regulated (BTpH) with NaOH, and used as feedstock.

3.2 Fermentation Products of HT and BT

Production of various fermentation products was analysed in samples from the hyper-thermophilic reactor (HT), the buffer tank (BT-NonR) and hygienised and pH adjusted samples from the buffer tank (BTpH). In Figure 3.5, most samples show that butyric acid was dominating in concentration, ranging from 17.09 g/L to 33.02 g/L. The exception was on day 1 in HT (Figure 3.5 (a)) and day 13 in BT-NonR (Figure 3.5 (c)), where the

concentration of all compounds were detected below 6.2 g/L. At day 13 butyric acid was still the dominating acid in BTpH.

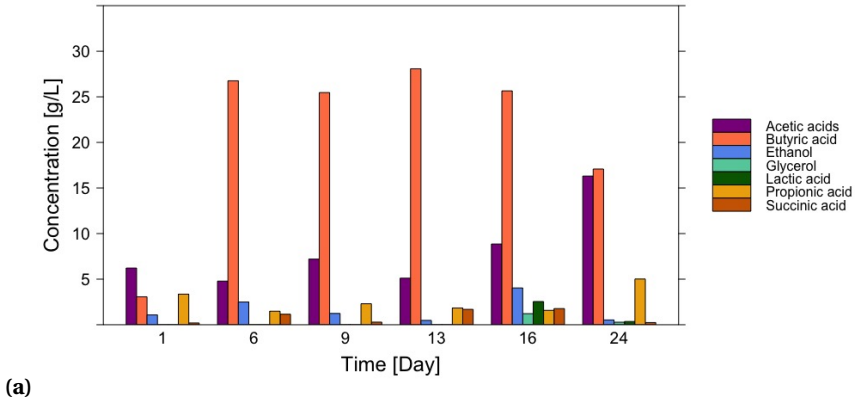
Acetic acid was detected in all reactors at most of the sampling days, except at day 1 for BTpH and day 9 for BT-NonR. The most stable production of acetic acid observed was in HT samples at concentrations from 4.79 g/L to 8.86 g/L. At day 24, HT also showed a peak in acetic acid content, with a detected concentration of 16.31 g/L. BT-NonR showed the overall highest concentrations of acetic acid observed at 16.65 g/L, 15.64 g/L and 17.07 g/L, for day 6, 16 and 24, respectively.

Of all treatments, HT showed more variation in fermentation products. Succinic acid was detected in all samples of HT at a maximum concentration of 1.77 g/L, but it was found in BT-NonR only on sampling day 13. From day 16 to 24, lactic acid was found in all samples from each waste treatment. In the same time period, glycerol was found in all samples of HT, while it was only shown present on day 16 in BTpH and BT-NonR.

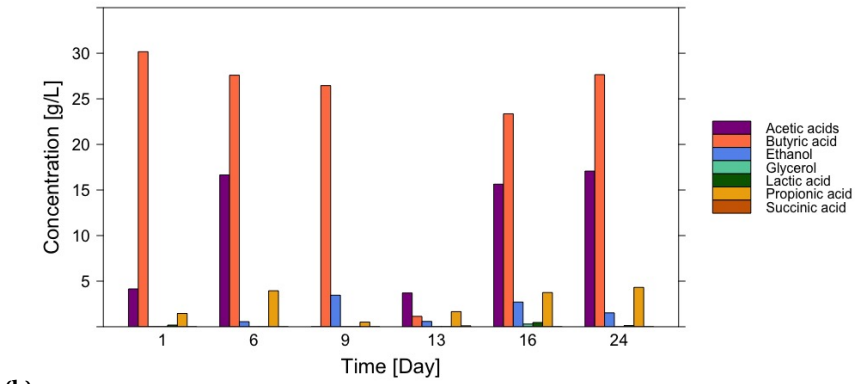
It should be noted that on some days, the quantified amounts of fermentation products were higher than the spectrophotometrically determined VFA concentration. For instance, on day 23, VFA concentration in BT was 23.7 g/L. At the same day, the amount of butyric acid alone was quantified to 29.9 g/L by HPLC. In addition to butyric acid, acetic acid and propionic acid was determined to 16.2 g/L and 3.8 g/L, respectively.

Table 3.6 show similar concentrations of total and soluble chemical oxygen demand (TCOD, SCOD) all treatments. The only exception was the concentration of TCOD in HT at day 13, where the concentration in HT was 58.5 g/L compared to 86.1 g/L and 92.4 g/L in BTpH and BT-NonR, respectively. An increase in concentration of both TCOD and SCOD was seen in all treatments after the addition of food waste (FW) to the pilot plant. After the addition of FW, the demand for NaOH (32%) in the pH adjustment of BT also increased from an average of 4 mL/L of BT to 18mL/L of BT.

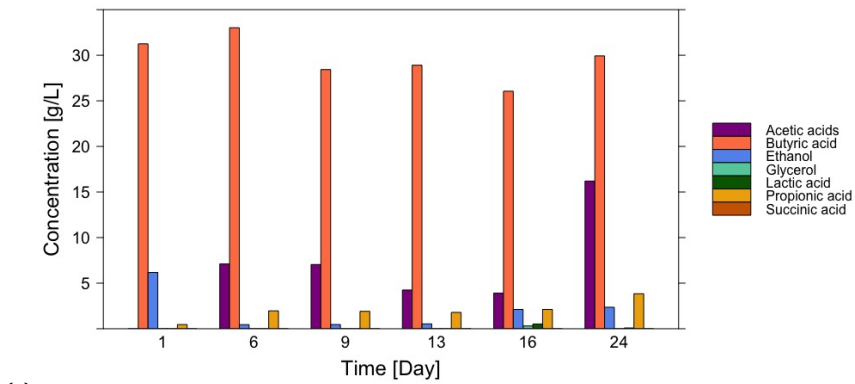
On day 16, FW was added to the buffer tank, and analyses were taken of both the pure FW and pure sludge before any pre-treatment. The fermentation profiles in Figure 3.6 shows the concentration of detected acids, sugars and alcohols. The sludge analysis showed a dominating content of butyric acid (20.07 g/L), while the FW had a more diverse acid profile. The FW had a SCOD and TCOD content of 20.1 and 78.8 g/L, respectively. Compared to this, concentrations in the sludge was 52.2 g/L and 175.7 g/L for SCOD and TCOD, respectively.



(a)



(b)



(c)

Figure 3.5: Biological waste was sampled from a pre-treatment plant. The profile of some fermentation products were analysed using HPLC of samples from the plant's hyperthermophilic reactor (HT) (Figure (a)) and the buffer tank (BT) (Figure (b)). Figure (c) shows the result of BT after a thermal hygienisation and pH treatment with NaOH.

Table 3.6: Concentrations (g/L) of soluble and total chemical oxygen demnad (SCOD and TCOD) in samples from pre-treated biological waste. Samples were collected from a buffer tank (BT) and a hyperthermophilic reactor (HT). In addition analyses were performed on pH-adjusted BT (BTpH).

	HT		BTpH		BT-NonR	
Day	SCOD	TCOD	SCOD	TCOD	SCOD	TCOD
1	20.4	75.3	18.1	69.7	17.9	74.2
2	19.4	78.4	18.8	73.7	N/A	N/A
6	21.2	76.3	19.9	78.8	20.1	78.8
9	24.0	83.0	19.9	76.3	21.4	83.3
13	22.7	58.5	19.5	86.1	18.7	92.4
16	43.5	121.8	35.7	129.5	37.5	128.1
21	45.3	115.5	37.7	128.5	41.4	123.2
23/24	41.6	131.3	41.0	133.7	43.2	120.4

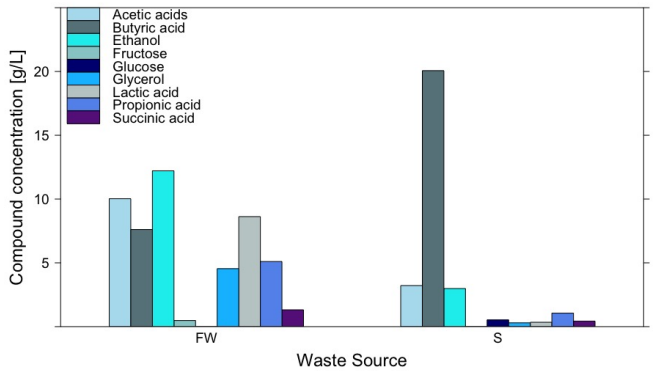


Figure 3.6: Pure food waste (FW) and sludge (S) was sampled before it was added to a pre-treatment plant. Samples were analysed with HPLC and the barcharts shows the concentrations of selected sugars, alcoholc and organic acids.

3.3 Alignment of Sequenced *T. Lepl 10* DNA

Polymerase chain reaction (PCR) was conducted to amplify specifc regions of 16S rDNA from *Thermotoga Lepl 10*. To investigate the PCR reactions, gel-electrophoresis was used to analyse the PCR products.

PCR product was confirmed in all reactions containing template DNA as shown in Figure 3.7. The bands labelled rt and rt-NTC were the result of a PCR reaction with broad range real-time PCR primers (RT-966F, RT-1089). In these reactions, a stronger band was observed for the rt-NTC than the reaction containing template DNA. This PCR product was therefore replaced with purified *Vibrio* DNA in the subsequent broad-range qPCR

assays. As also seen in the gel, some contamination was also present in the NTC of the EUB8f and 518R primers targeting variable region V1-V3 (Eu-NTC in Figure 3.7).

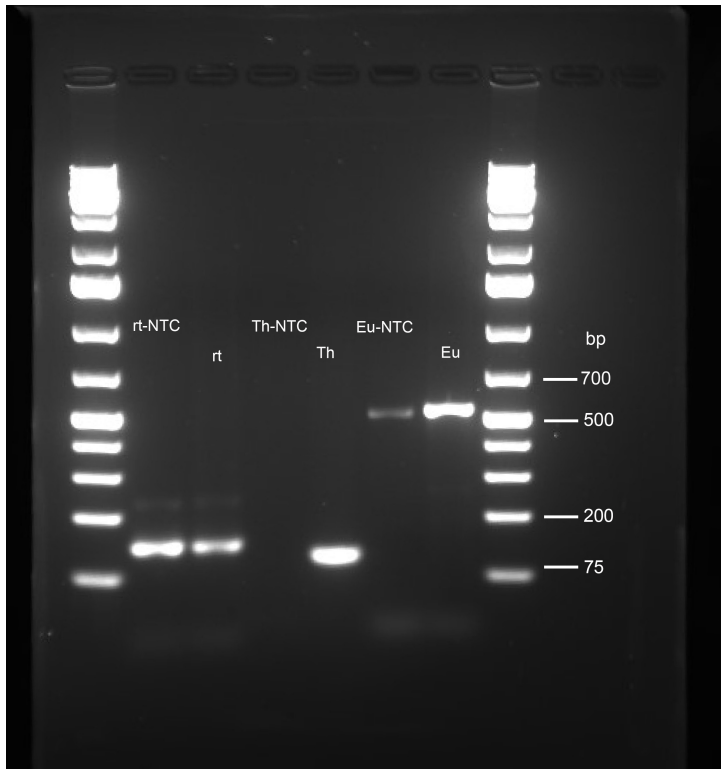


Figure 3.7: PCR product from isolated DNA from a pure-culture of *Thermotoga Lepl 10*. Broad-range real-time PCR (qPCR) primers (rt), Thermotoga qPCR-primers (Th) and a primer-pair for amplification of the variable reaction V1-V3 (Eu) in 16S rDNA were applied in the analysis. Each PCR reaction was conducted with a non-template control (NTC) using the corresponding primer-pair (rt, Th or Eu). The amplicons size variated between 100-510 base pairs (bp).

The purified PCR product from the V1-V3 amplification was sequenced by Sanger sequencing to validate the presence of *Thermotoga Lepl 10* in the pure-culture.

Apart from the primer regions at the end and beginning of the sequence, the chromatogram showed clear peaks. The first 20 base pairs (bp) and last 8 bp were removed from the sequence before using RDP Classifier and SeqMatch (RDP). The sequencing showed that *T. Lepl 10* belonged to the genus Thermotoga with a 98.4 % sequence match with *T. petrophila* (RKU10, AJ872269) and 97.0 % sequence match with *T. maritima* (M21774). Alignment of the isolated DNA with *T. petrophila* showed a mismatch of two bp in the analysed sequence. The sequence classification and matching from RDP, chromatogram and exact alignment with *T. petrophila* is added as Appendix I.

3.4 Quantification of Bacteria and *Thermotoga* spp. by Real-time PCR

Quantitative Real-Time PCR was conducted to quantify *Thermotoga Lepl 10* and the total amount of bacteria in samples from both the AD in CSTRs and the Reactor Study of HT and BT.

Standard curves

Two types of standard solutions were prepared, applying two different primer-pairs and pure-cultures. One primer-pair (RT-996F, RT-1089) was applied for broad-range detection of bacteria, the other for specific detection of *Thermotoga* spp. (*Thermotoga FWD, Thermotoga REV*). The DNA concentrations of the purified pure-cultures were 22.6 ng/µL and 4.62 ng/µL for *Vibrio* and *T. Lepl 10*, respectively. The standard solutions were made from this (Section 2.4.3).

The standard curves are shown in Figure 3.8. The five-fold dilution series used to make the standard curve in Figure 3.8 (a) was made with *T. Lepl 10* DNA-template concentrations ranging from 1 ng/µL to 0.0016 ng/µL. The assay was not able to detect concentrations of 1 ng/µL and 0.2 ng/µL, which is why the plot in Figure 3.8 (a) has only three data points. Therefore, the subsequent qPCR reactions were conducted with five-fold dilution series, with standard solutions ranging from 4.0×10^{-2} ng/µL to 6.4×10^{-5} ng/µL.

The amplification efficiency in quantification of *Thermotoga* spp. was calculated using Equation 2.4 to 48.96 % and 53.64 % for Figure 3.8 (a) and (b), respectively. The linear regression in Figure 3.8 (b) was made excluding the first data point. In the quantification of total bacteria, amplification efficiencies were calculated to 97.63 % and 99.52 % for Figure 3.8 (c) and (d), respectively.

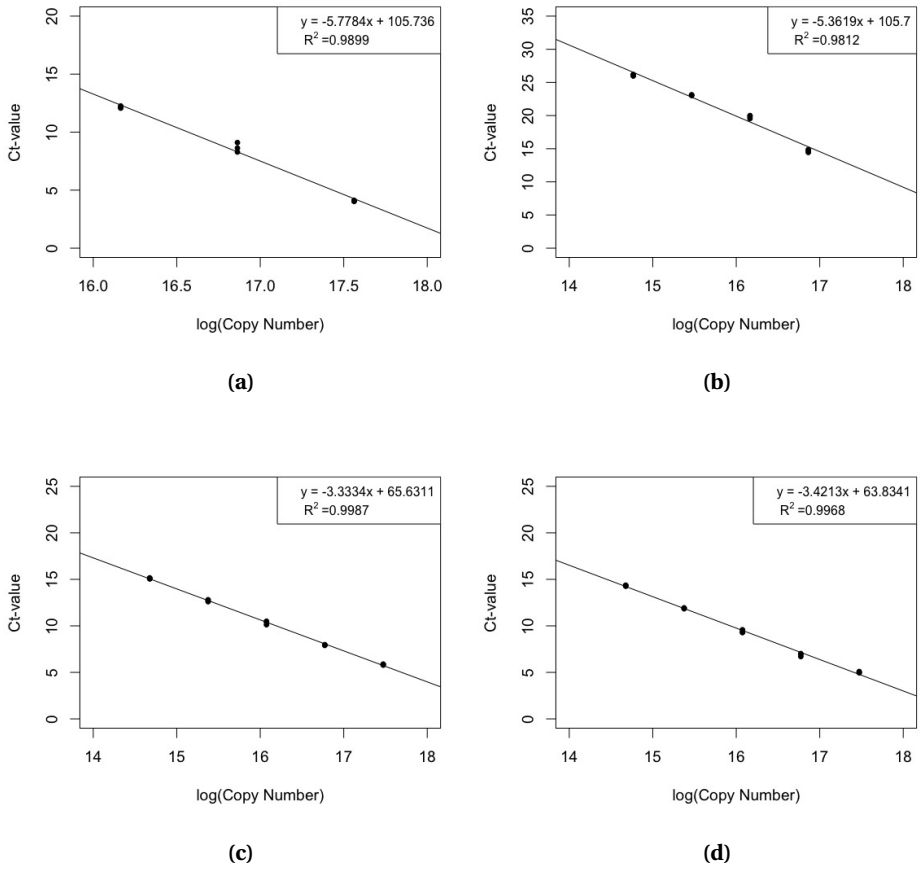


Figure 3.8: Standard curves from qantitative real-time PCR (qPCR). The linear regression of (a) and (b) were made applying the primers *Thermotoga FWD* and *Thermotoga REV* on pure *Thermotoga Lepl 10* DNA of known concentrations. The linear regression of (c) and (d) were made applying the primers RT-996F and RT-1089R on pure *Vibrio* DNA of known concentrations. The four curves were made in separate qPCR-reactions, targeting short 16S rDNA sequences.

Amplification plot and melt curve analysis

Figure 3.9 shows the amplification plots and melt curves from the qPCR analysis of feedstock, inoculum and digestate from the operation of CSTRs. The samples included were the substrates (HT and unhygienised BT), inoculum and digestate. In the amplification plot and melt curve, the grey lines to the right/at the top represents the standards. In addition, each coloured line is the result of an individual sample, and lines of the same colour belong to the same triplicate reaction. All amplification plots and melt curves

were produced by the QuantStudio 5 Real-Time PCR Analysis System.

In the broad-range assay (Figure 3.9 (a) and (b)), NTC had the highest peak in the melt curve, compared to all the unknown samples. As shown in Figure 3.9 (c) and (d), amplification of DNA was observed in nearly all samples analysed with the specific *Thermotoga* primer-pair (Thermotoga FWD, Thermotoga REV). Apart from NTC, amplification was observed in all samples (Figure 3.9), but in low concentrations compared to the standards. In the melt curve (Figure 3.9 (d)), some of the amplified samples showed melting temperatures that differed from the standards, and some samples showed multiple peaks in the melt curve.

The purpose of the broad-range assay was to use it as normalisation towards the quantification of *Thermotoga* spp. Because *Thermotoga* spp. was either not present or deviating within the triplicates, the copy number (CN) was not calculated for any of the assays.

Figure 3.10 shows the amplification plots and melt curves from the qPCR analysis of samples from the Reactor Study of HT and BT. The broad range primer-pair applied (Figure 3.10 (a) and (b)) shows amplification in all samples, including the NTC. Apart from a few deviating individual samples, the specific *Thermotoga* primer did not show any amplification in the analysis (3.10 (c) and (d)).

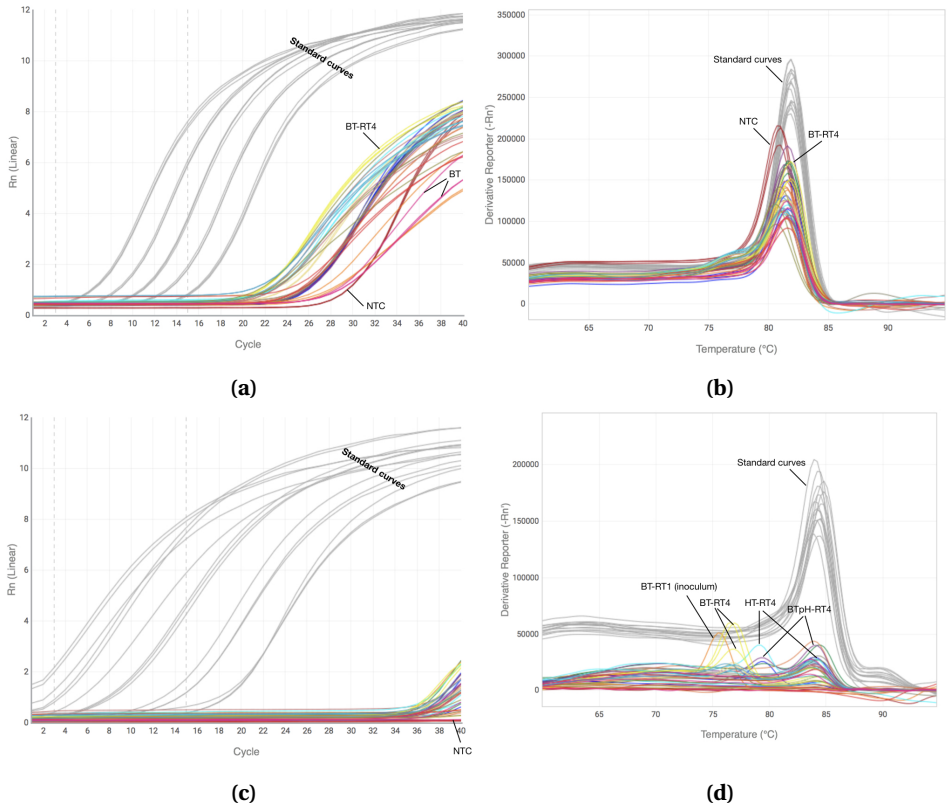


Figure 3.9: Amplification plot (a) and (c) and melt curve plots (b) and (d) from quantitative real-time PCR (qPCR) retrieved from the Design and Analysis app (Thermo Fisher Scientific). Analysis was conducted on samples from an anaerobic digestion experiment using lab-scale reactors (RT). The feedstock was food waste treated in a hyperthermophilic reactor (HT) and the buffer tank (BT). (a) and (b) are results from quantification of bacterial 16S rDNA of using the primer-pair RT-966F and RT-1089R. (c) and (d) are from quantification of 16S rDNA of *Thermotoga* spp. using *Thermotoga FWD* and *Thermotoga REV* as primer-pair.

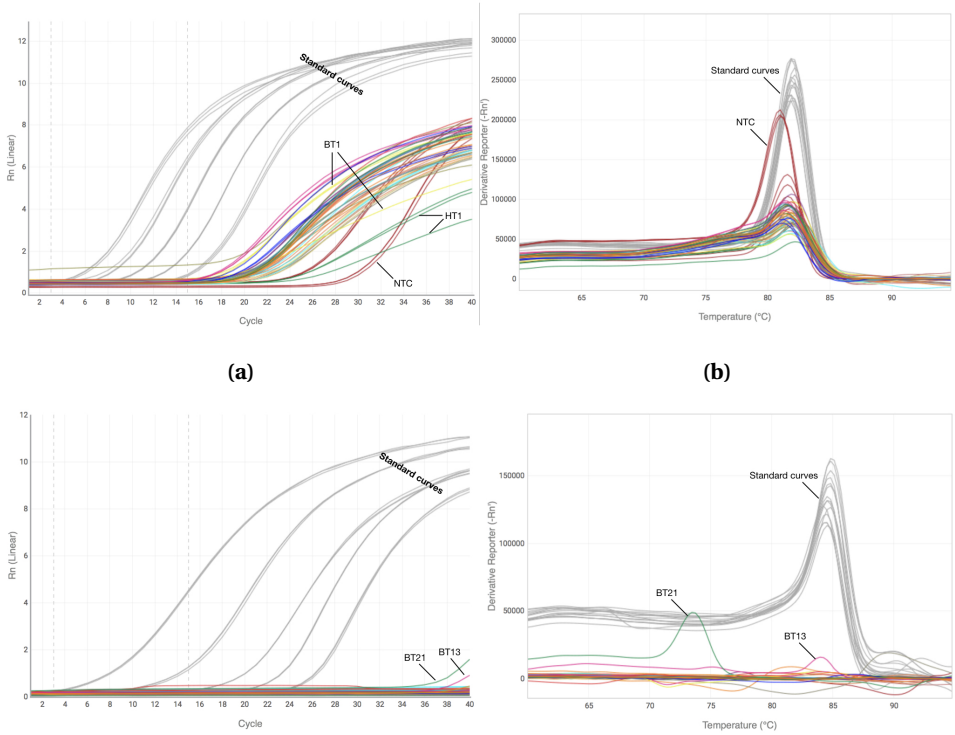


Figure 3.10: Amplification plot (a) and (c) and melt curve plots (b) and (d) from quantitative real-time PCR (qPCR) retrieved from the Design and Analysis app (Thermo Fisher Scientific). Biological waste was collected from a hyperthermophilic reactor (HT) and the buffer tank (BT). (a) and (b) are results from quantification of bacterial 16S rDNA of using the primer-pair RT-966F and RT-1089R. (c) and (d) are from quantification of 16S rDNA of *Thermotoga* spp. using *Thermotoga FWD* and *Thermotoga REV* as primer-pair. Only BT from day 13 and 21 showed any amplification in (c) and (d).

Chapter 4

Discussion

4.1 Evaluation of methods

4.1.1 Biogas Production in Continuous Stirred-Tank Reactors

One practical issue regarding operation of the continuous stirred-tank reactors (CSTR) operation, was the load of manual work. 12 reactors were run and fed semi-continuously throughout the whole period, and several sources of error were introduced.

The most common source of error was probably exposure to oxygen (O_2), which happened on several occasions during the experimental period. Anaerobic digestion (AD) is an obligate anaerobic process (Østgaard, 1995). Although a culture would never be purely anaerobic, consumption of oxygen would also demand consumption of substrate. The most frequent cause of O_2 exposure was the daily opening of the feed- and output-valves during feeding with the substrate.

During feeding, the gas valves leading to Unit C in the AMPTS system had to be closed. It was done in order to prevent accumulated gas in the reactor from being pressed into Unit C during feeding. At a few occasions after completion of feeding, the gas valves were not re-opened in certain reactors, which led to increased pressure due to accumulated gas inside the reactor. The elevated pressure eventually made feed- or output valve jump off, which exposed the digestate to O_2 and methane gas was not detected. In one case with one of the digesters with BT as feedstock (BT-RT2), half of the reactor liquid was also pressed out due to a high pressure inside the reactor. This required re-inoculation of BT-RT2, which is likely the cause of the distinct difference in fermentation

products between BT-RT2 and BT-RT3 (Figure 3.4). The new inoculum resulted in a shorter time for accumulation of VFA and other compounds in BT-RT2 compared to the other reators.

The CSTRs were manually fed with a syringe once a day during the experiment. More continuous feeding, would have lead to a more stable substrate concentration in the digesters. A more continuous process would have been possible if the system was more automatised. Another option for a more stable AD performance could have been to introduce an acclimatisation period in the change of feedstock from THP content to FW. Acclimatisation to new waste types can adapt the microbial community to toxic compounds, such as high concentrations of VFA or free ammonia nitrogen (Gerardi, 2003).

All these challenges affected the detected methane production and probably the quality of some digestate samples. At the same time, the CSTRs were operated with four replicates each, the obtained data are reliable.

4.1.2 Sampling Procedure in Reactor Study of HT and BT

After the CSTR experiment was completed, the preliminary results indicated the need for a closer study on the process in the hyperthermophilic reactor (HT). Samples were therefore taken from the buffer tank (BT) and HT over 24 days, and analysed with a selection of analytical methods. Several practical challenges could have affected the observed results.

One factor was the variation between the continuous and circulatory operation of the Hyperthermics™ pilot-plant. Ideally, the plant should be operated continuously with BT working mainly as pre-heating of the waste. BT is an anaerobic reactor keeping approximately 60 °C. Therefore, when the plant was on circulation, small or no amounts of fresh waste could be added to BT. This introduced a longer anaerobic thermophilic pre-treatment to the waste, likely to fermenting the waste to some extent already before it entered HT. This would reduce the amount of degradable matter in HT, and reduce a potential effect between the two treatments.

What might have reduced the observed effects of HT, is the evaporation of volatile compounds like short VFA or H_2. It seems likely that 80 °C would evaporate some compounds that in the full-scale plant would be transferred to the anaerobic digester through a gas outlet (Figure 2.1).

Also, at circulatory operation, the waste in HT received a prolonged hyperthermophilic treatment (80 °C) compared to the HT samples taken at continuous operation. However,

this did not seem to affect the production of fermentation products. Also, the measured total and soluble chemical oxygen demand (TCOD, SCOD) did not show any clear change between circulatory and continuous operation in any of the waste treatments (Table 3.6). The only distinct change in TCOD and SCOD was observed after the waste consisted of both sludge and FW. This indicates that the increase in both TCOD and SCOD was a result of this change.

The variation in parameters like operational mode and waste type, makes it difficult to make any certain conclusions on the obtained data. Still, it should be noted that the shifts between continuous and circulatory operation were unavoidable due to clogging of pipes and different safety considerations.

4.1.3 qPCR as Quantification Method of *Thermotoga* spp. and Total Bacteria

qPCR was chosen to quantify species of *Thermotoga* spp. and compare it to the total number of bacteria. As shown in Section 3.4, the assay could detect no or little product of *Thermotoga* spp. in any of the analysed samples. In samples from the operation of CSTRs, PCR product was detected in samples where no *Thermotoga* should be present (Figure 3.9 (c) and (d)). Both the BT substrate and inoculum samples showed small peaks in the detection, although with deviation between the triplicate reactions. Therefore, the detection of *Thermotoga* DNA in the HT substrate does not seem reliable. In the later sampling from HT, no *Thermotoga* spp. was amplified (Figure 3.10 (c) and (d)). The detection of bacteria in qPCR is dependent on the fluorescent signal reaching a certain intensity, and *Thermotoga Lepl 10* could potentially be present at low concentrations.

The standard curve from the quantification of *Thermotoga* spp. (Figure 3.8 (a) and (b)) showed low amplification efficiencies (48.7 % and 53.64 %) compared to the broad-range analysis (97.6 % and 99.5 %). It seems likely that the reduced efficiency is due to the applied primer-pair (Thermotoga FWD, Thermotoga REV), as this was the only difference from the broad-range quantification. Low amplification efficiency can be caused by several factors, as PCR is an enzymatic reaction and susceptible to inhibitors (Schrader et al., 2012). Inhibitors can be organic or inorganic and can originate from any stage of the sample preparation.

The low amplification effeiciency in the assay of *Thermotoga* spp. indicates that working conditions were not optimal. Annealing temperature at 60 °C was chosen as working temperature because this was the recommended temperature for the SYBR® Green Master Mix. Okonkwo et al., 2018 applied the same primers (Thermotoga FWD, Thermotoga

REV) on a pure-culture of *T. neapolitana*, and found that the amplicon had a low ΔG (- 14.69 kcal/mol). A low ΔG indicates a demand for high amounts of energy to break secondary structures in DNA, affecting the optimum temperatures of the qPCR. This could indicate that higher temperatures were needed to increase the efficiency.

Okonkwo et al., 2018 tested out annealing temperature at 48 °C, 53 °C, 55 °C, 58 °C, 60 °C and 65 °C. They ended up applying identical working temperatures as in this thesis (95 °C denaturation, 60 °C annealing and elongation), and achieved an amplification efficiency of 75-85 %. This indicates that the reaction mixture in this thesis should have been optimised further, by for instance adjusting reactant concentrations.

Low amplification efficiency might also be due to the less linear standard curves obtained in the *Thermotoga* spp. assay (Figure 3.8 (a) and (b)). Some data points deviated from the regression line in both curves, which also would affect the accuracy of the equation used in calculation of the amplification efficiency. In both the qPCR and regular PCR applying the broad-range bacterial primers (RT966F, RT1089R), resulted in amplified DNA in the NTC (Section 3.4), indicating some contamination in the reaction mixtures. This and the low amplification efficiency obtained in the *Thermotoga* spp. analysis, makes the assays less reliable in a quantification perspective. However, as seen in the amplification plots, the assays were able to detect DNA in the least concentrated standard (6.4×10^{-5} µg/L), and can therefore still be used as an indicator.

4.1.4 Analysis of Fermentation Profiles

In several of the HPLC chromatograms (Appendix H), a relatively high and unstable baseline was observed, which makes the analysis less reliable. The baseline noise indicates that some compounds might have been eluted without being separated by the column.

A source to baseline noise is commonly the detector and it can occur if the equilibrium time before each injection is too short(Vallat, 2007). Another contribution to decreased selectivity and background noise can be contamination of the HPLC column or adsorption of solvent impurities (Waters Corporation, 2016). This might lead to un-identified peaks, and could also be the cause of the broad curves observed in the chromatogram.

Because the samples were of complex composition, a more thorough sample preparation would have made the separation simpler and the quantification of the compounds more reliable. This was indicated by the digestate samples from HT-RT2 and HT-RT3, where the total VFA concentration in HT-RT (2.8 g/L and 4.8 g/L) was much higher than the

propionic acid concentrations (0.31 g/L). The spectrophotometric analysis of organic acids also measures acids of higher molecular weights than those included in the HPLC analysis. Therefore, some of the fatty acids (FA) in the samples might have been too long-chained for separation in HPLC.

Similar compounds can have similar retention times, which could lead to co-elution and un-identified peaks (Latasa et al., 1996). Co-elution can also lead to an over-estimation of the identified compounds. This could explain why the concentration of VFA measured sepctrophotometrically were in some cases lower than the acids quantified in HPLC. For instance, on day 23 of the reactor study of HT and BT, VFA concentration in BTpH was 23.7 g/L, while the concentration of butyric acid alone was 29.9 g/L in HPLC (Section 3.2)

Ethanol was detected in most samples analysed with HPLC, but the quantification might not be reliable, because ethanol was used as the injector needle wash. This might have lead to higher concentrations than the actual ethanol concentration in the samples.

All these factors make the quantification of compounds in HPLC less reliable. Therefore, the obtained results of the fermentation profiles will mostly be used as an indication in the further discussion.

4.2 Effect of Hyperthermophilic Pre-Treatment on Production of CH_4 and Digestate

The highest CH_4 gas production was observed in the anaerobic digesters with BTpH as feedstock (BTpH-RT) (Section 3.1.4). Even though the waste treated in the hyperthermophilic reactor (HT) had approximately the same concentration of volatile solids (VS) as BTpH (9.83 % in HT and 9.96 % in BTpH) and higher concentrations of both TCOD (205.60 g/L) and SCOD (88.56 g/L), the observed methane production was 24% lower in HT-RT compared to BTpH-RT. Kim et al., 2003 showed that a thermochemical pre-treatment with NaOH and 121 °C on waste activated sludge increased biogas production by 34.4 %. However, the pre-treatment in HT is also thermochemical, applying the same temperature (80 °C) and NaOH (32%) as in BTpH.

The reactors fed with BT substrate (BT-RT), showed a higher rate of increasing VFA concentration compared to the other two treatments (Figure 3.2). It should be noted that the pH of the non-treated BT substrate had an average pH of 4.00, in contrast to 6.23 and 6.18 for HT and BTpH substrates, respectively. The added NaOH in both

BTpH and HT would affect the total alkalinity (TA) of the substrate, and thereby also the VFA/TA ratios in the reactors. However, from day 16 to day 24, there was a sudden increase in VFA accumulation in both BTpH-RT and HT-RT. This might indicate a higher consumption rate of VFA and some degree of adaption in BT-RT compared to HT-RT and BTpH-RT.

The HT-RT showed a peak in propionic acid and ethanol as the only identified organic compounds (Figure 3.4). While BT-RT and BTpH-RT had a more diverse fermentation profile containing both acetic acid and butyric acid. It has been reported that thermophilic AD leads to an increased propionic acid production (Wijekoon, Visvanathan and Abeynayaka, 2011). The AD was conducted at 43 °C, which is between temperatures for mesophilic (30-40 °C) and thermophilic (50-60 °C) conditions (Kim, Ahn and Speece, 2002). In all digestate samples, the measured concentrations of propionic acid were 0.230-0.538 g/L, and the semi-thermophilic conditions might be the explanation for the propionic acid content in all reactors. However, this does not explain why the diversity in fermentation products were higher in BT-RT and BTpH-RT compared to HT-RT.

The acetogenic fermentation of VFA, ethanol and aromatic compounds to acetic acid has a positive change in Gibbs free energy ($\Delta G^\circ > 0$) (Khanal, 2011). Among the most common substrates for acetogenesis, propionic acid is the least thermodynamically favourable ($\Delta G^\circ = +76.1$). In presence of H_2, the slow degradation of propionic acid is emphasised, and will be inhibited by a pH_2 above 9×10^{-5} atm (Gourdon and Vermande, 1987). Therefore, the acetogenic bacteria are dependent on the symbiotic relationship with hydrogenotrophic methanogens (Khanal, 2011). Propionic acid is slowly degraded and converted to CH_4 compared to the more favourable acetic acid and butyric acid (Wijekoon, Visvanathan and Abeynayaka, 2011). HT-RT showed a lower methane yield compared to BT-RT and BTpH-RT (Section 3.1.4), which could be explained by differences in fermentation profiles of the digestate.

Another factor in the production of BTpH and HT was the demand for large amounts of NaOH (32 %) to maintain a suitable pH. Based on the amounts added to get a pH of 6.2 in BT, 1 m^3 of FW feedstock would demand 60 L of NaOH. This is expensive and NaOH is challenging to handle due to its highly alkaline properties. On day 16 of the Reactor Study of HT and BT, the waste in the pilot-plant contained 50 % sludge and 50 % FW, which demanded a lower amount of NaOH (Section 3.2). Based on this waste composition, only 18 L of NaOH would be needed per 1 m^3 of waste. Sludge has a higher buffer capacity than FW, and co-digestion sludge and FW is assumed to improve availability of essential nutrients (Zamanzadeh et al., 2017). In AD, co-digestion of sludge and FW could also give higher CH_4-yields and maintain a healthy and diverse microbial community (Braguglia et al., 2018).

To sum up, no increase in CH_4 production was observed in HT-RT, and was 24% lower than CSTRs with BTpH as feedstock. HT-RT also had propionic acid as the only VFA detected in the digestate, while BT-RT and BTpH-RT had a higher diversity in fermentation products. propionic acid can be degraded in AD, but has a higher inhibiting effect on the microbial community compared to acetic acid and butyric acid. While the waste composition in the pilot-plant was pure FW, large amounts of NaOH were needed to make BTpH. However, the demand decreased when the mixture was 50 % FW and 50 % sludge. The mixture of FW and sludge could also be more suitable for AD because sludge increases buffer capacity and improves the nutrient availability for the microbes.

4.3 Hyperthermophilic Pre-Treatment: Effect on Feedstock

Fermentation profiles were also studied in samples from HT and BT. It was studied both in the feedstock for the AD in CSTRs and in the Reactor Study of HT and BT.

The analysed BT feedstock for the CSTR operation was not pre-treated by hygienisation or with NaOH. This could explain the differences in the fermentation profiles (Table 3.4). High-temperature treatment of biological waste is a standard pre-treatment method before anaerobic digestion because it kills pathogens and hydrolyses complex carbon-compounds (Kim, Ahn and Speece, 2002). Due to increased hydrolysis from hygienisation, waste treated in HT would naturally get higher amounts of the short-chained fermentation products compared to un-hygienised BT. In the Reactor Study of BT and HT, content from BT was analysed both as hygienised/pH-adjusted (BTpH) and un-hygienised (BT-NonR). Between BTpH and BT-NonR, no major difference in fermentation products was observed, but HT indicated a slightly higher diversity (Figure 3.5).

The HT feedstock in the operation of CSTR, contained high concentrations of glycerol and lactate (17.8 and 23.6 g/L). In the reactor study, production of glycerol and lactate were observed as fermentation products after day 16 of the sampling period (Section 3.2). Before day 16, only sludge was treated in the pilot-plant. After this, the treated waste was a mixture with 50% sludge and 50% FW. The fermentation profiles of pure FW and sludge (Figure 3.6) showed a glycerol and lactate concentration at 4.5 g/L and 8.6 g/L, respectively. In BT, the concentrations were at 0.3 g/L and 0.4 g/L for glycerol and lactate, respectively. This indicates that FW introduced the observed change of these fermentation products after day 16.

Glycerol was only produced in samples from HT, with one exception at day 16, where it was also identified in BTpH and BT-NonR. HT was also the only treatment with succinate

in the feedstock (Table 3.4 and Figure 3.5). Compared to butyric acid and acetid acid, the observed concentrations of succinate and glycerol in HT were low. Still, it could be an indicator of how the high temperature affected the waste. Both glycerol and succinate can be precursors for propionic acid, which means they are degradable in AD, but they can also introduce inhibitory effects to the system (Viana et al., 2012; Wijekoon, Visvanathan and Abeynayaka, 2011) (Section 4.1.1). As seen in the AD, propionic acid was the only identified and quantified acid in HT-RT. Glycerol and succinate could potentially be one of the sources to propionic acid and the lower production of CH_4 from HT-RT.

Acetic acid and butyric acid are known to be more favourable VFA in AD compared to propionic acid, and acetic acid should be the main product when *T. Lepl 10* ferments carbohydrates (Frock, Notey and Kelly, 2010; Wijekoon, Visvanathan and Abeynayaka, 2011). However, no improvement in concentrations of acetic acid or butyric acid was observed after treatment in HT. This agrees with the qPCR analysis, where no *Thermotoga* spp. could be confirmed by the assay.

To conclude, the waste collected from HT showed an overall higher diversity in fermentation products compared to BT and BTpH. It is likely to assume that this difference was due to a longer hyperthermophilic pre-treatment in HT compared to the one hour hygienisation applied to BTpH. Also, because no presence *Thermotoga* could be verified above the detection limit of the qPCR assays, it makes sense that no improvement in the substrate or anaerobic digestion could be observed.

4.4 *Thermotoga Lepl 10* in Full-Scale Waste Treatment

The qPCR analysis could not detect or quantify *Thermotoga* spp. in any samples. Potential species of *T. Lepl 10* dispersed in the medium would therefore be of concentrations below the most diluted standard. Still, the bacterium could be present below the limit of detection.

It has been shown that *T. maritima* can make biofilms in lab-scale continuous culture (Frock, Notey and Kelly, 2010), and the sequenced pure-culture of *T. Lepl 10* proved to be closely related to *T. maritima* (97.0%) in the alignment (Appendix I). Therefore, there is a chance that *T. Lepl 10* formed a biofilm on the walls of the hyperthermophilic reactor. Biofilms can be used in waste treatment, but then, a reactor configuration with a high surface-to-substrate ratio would be necessary (Sehar and Naz, 2016).

The hyperthermophilic conditions of the pilot-plant introduces selective living condi-

tions, which should reduce the competition. On the other hand, the pre-treatment is dependent on one single species. It therefore seems logic that the system will become more fragile if instability occurs compared to a system based on a co-culture or a species rich microbial community. Biological waste is heterogeneous material, and will definitely introduce instability due to natural variation in composition. This was seen when the actual OLRs of the CSTRs were calculated (Table 3.5). For instance, at day 3 and 5, the volumetric OLR was the same (90 mL), but differed in terms of VS (8.80 and 12.17, respectively).

A challenge in the hyperthermophilic pilot-plant seems to be to establish a stable population of *T. Lepl 10* that is also suspended in the liquid waste. Other species of *Thermotoga* are known to be resistant and efficient in adaptability to variations in growth conditions such as substrate types, high OLR and low HRT (Pradhan et al., 2015). *Thermotoga* spp. therefore seem like ideal organisms in treatment of heterogenous substrates such as biological waste. Why this has not been the case in this study is hard to determine, but might be due to the challenges in keeping operation stable.

In conclusion, even though *Thermotoga* spp. was not detected in the qPCR assay, the bacterium could be present in a biofilm. The challenge could therefore be to get it suspended into the liquid waste. As operational conditions were unstable during the sampling period, this might have caused unfavorable growth conditions to the *T. Lepl 10* in HT.

4.5 Conclusions and Future Perspectives

AD has become a well established technology in treatment of organic waste. By making human waste into a renewable energy source, AD is an important contributor in the reduction of green house gas emission. Therefore, research and development on new technological solutions for AD is important.

What was not tested in the thesis, was the production or effect of hydrogen gas (H_2). First, production of H_2 is interesting as a substrate in AD, and alone as a source to bioenergy. Therefore, further research on microbial H_2 production from waste is interesting and important. Second, if the full-scale H_2 production from *Thermotoga Lepl 10* becomes a reality, the effect on AD also needs to be tested. On one hand, hydrogen could increase the rate of AD by acting as a substrate for hydrogenotrophic methanogens and other H_2-consuming microorganisms. At the same time, it could also inhibit H_2-producing microorganisms, preventing the conversion of propionic acid to acetic acid (Gourdon

and Vermande, 1987).

Even though the experimental part of the thesis has introduced many challenges, it has also emphasized some of the difficulties of up-scaling from small-scale to industrial production. In laboratory scale, *Thermotoga* species are well tested as a potential organism in waste treatment (Frock, Notey and Kelly, 2010; Pradhan et al., 2015). Therefore, if a more stable operation of the Hyperthermics™ pilot-plant becomes possible, it will be easier to determine other limiting factors and make more favourable growth conditions for the bacterium.

In this thesis, the hyperthermophilic pre-treatment of FW did not show any positive effect on anaerobic digestion. Also, no *Thermotoga* spp. could be detected in any samples. However, based on lab-scale studies in the past, *Thermotoga* spp. could be species with a good future potential in biological waste treatment. When a more stable operation of the pilot-plant is accomplished, it will also be easier to determine other limiting factors and establish a more stable bacterial culture of *T. Lepl 10*.

Chapter 5

Conclusion

In this thesis, biological waste was collected from a hyperthermophilic pre-treatment pilot-plant. The purpose of the pre-treatment was to utilise the hyperthermophilic properties of the bacterium *Thermotoga Lepl 10*. The collected waste was used as feedstock in lab-scale anaerobic digestion (AD). Methane gas was monitored with automatic methane potential test system (AMPTS). Fermentation products of feedstock and digestate were studied applying spectrophotometric methods (Hach) and high-performance liquid chromatography (HPLC). Quantiative real-time PCR (qPCR) was conducted to find the amount of *Thermotoga* spp. compared to the total number of bacteria in the samples.

In this thesis, the technology did not improve AD in terms of increased CH_4 production or digestate quality. Also, the presence of *Thermotoga* spp. could not be confirmed. However, compared to buffer tank content, a slightly higher variation in the fermentation profile of the hyperthermophilic pre-treatment was observed. This thesis has emphasised that up-scaling from lab-scale to industrial production is challenging. However, *Thermotoga* species have shown promising results in other lab-scale trials, and a future application in full-scale waste treatment seems likely.

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Appendix A

DNeasy® PowerSoil® Kit

The DNeasy® PowerSoil® Qiagen was used for extraction of genomic DNA from the Thermotoga pure-culture and all the unknown samples from the biogas/Hyperthermics plant.

Quick-Start Protocol June 2016

DNeasy® PowerSoil® Kit

The DNeasy PowerSoil Kit can be stored at room temperature (15–25°C) until the expiry date printed on the box label.

Further information

- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- 2 ml collection tubes are provided.

1. Add 0.25 g of soil sample to the PowerBead Tube provided. Gently vortex to mix.
2. Add 60 µl of Solution C1 and invert several times or vortex briefly.

   Note: Solution C1 may be added to the PowerBead tube before adding soil sample
3. Secure PowerBead Tubes horizontally using a Vortex Adapter tube holder (cat. no. 13000–V1–24).
4. Vortex at maximum speed for 10 min.

   Note: If using the 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min.
5. Centrifuge tubes at 10,000 x *g* for 30 s.
6. Transfer the supernatant to a clean 2 ml collection tube.

   Note: Expect between 400–500 µl of supernatant. Supernatant may still contain some soil particles.
7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.

Note: You can skip the 5 min incubation. However, if you have already validated the DNeasy PowerSoil extractions with this incubation we recommend you retain the step.

8. Centrifuge the tubes for 1 min at 10,000 x *g*.
9. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml collection tube.
10. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.

    Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with this incubation we recommend you retain the step.
11. Centrifuge the tubes for 1 min at 10,000 x *g*.
12. Avoiding the pellet, transfer up to 750 µl of supernatant to a clean 2 ml collection tube.
13. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 s.
14. Load 675 µl onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard flow through.
15. Repeat step 14 twice, until all of the sample has been processed.
16. Add 500 µl of Solution C5. Centrifuge for 30 s at 10,000 x *g*.
17. Discard the flow through. Centrifuge again for 1 min at 10,000 x *g*.
18. Carefully place the MB Spin Column into a clean 2 ml collection tube. Avoid splashing any Solution C5 onto the column.
19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-Free PCR Grade Water for this step (cat. no. 17000–10).
20. Centrifuge at room temperature for 30 s at 10,000 x *g*. Discard the MB Spin Column. The DNA is now ready for downstream applications.

    Note: Solution C6 is 10 mM Tris-HCl, pH 8.5. We recommend storing DNA frozen (–20° to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA see the Hints & Troubleshooting Guide.

Appendix B

QIAquick® PCR Purification Kit

PCR-products (Section 2.4.3) were purified with the QIAquick® PCR purification Kit.

July 2018

Quick-Start Protocol

QIAquick® PCR Purification Kit
QIAquick® PCR & Gel Cleanup Kit

The QIAquick PCR Purification Kit and the QIAquick PCR & Gel Cleanup Kit (cat. nos. 28104, 28106, 28506 and 28115) can be stored at room temperature (15–25°C) for up to 12 months if not otherwise stated on label.

Further information

- *QIAquick Spin Handbook*: **www.qiagen.com/HB-1196**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

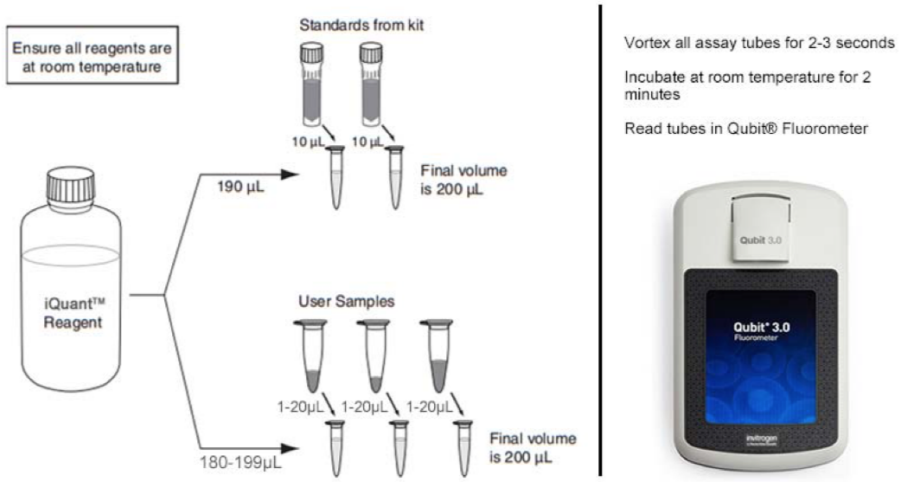
- This protocol is for the purification of up to 10 µg PCR products (100 bp to 10 kb in size).
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH ≤7.5. The adsorption of DNA to the membrane is only efficient at pH ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I; do not add pH indicator I to buffer aliquots.
- Symbols: ● centrifuge processing; ▲ vacuum processing.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ● a provided 2 ml collection tube or into ▲ a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and ● centrifuge for 30–60 s or ▲ apply vacuum to the manifold until all the samples have passed through the column. ● Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 µl Buffer PE to the QIAquick column ● centrifuge for 30–60 s or ▲ apply vacuum. ● Discard flow-through and place the QIAquick column back into the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix C

iQuant™ quantitation assay and Qubit® 3.0 Fluorometer

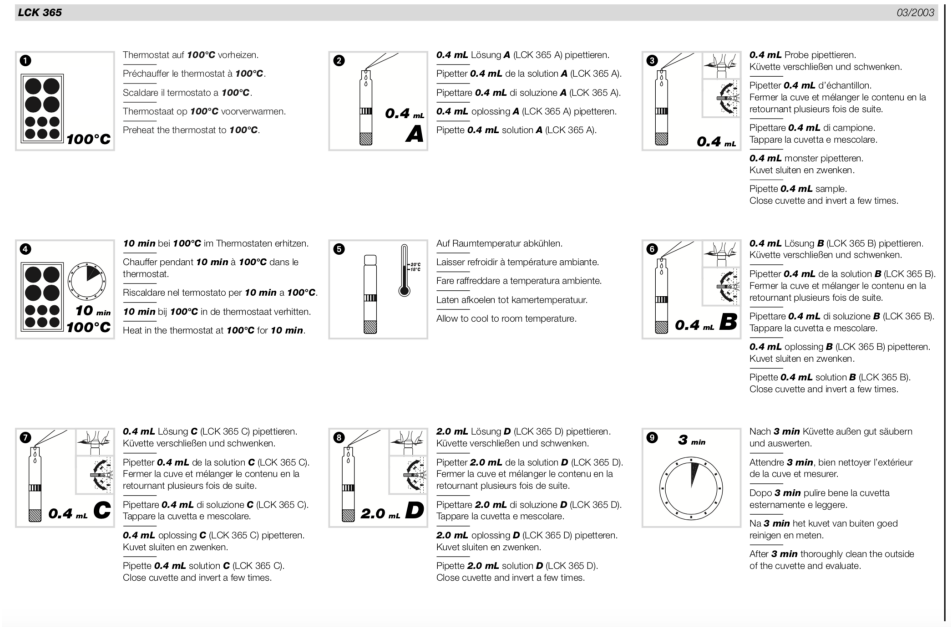
The DNA concentration of the purified PCR products (Section 2.4.3) were determined with iQuant™ HS dsDNA quantitation assay with Qubit® 3.0 Fluorometer. The workflow is shown below.



Appendix D

Protocol LCK 365, Organic Acids

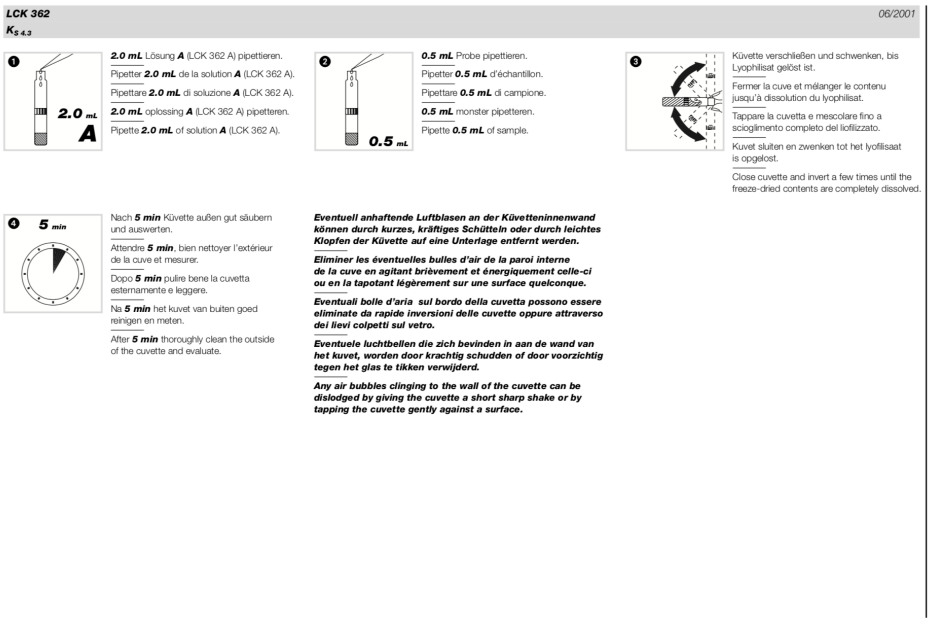
LCK 365, Organic Acids cuvette test HACH was used in spectrophotometric analyses of VFA for the whole thesis. In some cases, samples were diluted to ensure the concentration was within the measuring range as stated in the protocol. In step 9, "evaluate" means to put the sample vial into the spectrophotometer for concentration measurement.



Appendix E

Protocol LCK 362, Acid Capacity

LCK 362, Acid Capacity cuvette test HACH was applied on samples to study the total alkalinity of the selected samples.



LCK 362
$K_{S\,4.3}$

06/2001

❶

2.0 mL Lösung ***A*** (LCK 362 A) pipettieren.

Pipetter ***2.0 mL*** de la solution ***A*** (LCK 362 A).

Pipettare ***2.0 mL*** di soluzione ***A*** (LCK 362 A).

2.0 mL oplossing ***A*** (LCK 362 A) pipetteren.

Pipette ***2.0 mL*** of solution ***A*** (LCK 362 A).

❷

0.5 mL Probe pipettieren.

Pipetter ***0.5 mL*** d'échantillon.

Pipettare ***0.5 mL*** di campione.

0.5 mL monster pipetteren.

Pipette ***0.5 mL*** of sample.

❸

Küvette verschließen und schwenken, bis Lyophilisat gelöst ist.

Fermer la cuve et mélanger le contenu jusqu'à dissolution du lyophilisat.

Tappare la cuvetta e mescolare fino a scioglimento completo del liofilizzato.

Kuvet sluiten en zwenken tot het lyofilisaat is opgelost.

Close cuvette and invert a few times until the freeze-dried contents are completely dissolved.

❹

Nach ***5 min*** Küvette außen gut säubern und auswerten.

Attendre ***5 min***, bien nettoyer l'extérieur de la cuve et mesurer.

Dopo ***5 min*** pulire bene la cuvetta esternamente e leggere.

Na ***5 min*** het kuvet van buiten goed reinigen en meten.

After ***5 min*** thoroughly clean the outside of the cuvette and evaluate.

Eventuell anhaftende Luftblasen an der Küvetteninnenwand können durch kurzes, kräftiges Schütteln oder durch leichtes Klopfen der Küvette auf eine Unterlage entfernt werden.

Eliminer les éventuelles bulles d'air de la paroi interne de la cuve en agitant brièvement et énergiquement celle-ci ou en la tapotant légèrement sur une surface quelconque.

Eventuali bolle d'aria sul bordo della cuvetta possono essere eliminate da rapide inversioni delle cuvette oppure attraverso dei lievi colpetti sul vetro.

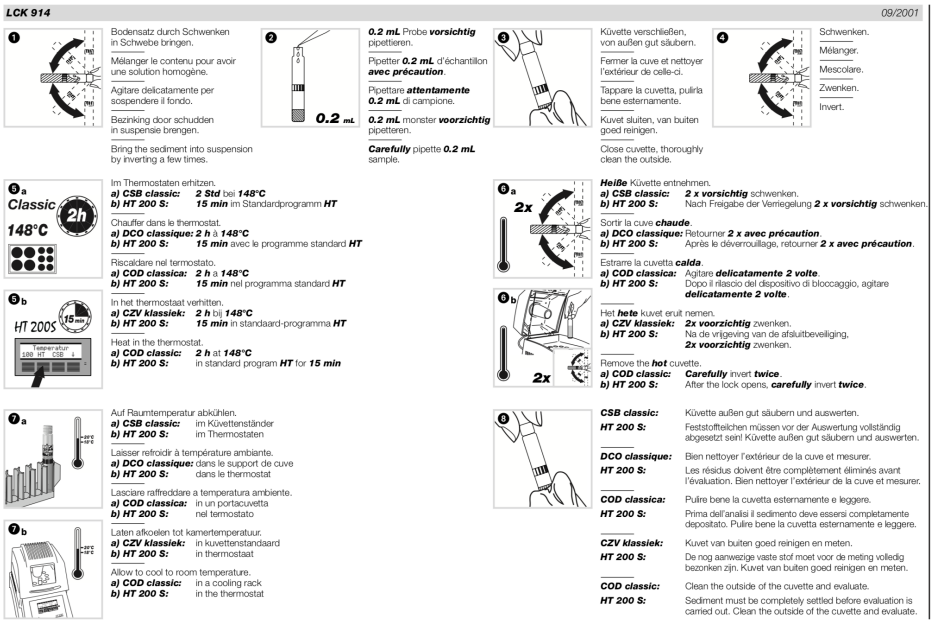
Eventuele luchtbellen die zich bevinden in aan de wand van het kuvet, worden door krachtig schudden of door voorzichtig tegen het glas te tikken verwijderd.

Any air bubbles clinging to the wall of the cuvette can be dislodged by giving the cuvette a short sharp shake or by tapping the cuvette gently against a surface.

Appendix F

Protocol LCK , Chemical Oxygen Demand

LCK 914, COD cuvette test (HACH) was used to analyse total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD). In TCOD analysis, uncentrifuged material was analysed. In analysis of SCOD, the sample was centrifuged before the procedure.

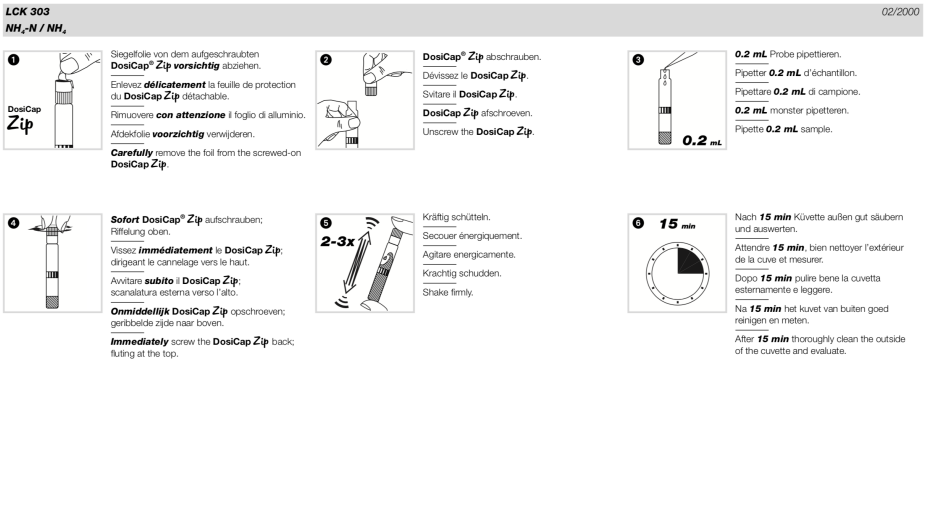


Appendix G

Protocol LCK 302 and LCK 303,

Ammonium-Nitrogen

LCK 302 HACH and LCK 303 HACH, Ammonium cuvette test, were used for monitoring the amount of ammonium in the selected samples. LCK 302 and LCK 303 had measuring ranges 47-130 mg/L NH_4−N and 2.0-47 mg/L, respectively.



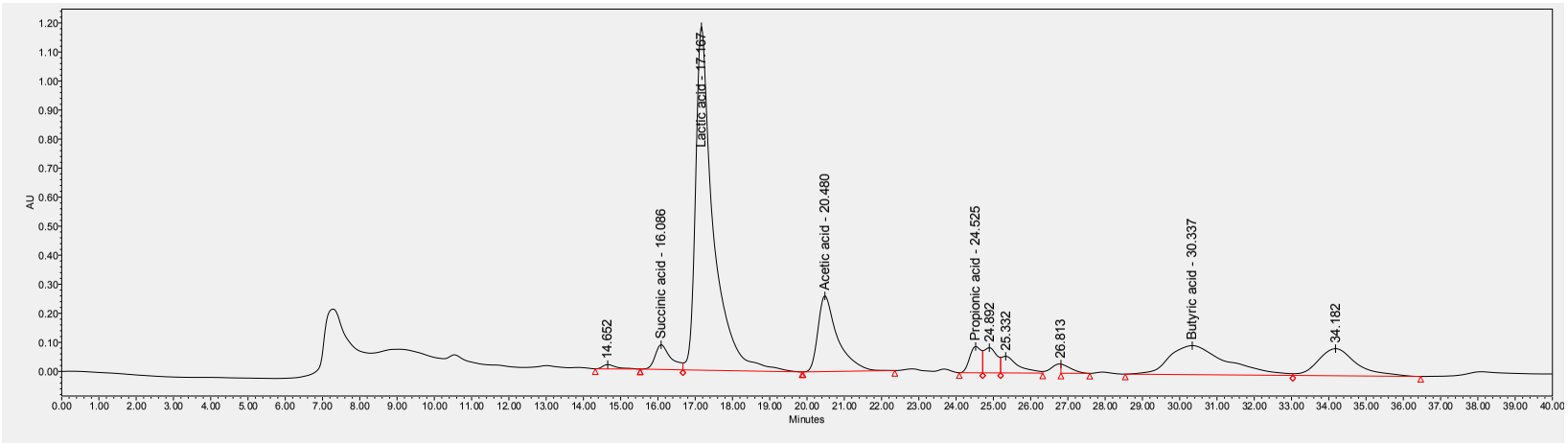
Appendix H

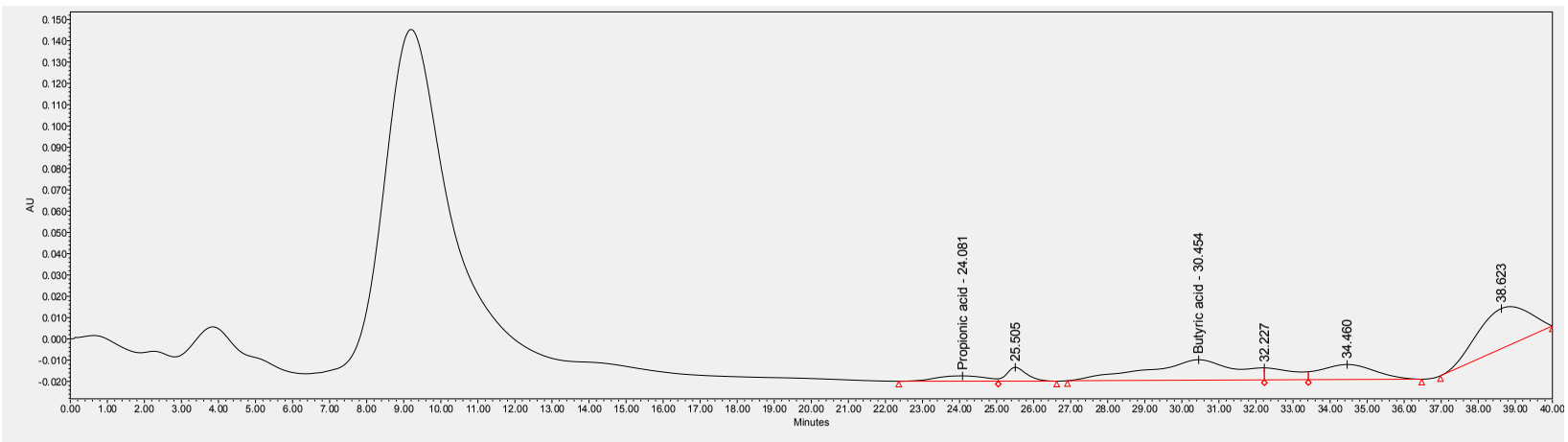
HPLC Peak Diagrams

H.1 CSTR substrates, inoculum and digestate

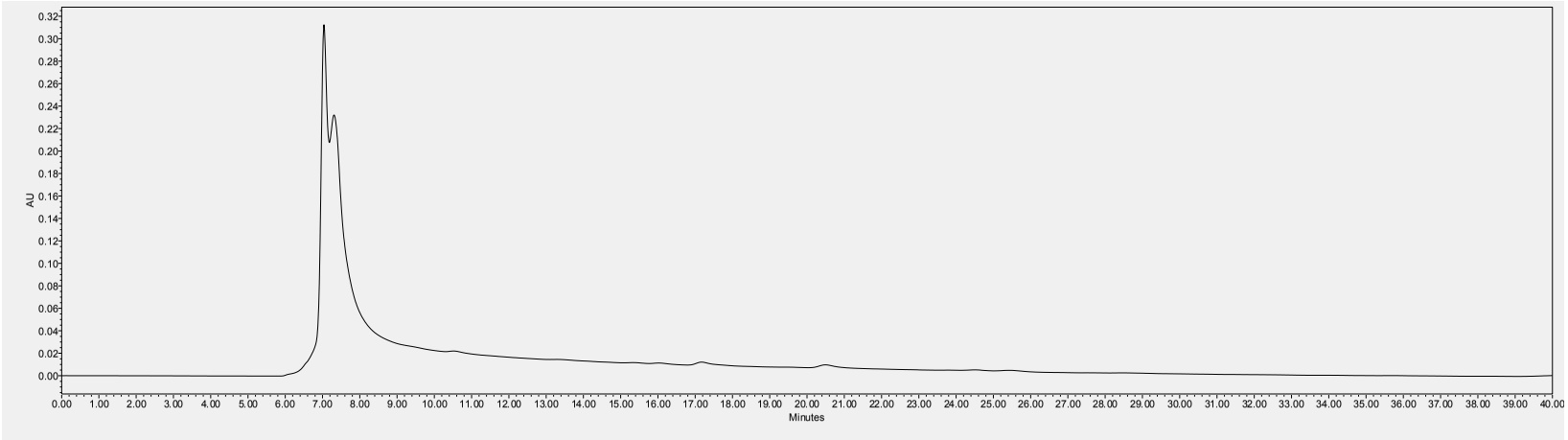
Below, the high performance liquid chromatography (HPLC) peak diagrams for the samples from the hyperthermophilic reactor (HT) and buffer tank (BT) are shown. In addition peak diagrams from the analysis of the inoculum and digestate are added. The experiment was conducted with 12 digesters (RT), and three different substrate treatments, giving four replicates of each feed type. Samples from two of the four replicates were analysed using HPLC. BTpH-RT is the inoculum/digestate from reactors fed with a pH regulated BT substrate.

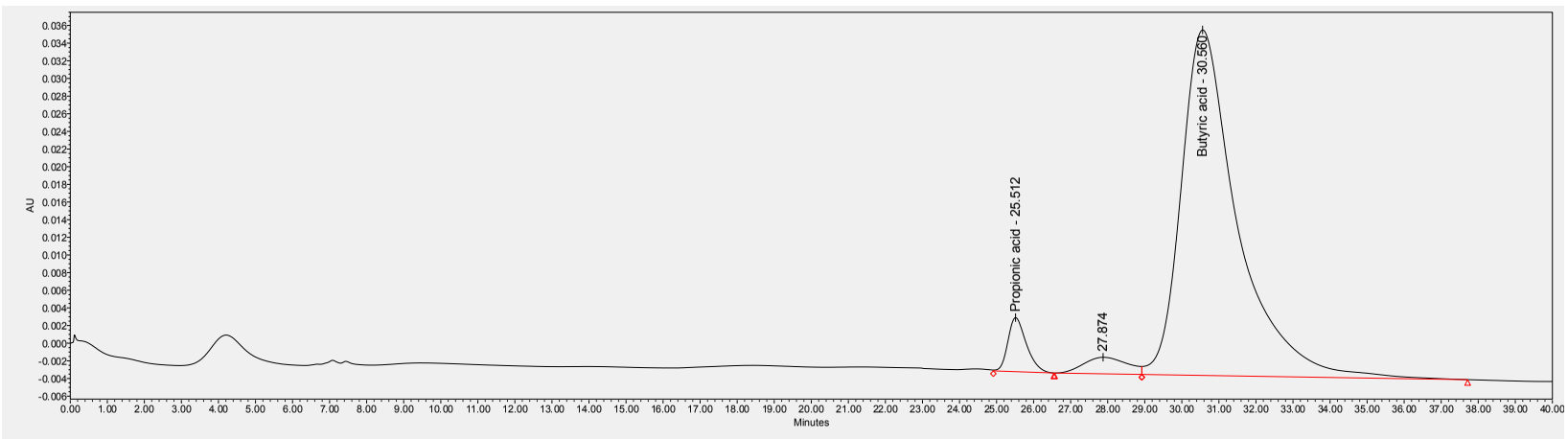
Substrate from HT and BT



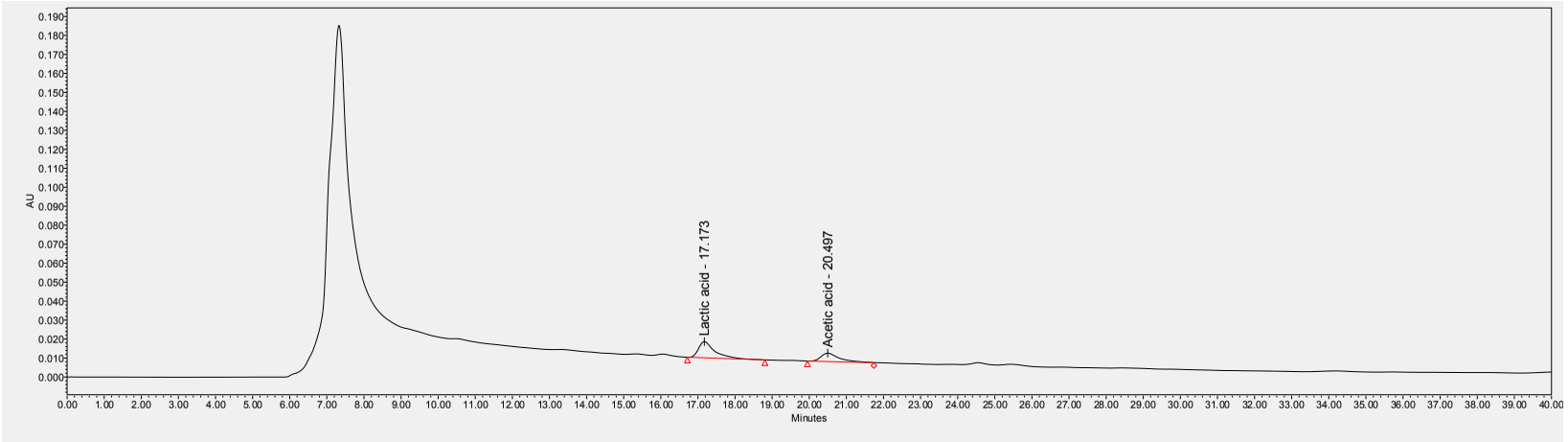


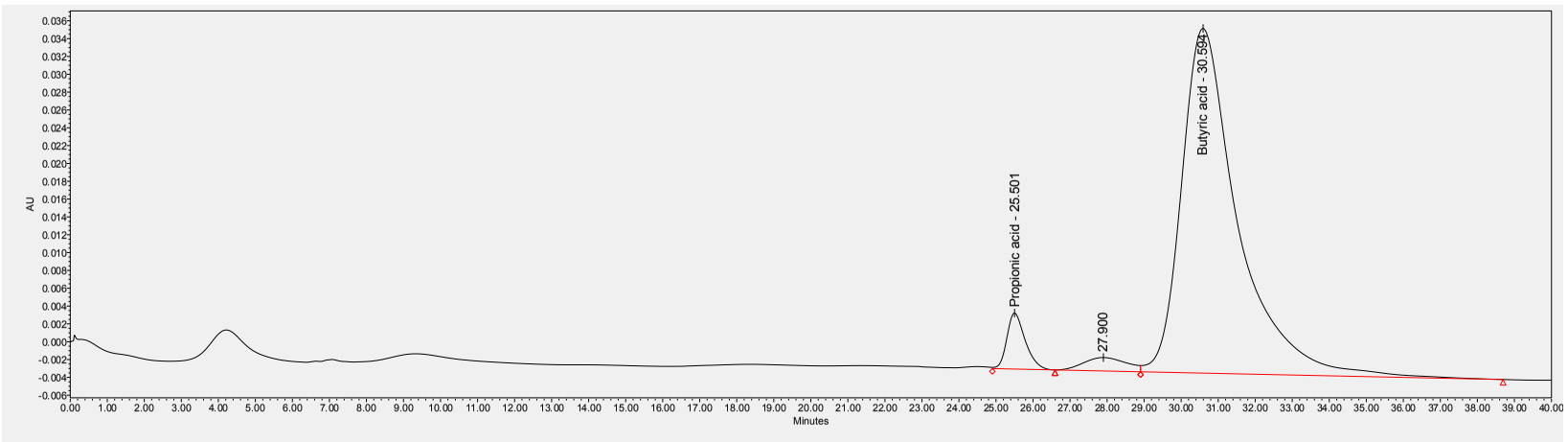
Inokulum HT-RT2 and HT-RT3



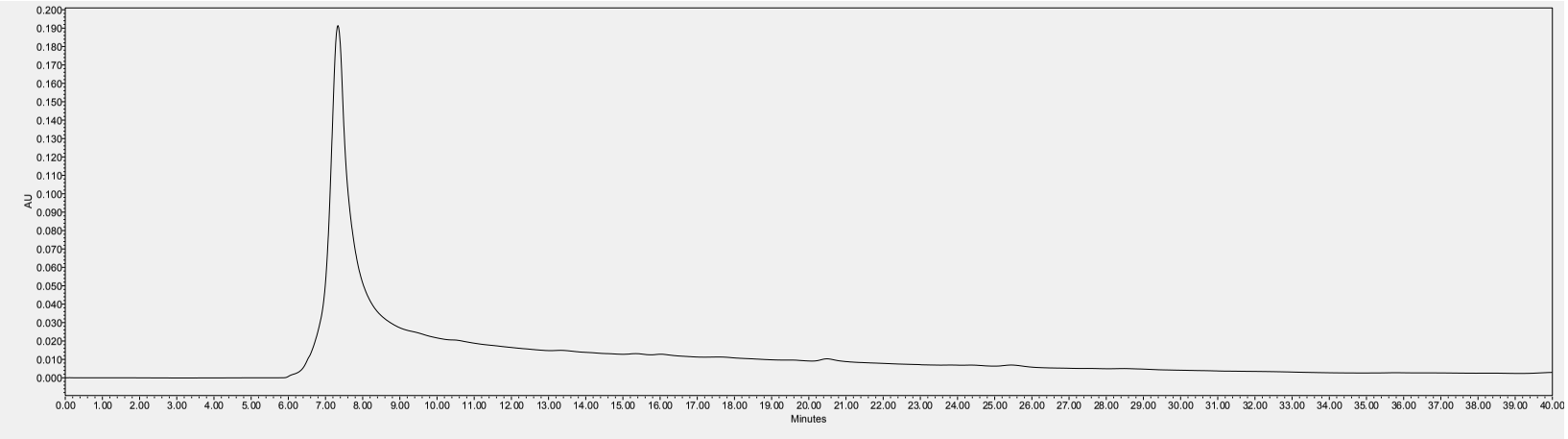


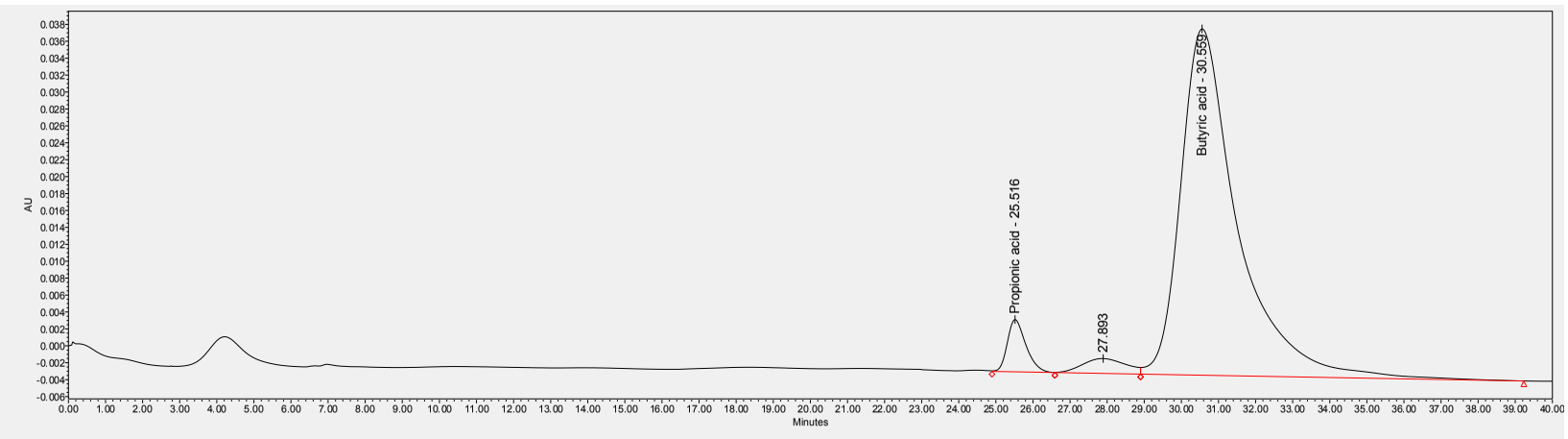
Inokulum BT-RT2 and BT-RT3



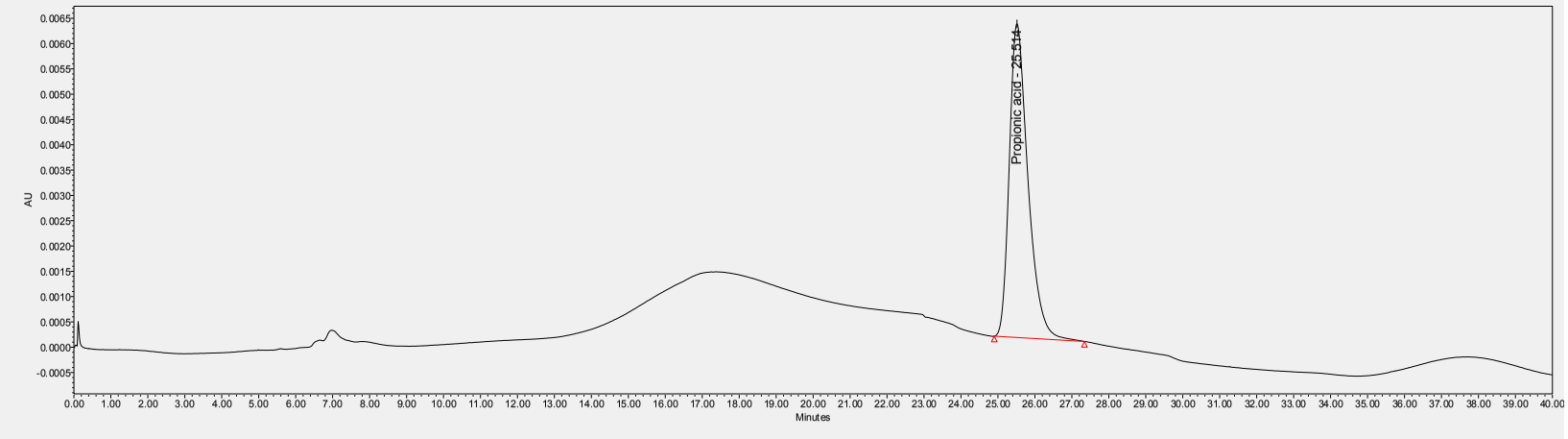


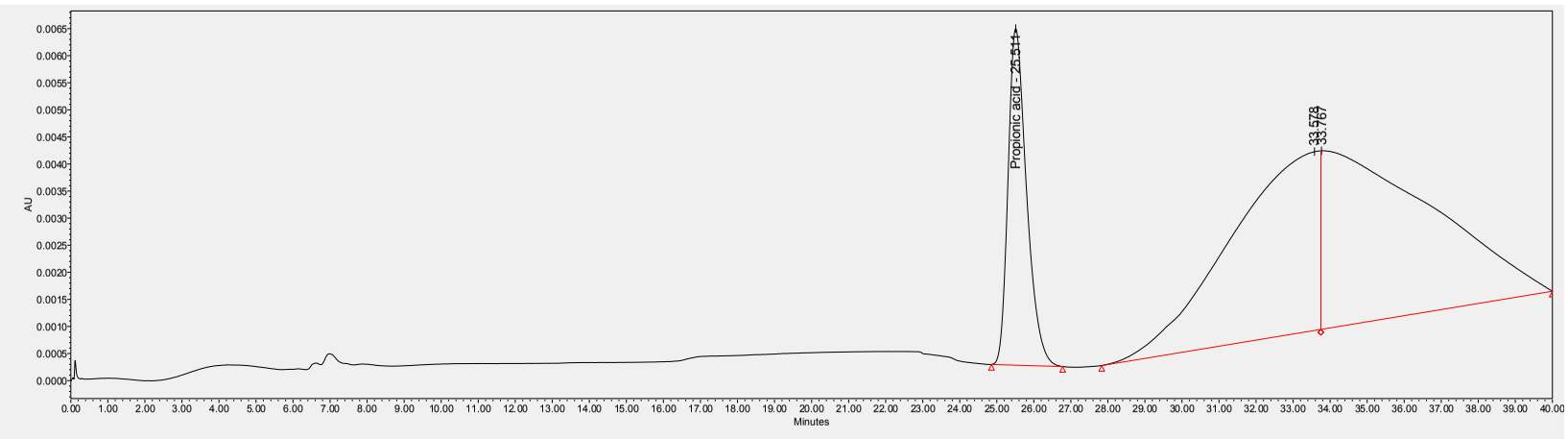
Inokulum BTpH-RT2 and BTpH-RT3



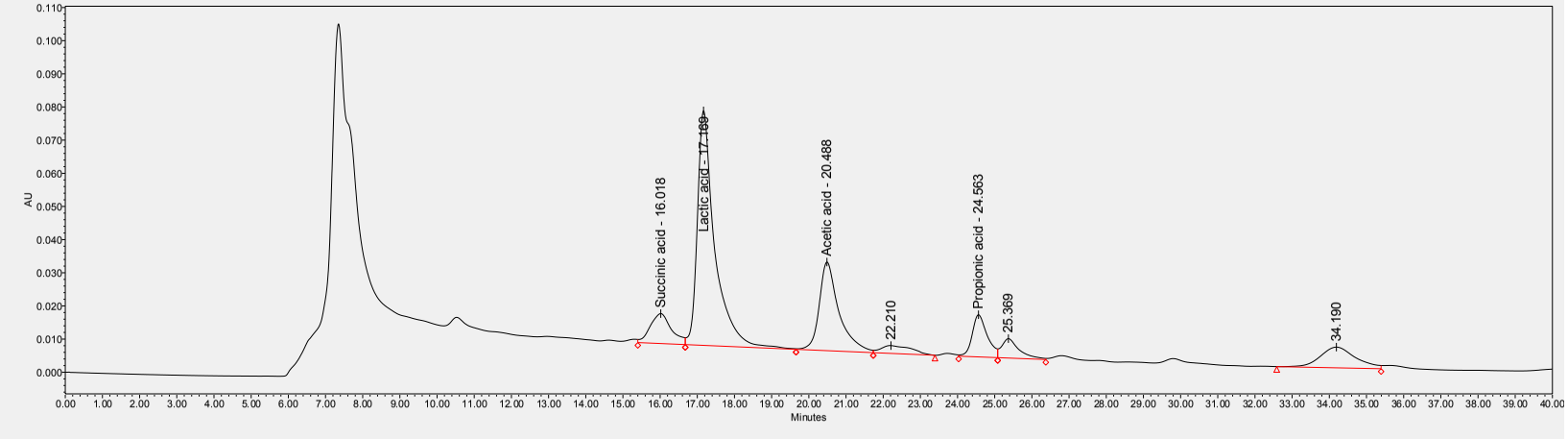


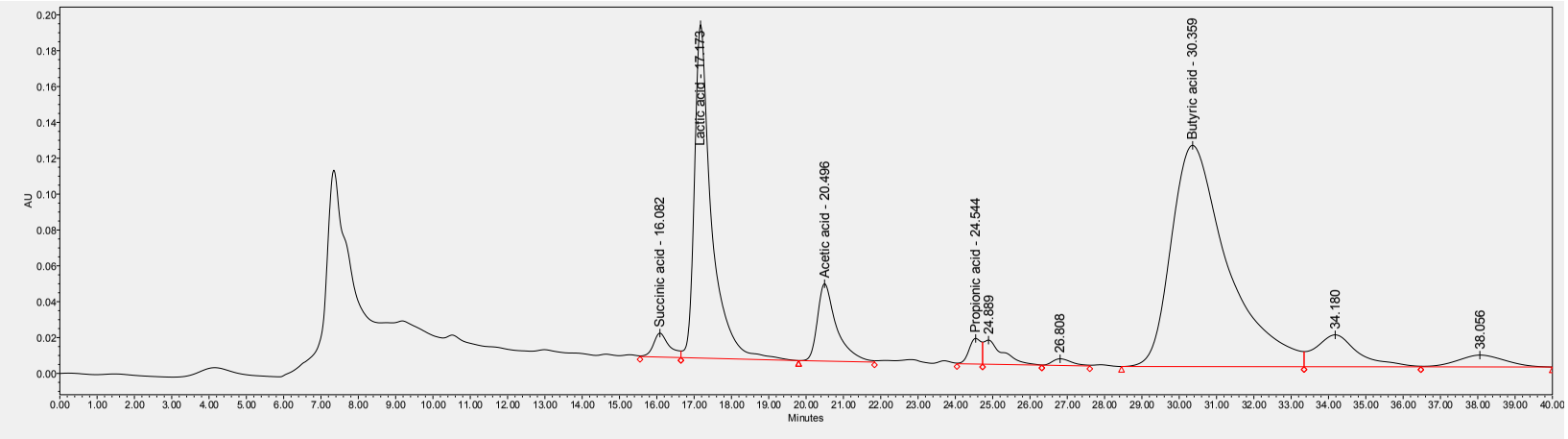
Digestate after HRT from HT-RT2 and HT-RT3



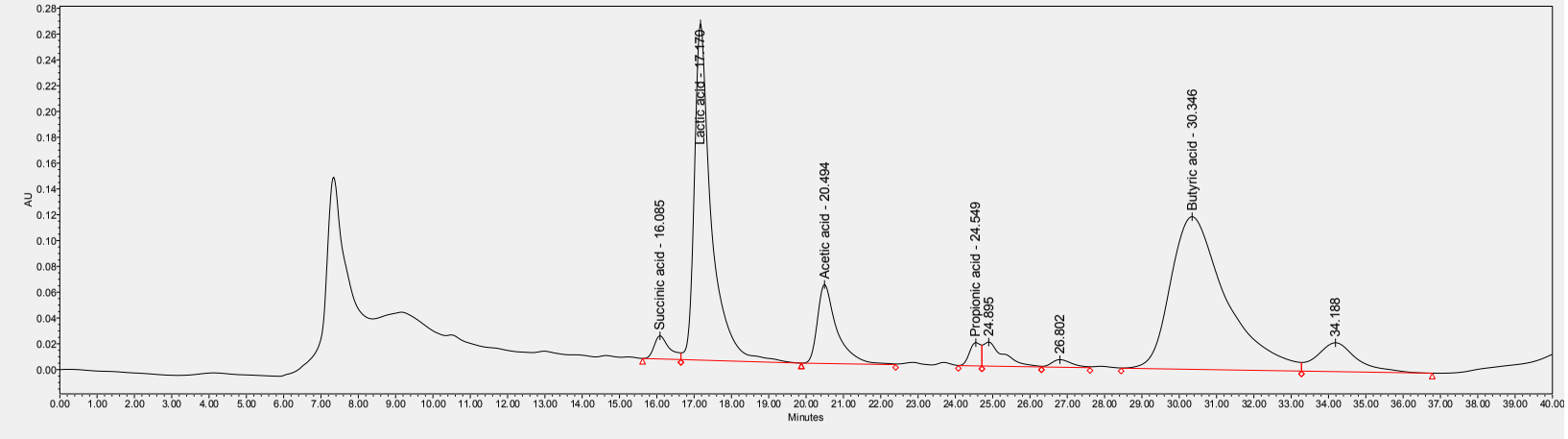


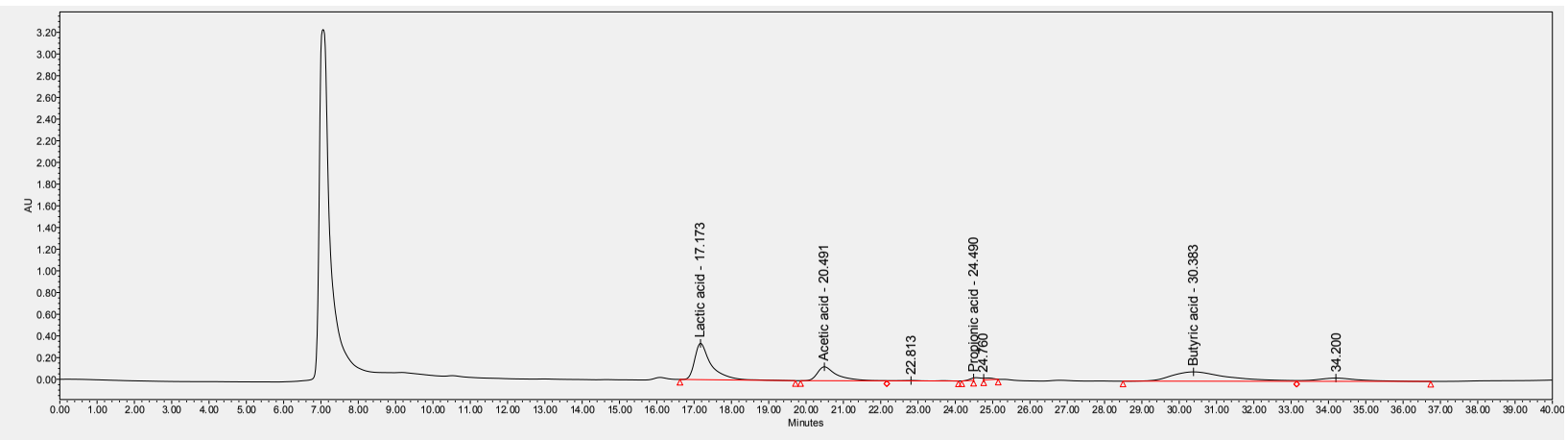
Digestate after HRT from BT-RT2 and BT-RT3





Digestate after HRT from BTpH-RT2 and BT-pHRT

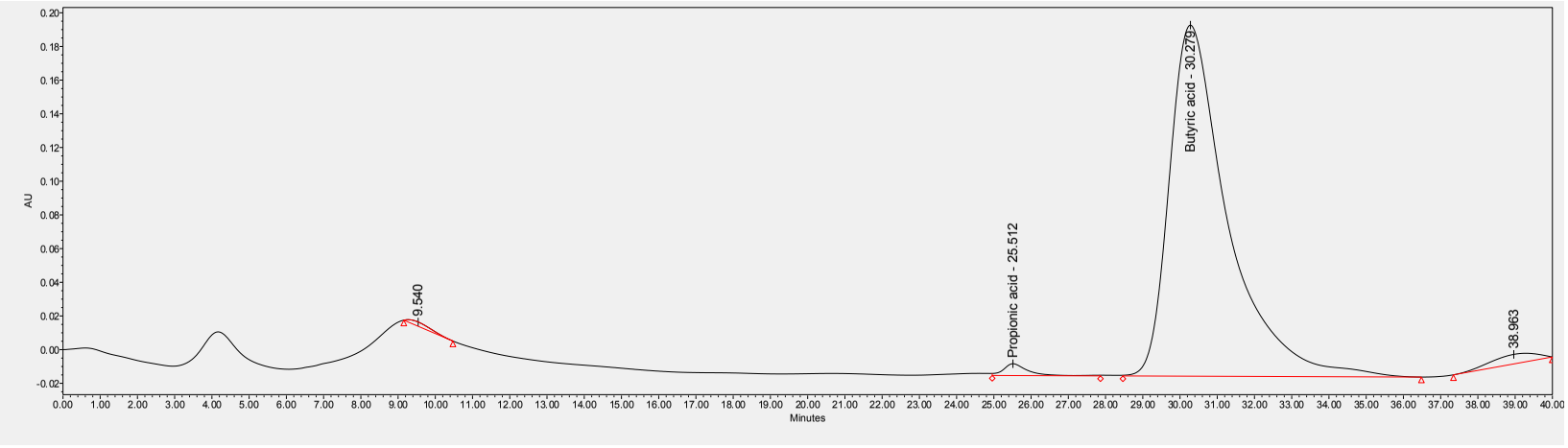


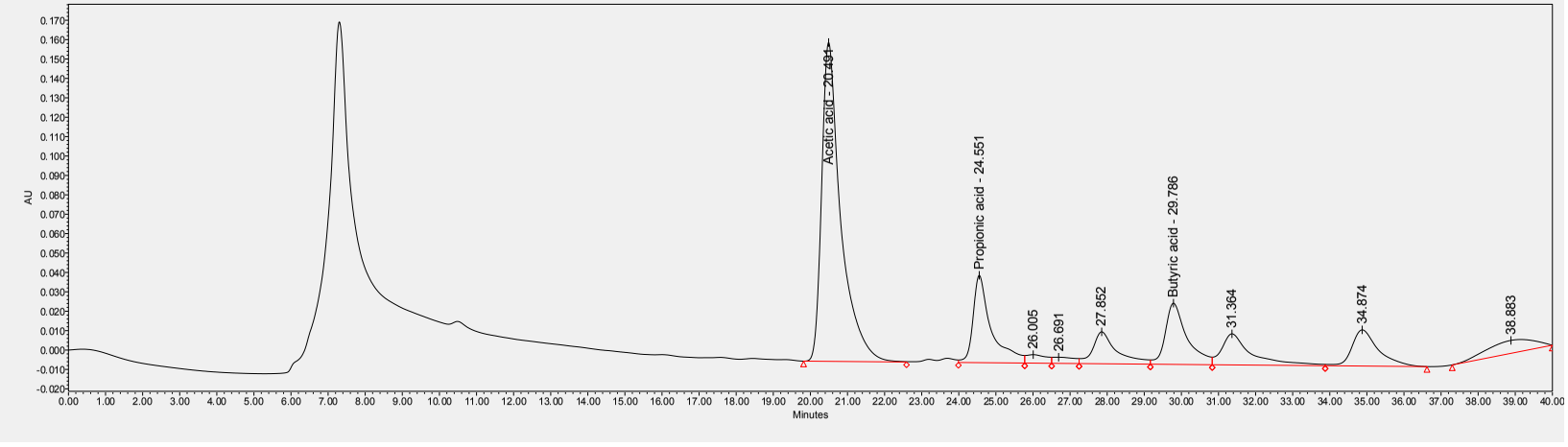


H.2 Degradation study

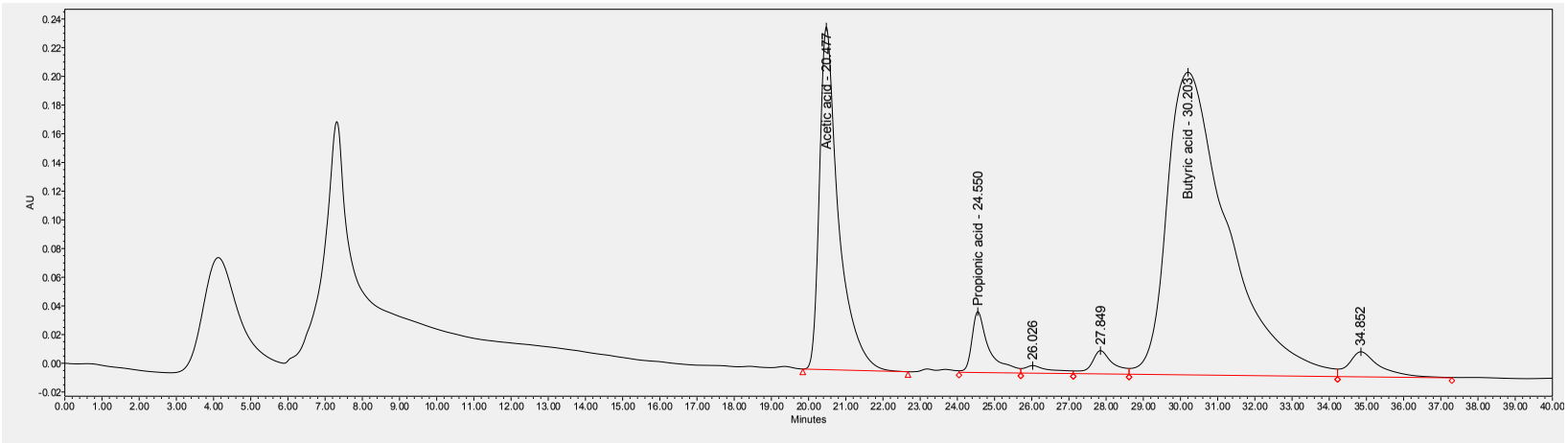
HPLC was also used to study the change in acid content over time in HT and BT. Both non-regulated (NonR) BT content and hygienised and pH-regulated BT content (BT-pH) was analysed. At day 16 of the sampling period, pure food waste and pure sludge was also sampled for HPLC analysis.

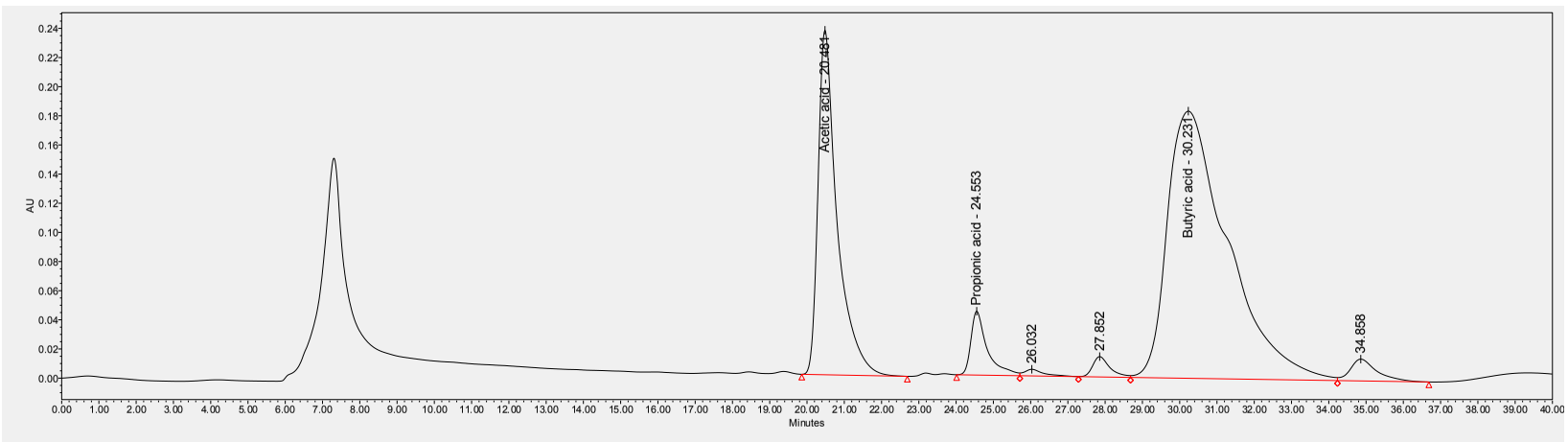
BTpH1 and BTpH2



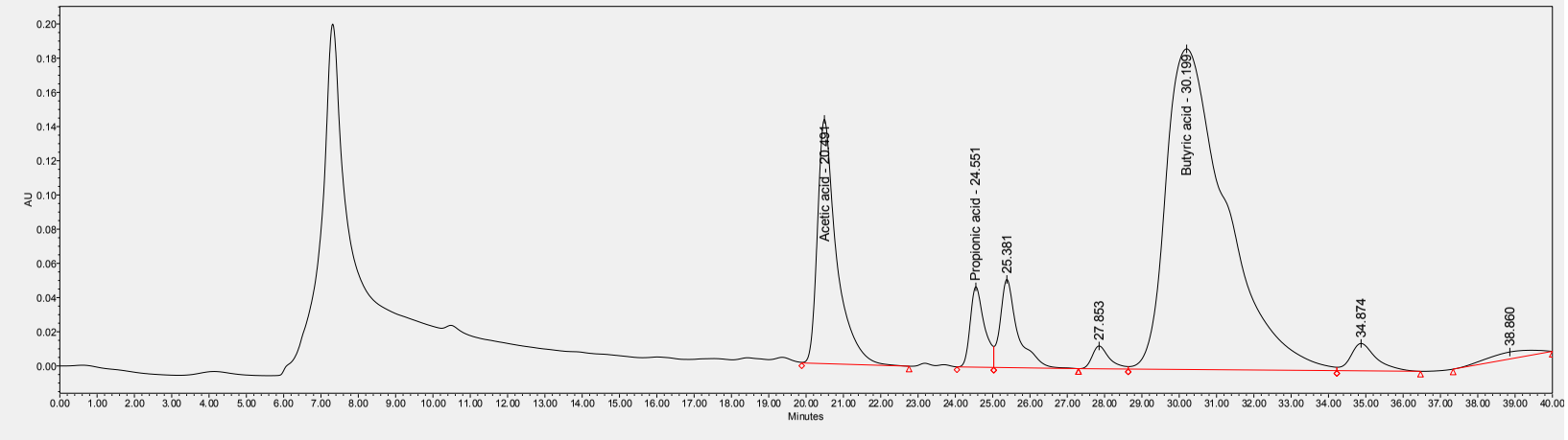


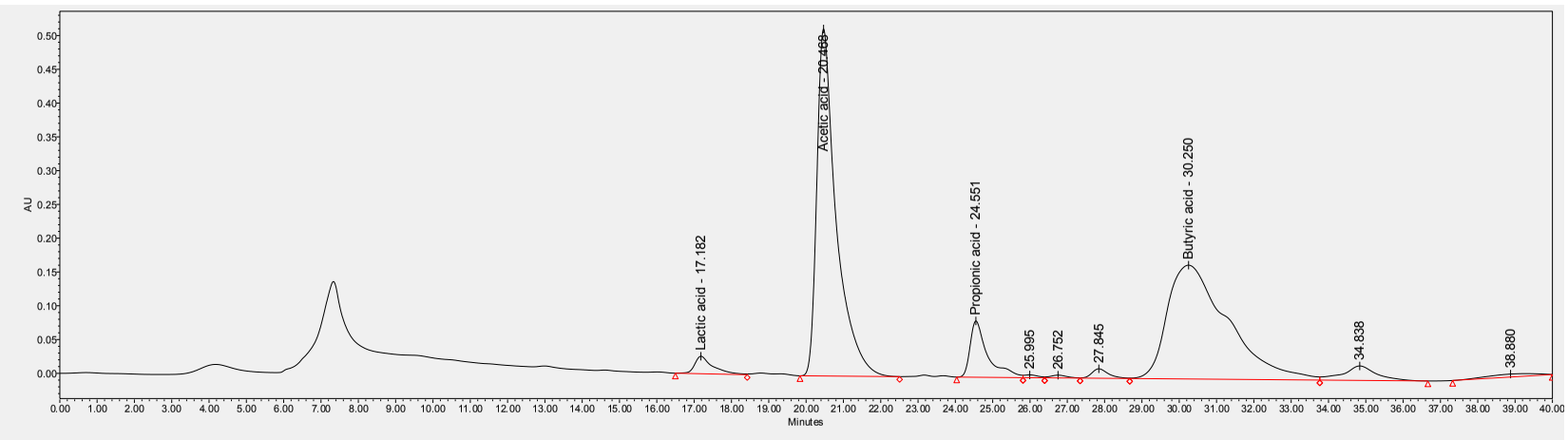
BTpH3 and BTpH4



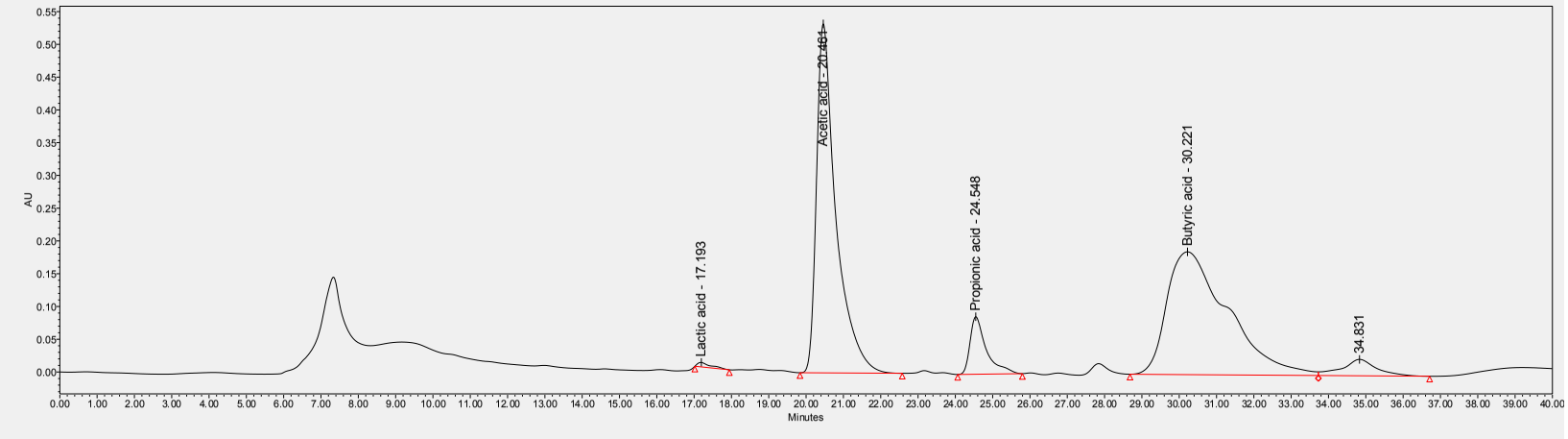


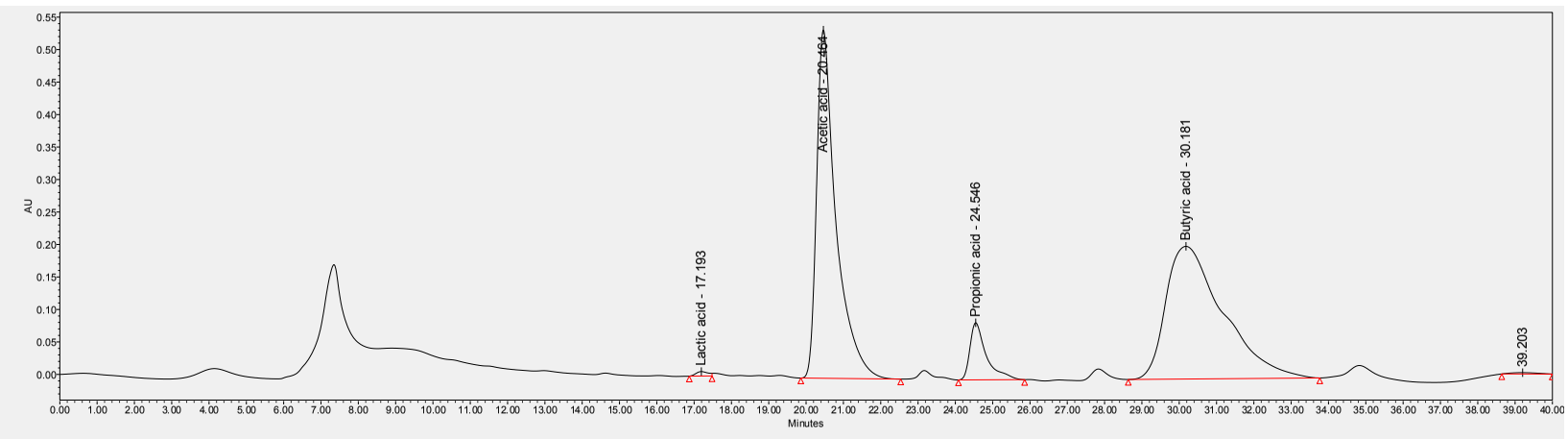
BTpH5 and BTpH6



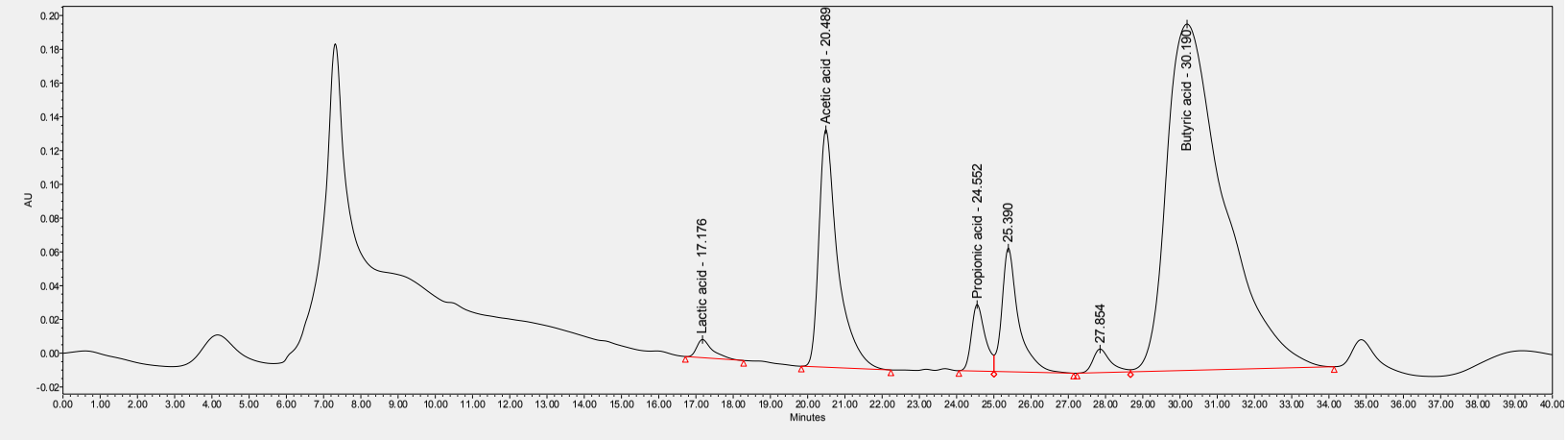


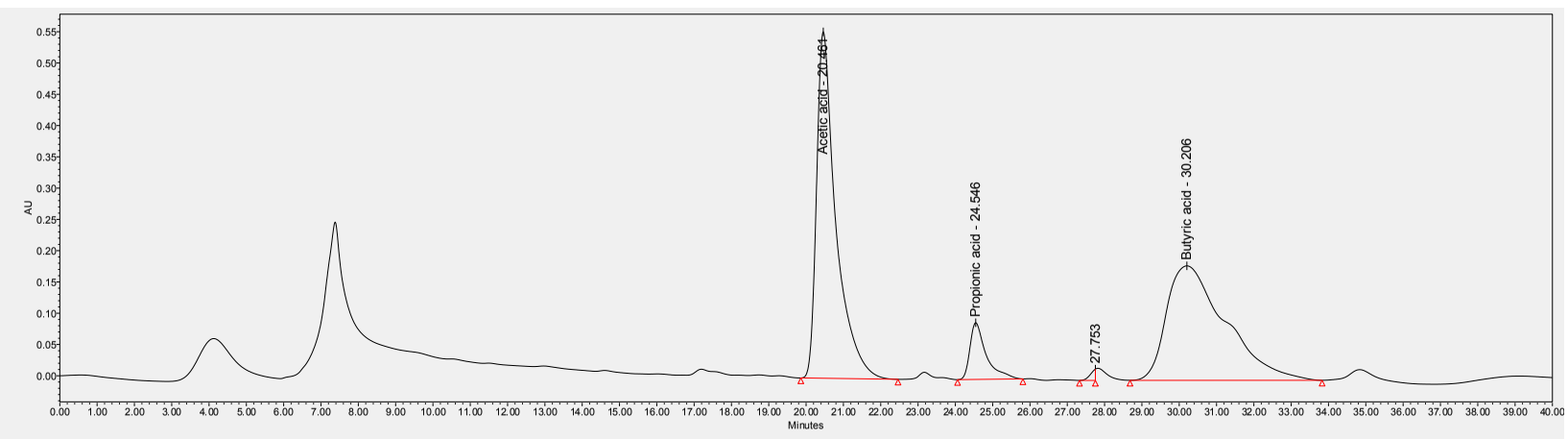
BTpH7 and BTpH8



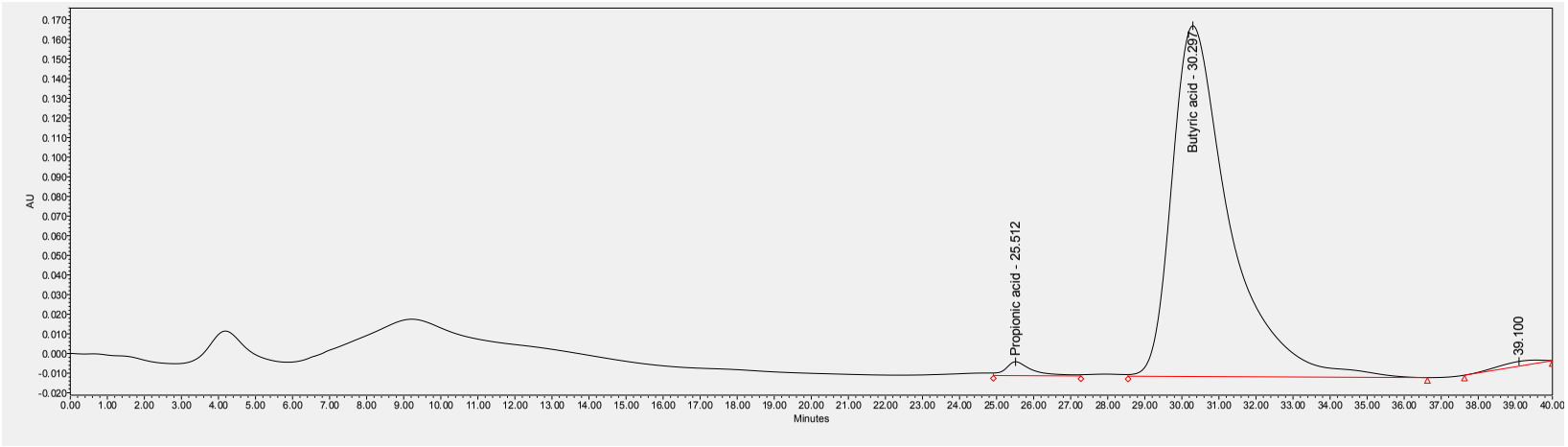


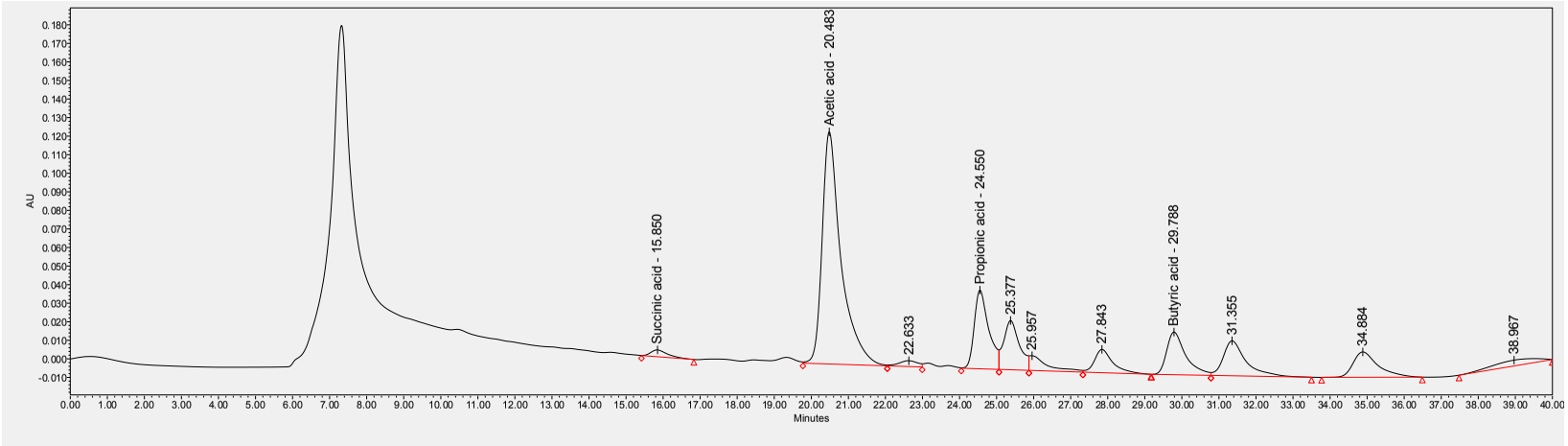
BT-NonR1 and BT-NonR3 (number 2 disappeared at the plant)



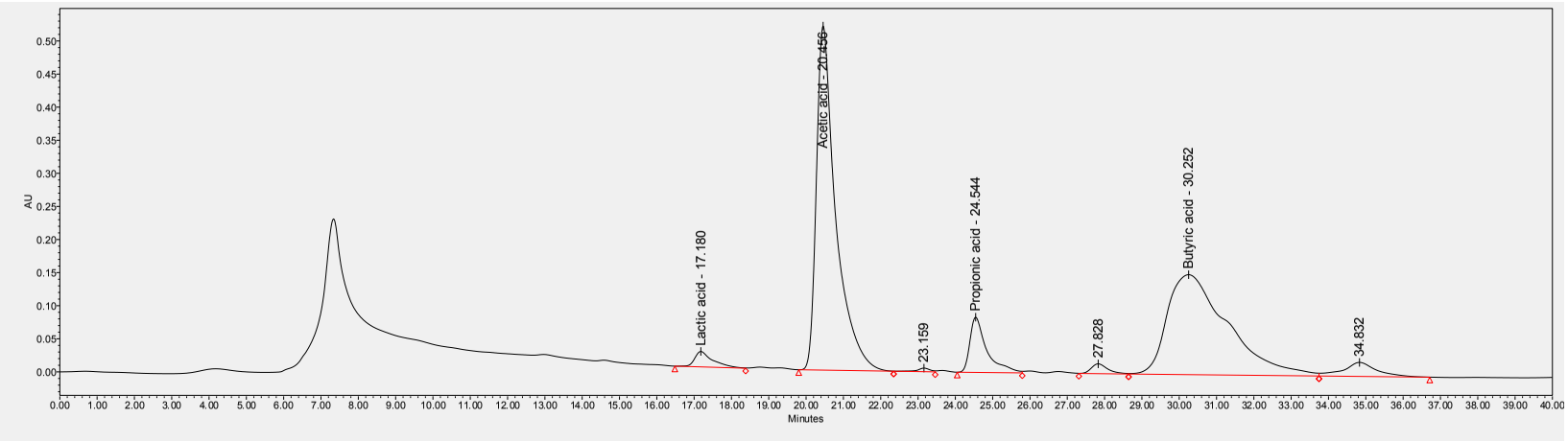


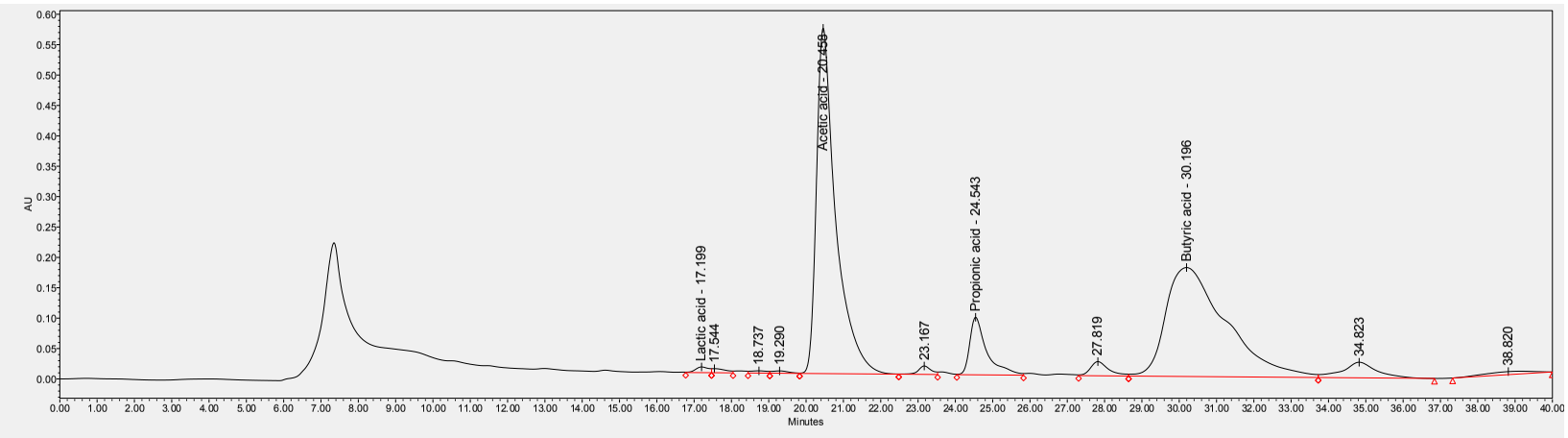
BT-NonR4 and BT-NonR5



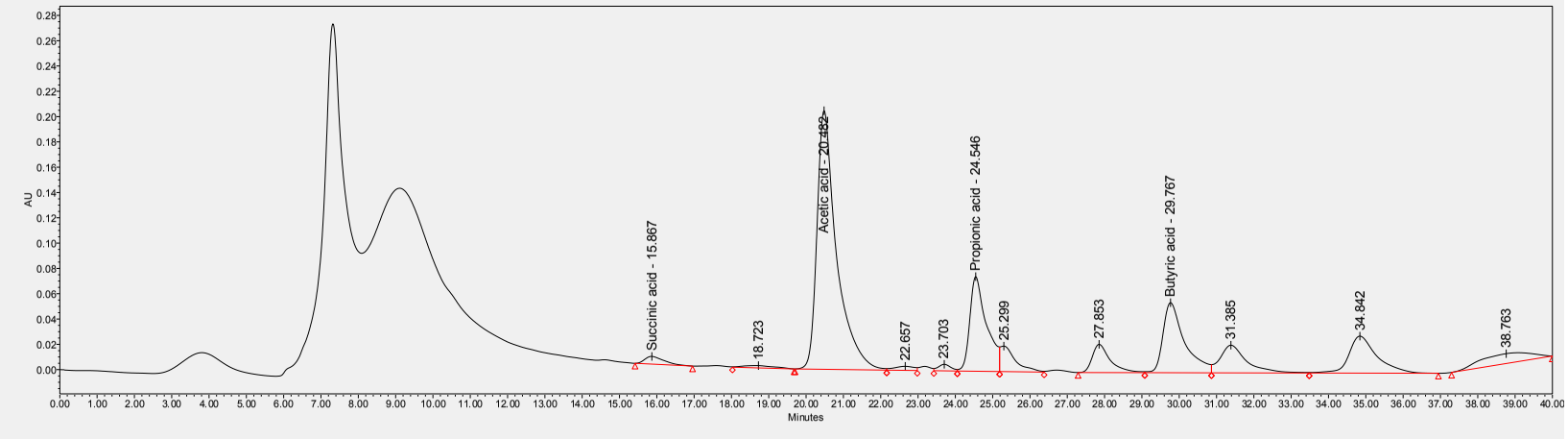


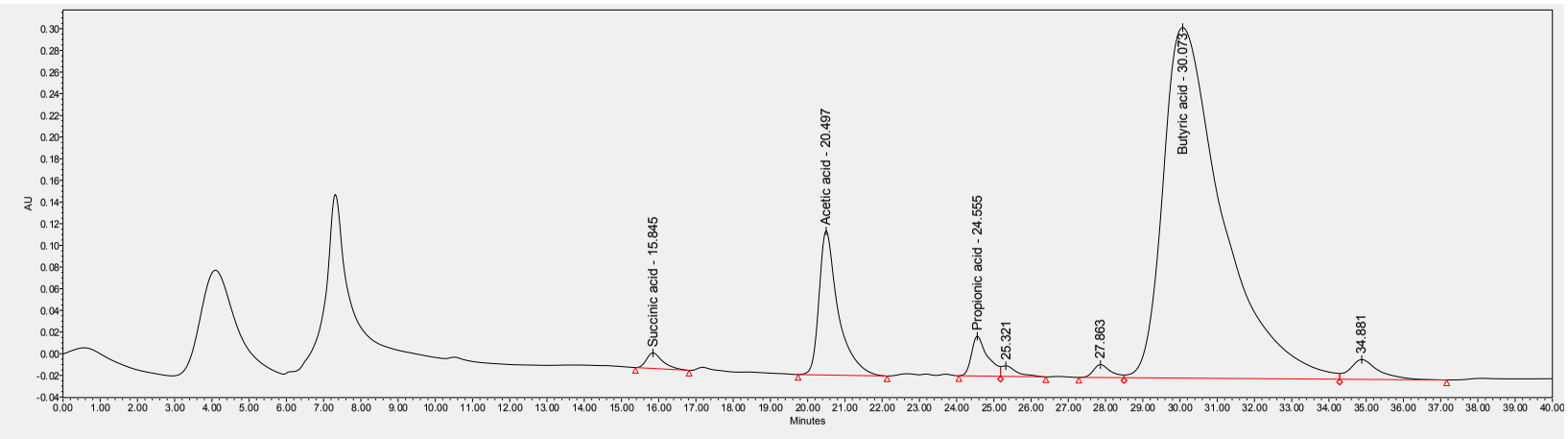
BT-NonR6 and BT-NonR8 (Tube with sample 7 opened during transport)



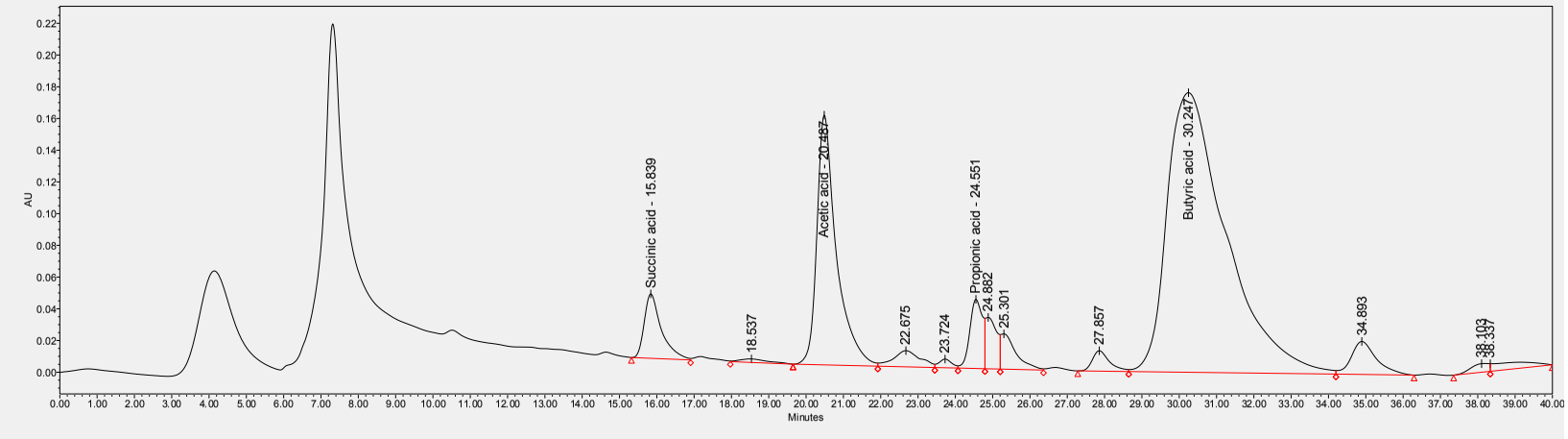


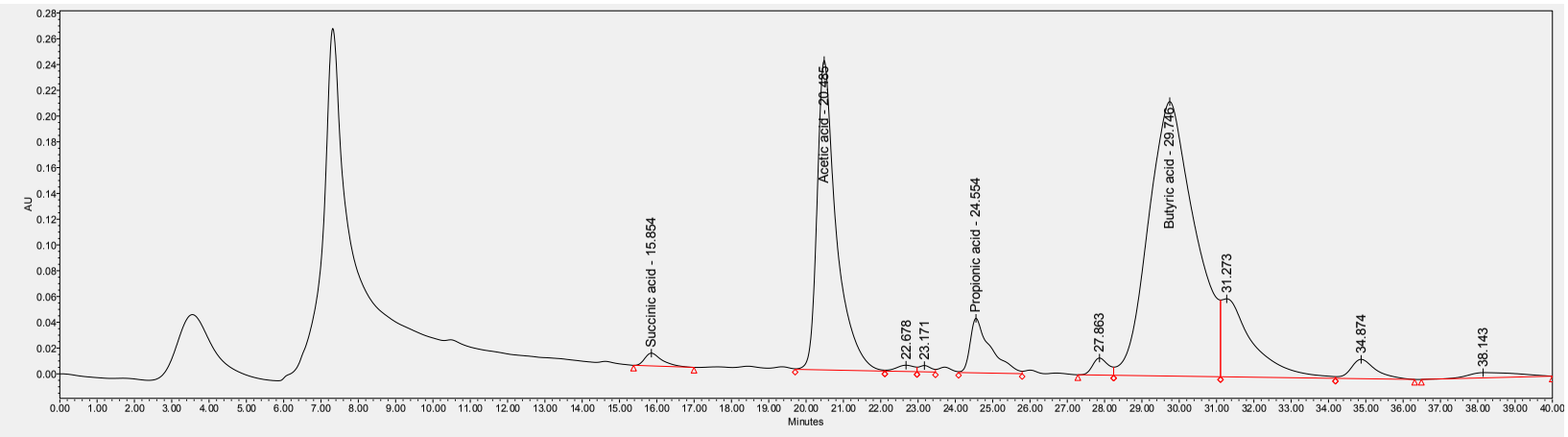
HT1 and HT2


AU
Minutes
Succinic acid - 15.867
18.723
Acetic acid - 20.482
22.657
23.703
Propionic acid - 24.546
25.299
27.853
Butyric acid - 29.767
31.385
34.842
38.763

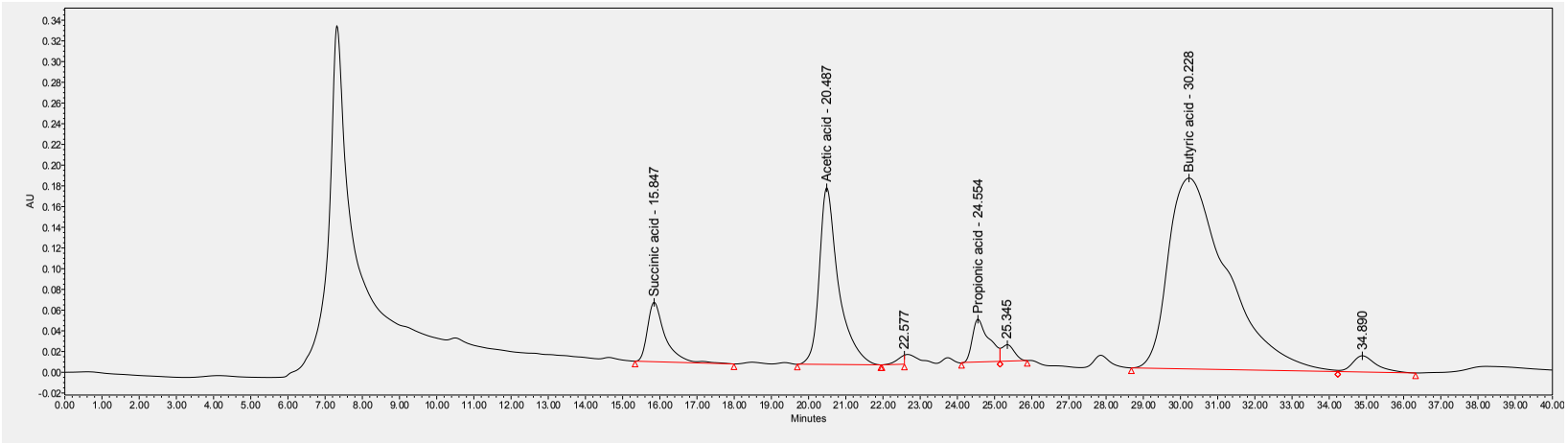

AU
Minutes
Succinic acid - 15.845
Acetic acid - 20.497
Propionic acid - 24.555
25.321
27.863
Butyric acid - 30.073
34.881

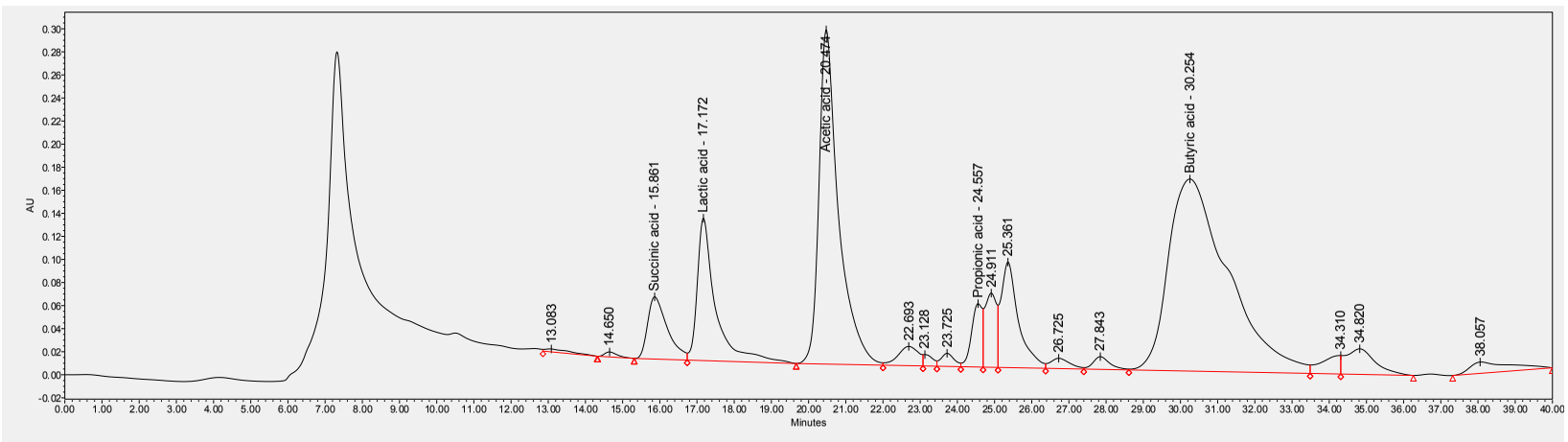
HT3 and HT4


AU
Minutes
Succinic acid - 15.839
Acetic acid - 20.487
Propionic acid - 24.551
Butyric acid - 30.247
18.537
22.675
23.724
24.882
25.301
27.857
34.893
38.103
38.337

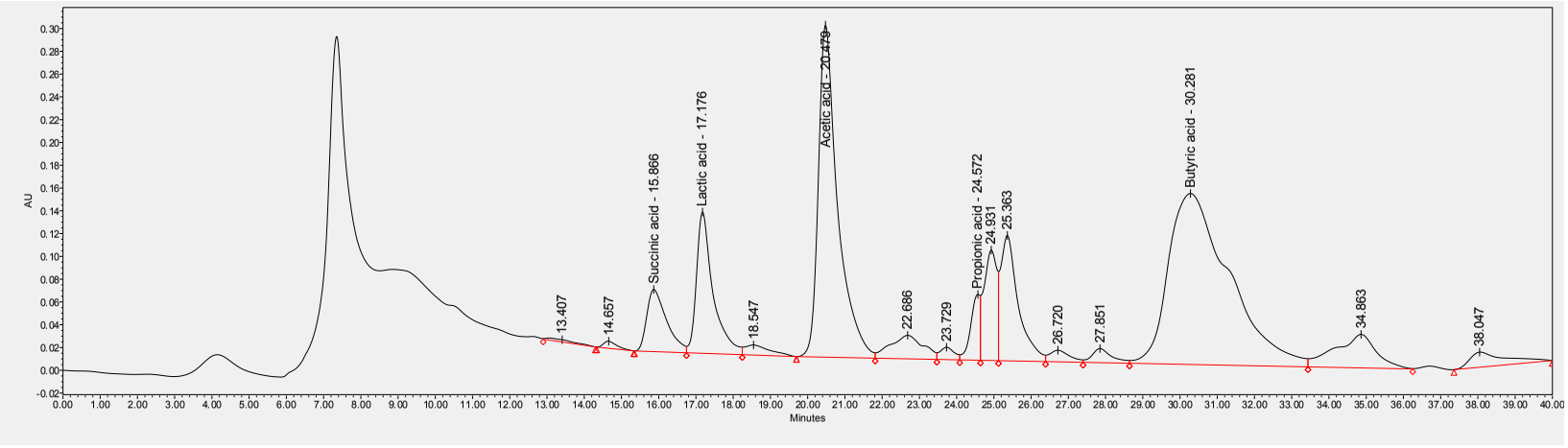

AU
Minutes
Succinic acid - 15.854
Acetic acid - 20.485
Propionic acid - 24.554
Butyric acid - 29.746
22.678
23.171
27.863
31.273
34.874
38.143

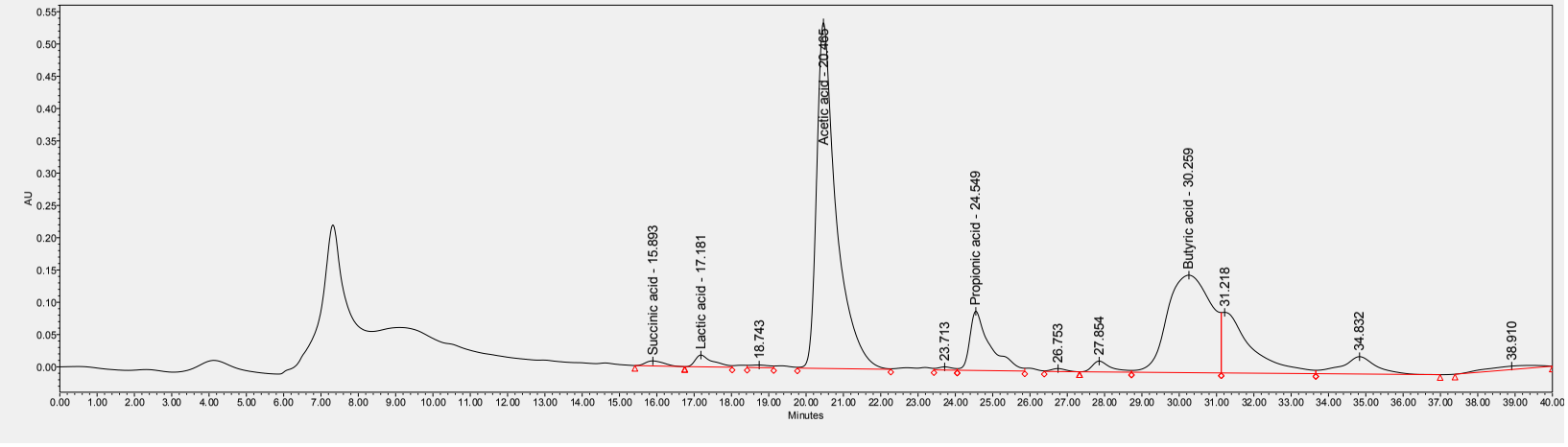
HT5 and HT6


AU
Minutes
Succinic acid - 15.847
Acetic acid - 20.487
22.577
Propionic acid - 24.554
25.345
Butyric acid - 30.228
34.890

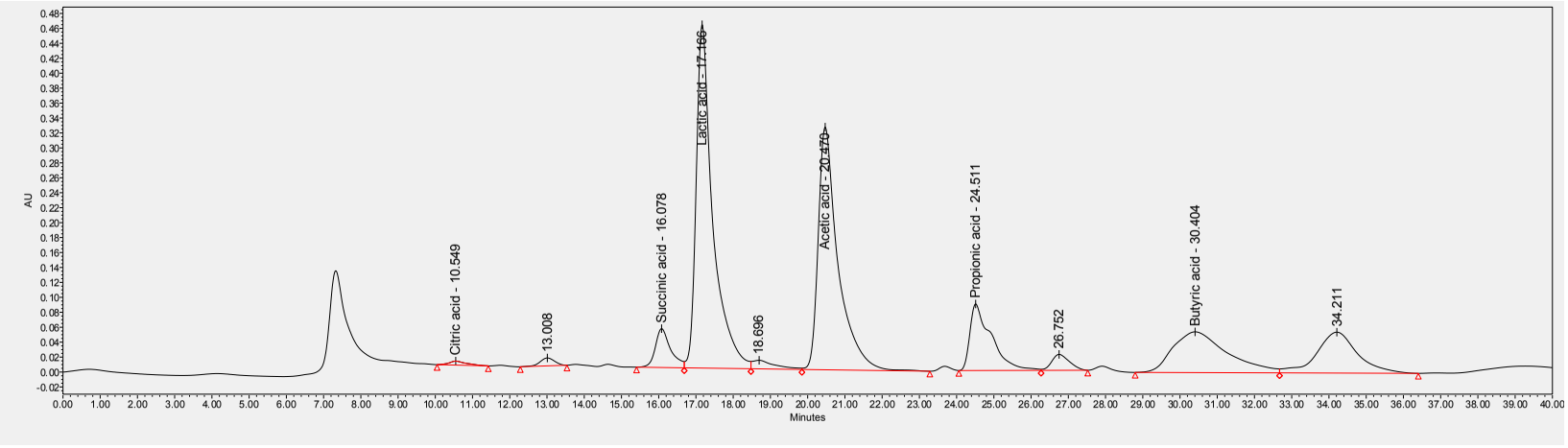

AU
Minutes
13.083
14.650
Succinic acid - 15.861
Lactic acid - 17.172
Acetic acid - 20.474
22.693
23.128
23.725
Propionic acid - 24.557
24.911
25.361
26.725
27.843
Butyric acid - 30.254
34.310
34.820
38.057

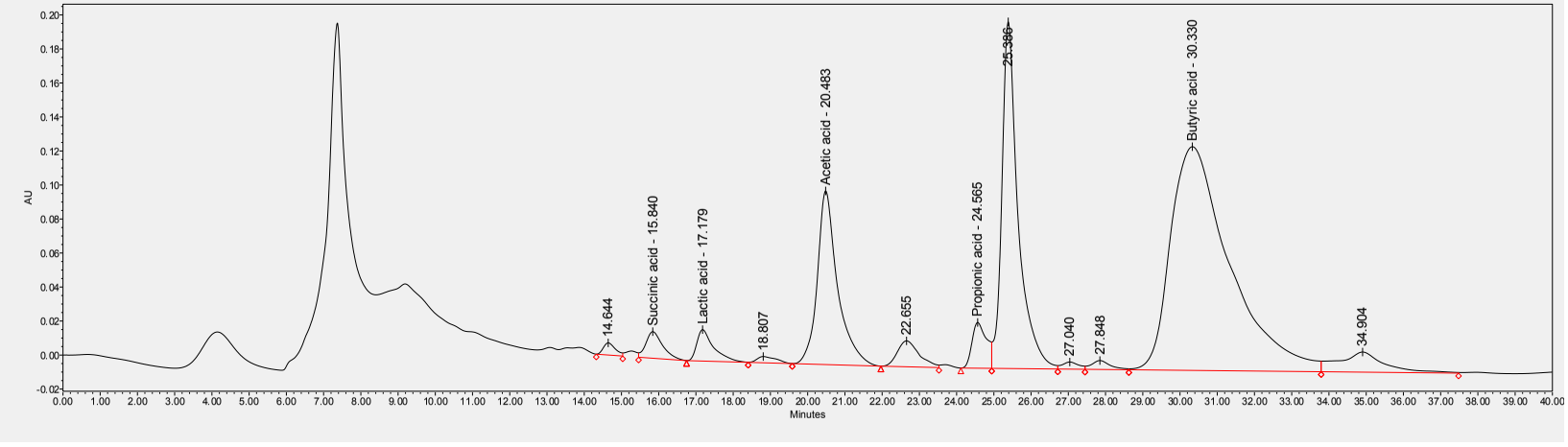
HT7 and HT8


AU
Minutes
13.407
14.657
Succinic acid - 15.866
Lactic acid - 17.176
Acetic acid - 20.479
18.547
22.686
23.729
Propionic acid - 24.572
24.931
25.363
26.720
27.851
Butyric acid - 30.281
34.863
38.047


AU
Minutes
Succinic acid - 15.893
Lactic acid - 17.181
18.743
Acetic acid - 20.485
23.713
Propionic acid - 24.549
26.753
27.854
Butyric acid - 30.259
31.218
34.832
38.910

Food substrate (top) and sludge (bottom) from day 16





Appendix I

Sanger Sequencing Chromatogram and Alignment

I.1 Total sequence

The primer-pair Eub8f (forward primer) and 518R (reverse primer) were used to amplify the variable region 3 and 4 in the 16S rDNA gene of *Thermotoga Lepl 10*. The PCR product was purified and sent to Sanger sequencing as shown in the chromatogram of I.1. The un-edited sequence was determined to:

5' - *T GGC ATG GCG GCT GCT ACC ATG CAG TCG AGC GGG GGA ACT CCC TTC GGG GAG GAG TAC CCA GCG GCG GAC GGG TGA GTA ACA CGT GGG TAA CCT GCC CTC CGG AGG GGG ATA ACC AGG GGA AAC CCT GGC TAA TAC CCC ATA CGC TCC ATC AAC GCA AGT TGG TGG AGG AAA GGG GCG TTT GCC CCG CCG GAG GAG GGG CCC GCG GCC CAT CAG GTA GTT GGT GGG GTA ACG GCC CAC CAA GCC GAC GAC GGG TAG CCG GCC TGA GAG GGT GGT CGG CCA CAG GGG CAC TGA GAC ACG GGC CCC ACT CCT ACG GGA GGC AGC AGT GGG GAA TCT TGG ACA ATG GGG GAA ACC CTG ATC CAG CGA CGC CGC GTG CGG GAC GAA GCC CTT CGG GGT GTA AAC CGC TGT GGC GGG GGA AGA ATA AGG TAG GGA GGG AAT GCC CTA CCG ATG ACG GTA CCC CGC TAG AAA GCC CCG GCT AAC TAC GTG CCA ACA GC* - 3'

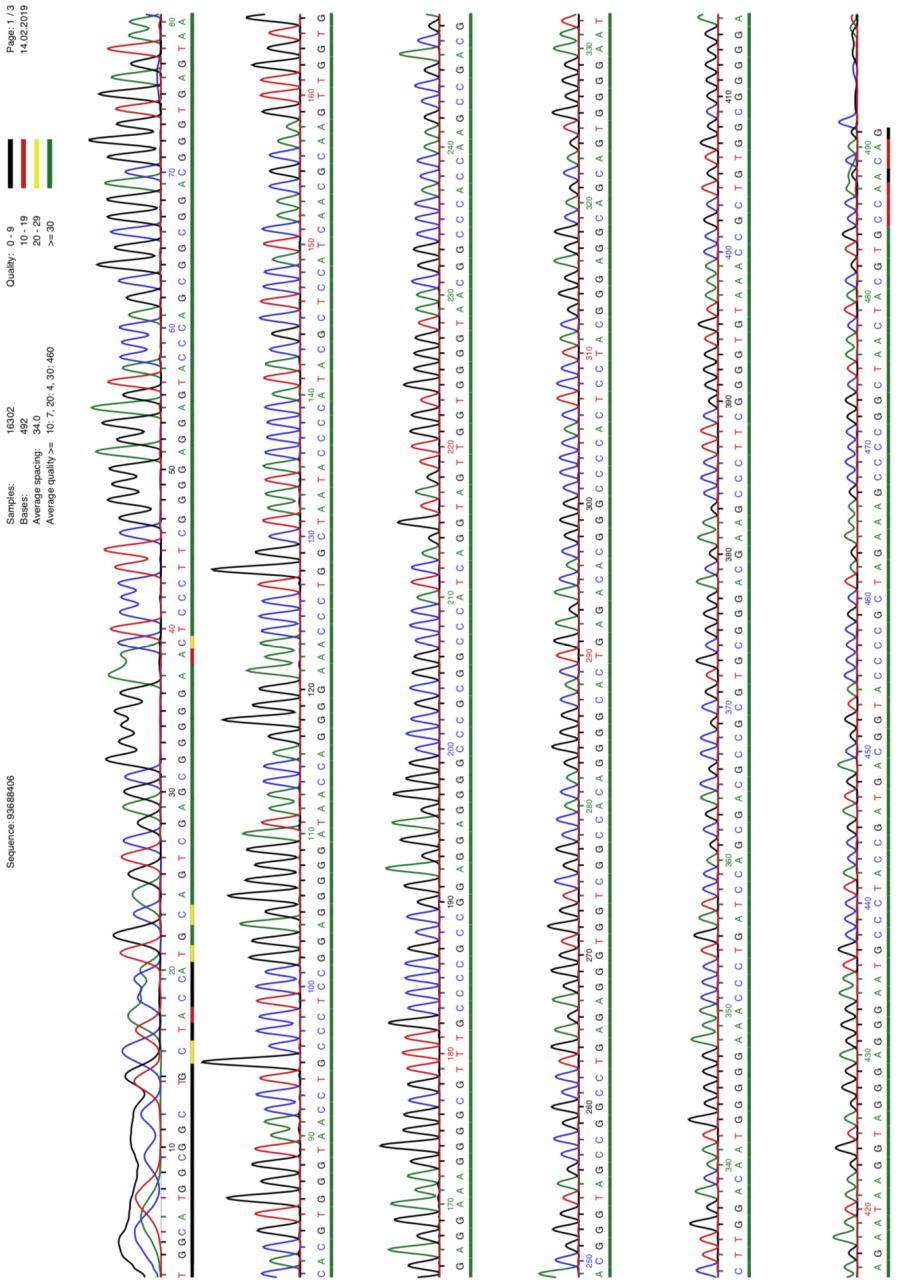


Figure I.1: Sequence result from Sanger sequencing of variable region 3 and 4 in 16S rDNA of *T. Lepl 10*.

Sequence matching

RDP classification and sequence matching (RDP) was used to classify the sequenced DNA as shown below.

Query Submit Date: Wed Mar 27 06:18:20 EDT 2019

Match hit format: short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full name. More help is available.

Lineage:

Results for Query Sequence: seqmatch_seq, 435 unique oligos

rootrank Root (20) (match sequences)
- domain Bacteria (20)
  - phylum "Aquificae" (1)
    - class Aquificae (1)
      - order Aquificales (1)
        - family Aquificaceae (1)
          - genus Hydrogenivirga (1)
            - S000652657 not_calculated 0.508 1321 Hydrogenivirga okinawensis (T); LS12-2 (= JCM 13302 = DSM 17378); AB235314
  - phylum "Chloroflexi" (1)
    - class Thermoflexia (1)
      - order Thermoflexales (1)
        - family Thermoflexaceae (1)
          - genus Thermoflexus (1)
            - S003718757 not_calculated 0.554 1320 Thermoflexus hugenholtzii (T); JAD2; KC526151
  - phylum "Thermotogae" (13)
    - class Thermotogae (13)
      - order Thermotogales (13)
        - family Thermotogaceae (9)
          - genus Thermotoga (6)
            - S000383207 not_calculated 0.936 1329 Thermotoga neapolitana (T); DSM 4359; AB039768
            - S000436057 not_calculated 0.970 1401 Thermotoga maritima (T); M21774
            - S000438744 not_calculated 0.559 1356 Thermotoga hypogea (T); SEBR 7054; U89768
            - S000539708 not_calculated 0.956 1404 Thermotoga naphthophila (T); RKU10; AJ872268
            - S000539710 not_calculated 0.984 1402 Thermotoga petrophila (T); RKU10; AJ872269
            - S004459158 not_calculated 0.579 1399 Thermotoga caldifontis (T); AZM44c09; AP014509

Alignment with *Thermotoga petrophila*

The first 20 basepairs (bp) and last 8 bp of the known sequence were removed to avoid including the primers, and to get a more reliable alignment.

```
T. petrophila        40 cggcgtgcctaacacatgcaagtcgagcgggggaaactcccttcggggaggagtacccagcggcggacgg
T. Lepl 10            1 ----------------....-.............-................................

T. petrophila       110 gtgagtaacacgtgggtaacctgccctccggagggggataaccaggggaaaccctggctaataccccata
T. Lepl 10           53 ............................................................................

T. petrophila       180 cgctccatcaacgcaagttggtggaggaaaggggcgtttgccccgccggaggaggggcccgcggcccatc
T. Lepl 10          123 ............................................................................

T. petrophila       250 aggtagttggtggggtaacggcccaccaagccgacgacgggtagccggcctgagagggtggtcggccaca
T. Lepl 10          193 ............................................................................

T. petrophila       320 ggggcactgagacacgggccccactcctacgggaggcagcagtggggaatcttggacaatgggggaaacc
T. Lepl 10          263 ............................................................................

T. petrophila       390 ctgatccagcgacgccgcgtgcgggacgaagcccttcggggtgtaaaccgctgtggcgggggaagaataa
T. Lepl 10          333 ............................................................................

T. petrophila       460 ggtagggagggaatgccctaccgatgacggtaccccgctagaaagccccggctaactacgtgccagcagc
T. Lepl 10          403 ............................................................--------
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Vilde Holen

Effects of a Hyperthermophilic Pre-Treatment in Anaerobic Digestion

NTNU
Kunnskap for en bedre verden

Lindum