


Master thesis

The negative effect of carbohydrates on protein degradation in the acid-phase of anaerobic digestion of protein-rich wastewater: a microbial composition study

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The negative effect of carbohydrates on protein degradation in the acid-phase of anaerobic digestion of protein-rich wastewater: a microbial composition study

By

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Time flies. With the year coming to an end, my study journey in TUD is also close to the end. Looking back to 2020, two keywords appear in my mind, COVID-19 and thesis. Although these two things present a great challenge, I am never alone. Here, I would like to address my sincerest gratitude to my supervisors, colleagues, family, and friends.

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Abstract

Anaerobic digestion (AD) is a promising technology to process protein-rich wastewater. However, incomplete protein degradation during the acidification of organic matter is frequently observed when carbohydrates are present. Literature reports little information to explain the mechanisms behind this phenomenon. This MSc study investigated the relationship between microbial composition and the negative effect of carbohydrates on anaerobic protein degradation in the acid-phase by restricting the carbon source to a mixed culture in a continuously stirred tank anaerobic reactor. Two continuously stirred anaerobic reactors fed with restrictive protein and/or carbohydrate substrates were operated and compared in the aspects of acidification, deamination, protease activity, and microbial composition. Results showed that the deamination degree in the protein-feeding reactor decreased from 77% to 15% and the acidification degree decreased from 75% to 34% when the restrictive carbon source shifted from proteins to mixtures of proteins and carbohydrates. A decrease in protease activity was also observed. Anaerobic protein degradation was significantly retarded by the presence of carbohydrates. Results of the microbial composition analysis showed that generalists (i.e. microorganisms that can ferment both proteins and carbohydrates) that preferentially consumed carbohydrates were the predominant populations in the microbial community when carbohydrates were present as additional substrates. Therefore, the observed negative effect of carbohydrates on protein degradation in acid-phase could be mainly attributed to the preferential substrate utilization feature of generalists. Further research on the metabolism functional analysis of the microbial community should be employed to confirm this hypothesis. Overall, this study offers a better understanding of the mechanisms behind the negative effect of carbohydrates on protein degradation, which can provide some hints of the design of the anaerobic digestion process of protein-rich wastewater.

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COD	Chemical oxygen demand
CSTR	Continuous-flow stirred tank reactor
C2	Acetic acid
C3	Propionic acid
C4	Butyric acid
C5	Valeric acid
C6	Caproic acid
GC	Gas chromatography
HRT	Hydraulic retention time
iC4	Iso-butyric acid
iC5	Iso-valeric acid
OLR	Organic loading rate
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
SKN	Soluble Kjeldahl nitrogen
TAN	Total ammonia nitrogen
TKN	Total Kjeldahl nitrogen
TN	Total nitrogen
TSS	Total suspended solids
UPGMA	Unweighted Pair-group Method with Arithmetic Mean
VFA	Volatile fatty acid
VSS	Volatile suspended solids

Chapter 1. Background

Proteins, important biological macromolecules to all living things, are common organic compounds that end up in the wastewater. Especially from the food production industry such as dairy and meat processing, the discharged effluents are in relatively high content of proteins, as well as carbohydrates and lipids, resulting in a high organic load to the wastewater treatment systems (Ahmad et al., 2019; Bustillo-Lecompte and Mehrvar, 2015; Carvalho et al., 2013; Demirel et al., 2005).

To treat such protein-rich wastewater, anaerobic digestion is preferred due to its advantages of less energy consumption, less excess sludge production, and recovery of energy in the form of biogas (Ahmad et al., 2019; Bustillo-Lecompte and Mehrvar, 2015; Demirel et al., 2005).

Incomplete protein degradation was commonly reported in the anaerobic digestion of protein-rich wastewater, giving rise to problems such as lower effluent quality, lower biogas production, foaming, and sludge deterioration (Duong et al., 2019; Ganidi et al., 2009; Hassan and Nelson, 2012; Perle et al., 1995; Prazeres et al., 2012).

The anaerobic digestion process of organic matter is theoretically divided into four steps: (i) hydrolysis, (ii) acidogenesis, (iii) acetogenesis, and (iv) methanogenesis (Gujer and Zehnder, 1983; van Lier, Mahmoud, and Zeeman, 2008). In practice, the four steps can be generalized in two phases, namely acid-phase and methane-phase (Göblös et al., 2008; Ince, 1998). The acid-phase comprises steps of hydrolysis and acidogenesis, in which complex materials are decomposed and converted into simple molecules by hydrolytic-acidogenic microorganisms (Ince, 1998). The methane-phase involves steps of acetogenesis and methanogenesis, in which products of the acid-phase are further converted into biogas by acetogenic and methanogenic microorganisms (Göblös et al., 2008; Ince, 1998; Yu and Fang, 2001).

A good knowledge of protein degradation in the acid-phase is important to the overall anaerobic process of protein-rich wastewater treatment since the acid-phase provides substrates for the methane-phase. Previous studies reported that the presence of carbohydrates can retard the acidogenic fermentation of protein (Breure et al., 1986a, 1986b; Tommaso et al., 2003; Yu and Fang, 2001). For example, the presence of glucose can reduce the degree of gelatin degradation from 82% to 46% in a gelatin-adapted anaerobic chemostat when a dilution rate of 0.2 h^{-1}, pH of 7, and temperature of 30 ℃ were applied (Breure et al., 1986b). However, the mechanism behind such a phenomenon is not well explored in those previous studies and remains a knowledge gap. Narrowing this knowledge gap could provide a better understanding of the microbial process in anaerobic digestion of protein-rich wastewater treatment.

The performance of the microbial process of wastewater treatment is the consequence of the ecology of associated microorganisms (Rittmann, 2010). Accordingly, the negative

effect of carbohydrates on protein degradation in the acid-phase can be regarded as the outcome of the ecology of associated microorganisms. Therefore, an insight into the microbial community might shed some light on the mechanism behind the negative effect of carbohydrates on protein degradation.

Chapter 2. Literature study

A literature study is a good approach to provide information on what is known and what is unknown. The unknown information leads us to the objective of this MSc research. The known information provides prior knowledge for the research methods.

In this chapter, a literature review covering the following aspects were addressed: 1) pathways of protein degradation in the acid-phase; 2) protein-degrading microorganisms; 3) operational conditions of the acid-phase 4) acid-phase bioreactor configuration; 5) available techniques for microbial community analysis.

2.1. The state of knowledge

Based on the literature review, the state of the knowledge regarding protein degradation in the acid-phase of anaerobic digestion of protein-rich wastewater can be summarized as follows:

(1) From the standpoint of microbiologists: the pathways of protein degradation and some relevant microorganisms have been investigated. In the acid-phase, the degradation of protein proceeds in two steps: protein hydrolysis and amino acid fermentation, of which pathway is dependent on the type of microorganisms present. The ability of the microorganisms to convert protein determines the protein conversion efficiency of the protein-rich wastewater treatment, which is highly related to the abundance of particular protein degrading microorganisms present in the system. Thus, a good knowledge of the microbial composition is important to protein degradation in the acid-phase. However, studies on the microbial composition of anaerobic protein degradation in the acid-phase are relatively limited.

(2) From the standpoint of wastewater treatment engineers: intensive studies have been performed on the protein degradation in the acid-phase of anaerobic digestion of protein-rich wastewater. The topics of these studies are concentrated on the operational conditions, nutritional conditions, and the application of different bioreactor configurations. However, little knowledge is available to link the microbial process performance in protein conversion with the microbial community.

(3) Various techniques that are based on selective culture or molecular biology have been developed to characterize the microbial community. Currently, the mainstream method for analyzing microbial composition in wastewater treatment is 16S rRNA gene-based amplicon sequencing. This method is regarded as a high-throughput molecular technique and can characterize the microbial composition more efficiently and reliably.

Therefore, the microbial composition in the acid-phase of anaerobic protein degradation is of interest to be studied. The viable method can be chosen as 16S rRNA gene-based amplicon sequencing.

2.2. Pathways of protein degradation in the acid-phase

In the acid phase, the degradation of protein proceeds in two steps: protein hydrolysis and amino acid fermentation, of which pathways are dependent on the microorganisms present.

Protein hydrolysis

Proteins are large and complex molecules consisting of amino acid units linked one to another by peptide bonds, which cannot be directly assimilated by microorganisms. In an anaerobic environment, proteins can be hydrolyzed into peptides and amino acids by extracellular enzymes secreted by proteolytic microorganisms (Gujer and Zehnder, 1983; McInerney, 1988).

Amino acid fermentation

Amino acids, the end product of protein hydrolysis, can be further converted into simple molecules: volatile fatty acids, ammonium, H_2, and CO_2 (Barker, 1981; Pavlostathis and Giraldo-Gomez, 1991). The pathways of amino acid fermentation are complex and diverse, which involves oxidation and reduction reactions between one or more amino acids or non-nitrogenous compounds derived from amino acids (Barker, 1981).

Two general pathways of amino acid fermentation have been characterized. One is via Stickland reactions, which are coupled with oxidation-reduction reactions between pairs of amino acids. The other can be denoted as non-Stickland reactions, in which uncoupled amino acids are oxidatively deaminated and decarboxylated to the corresponding carboxylic acids (Barker, 1981; McInerney, 1988). Reaction (1) and reaction (2) shown below are examples of Stickland reaction and non-Stickland reaction, respectively.

$$\text{alanine} + 2\text{glycine} + 3H_2O \rightarrow 3\text{acetate}^- + 3NH_4^+ + HCO_3^- + H^+ \quad (1)$$

$$4\text{glycine} + 4H_2O \rightarrow 3\text{acetate}^- + 2HCO_3^- + 4NH_4^+ + H^+ \quad (2)$$

Reaction (1): alanine and glycine are coupled together to carry out the Stickland reaction, in which alanine serves as the electron donor and glycine functions as the electron acceptor. Reaction (2): the single amino acid glycine is deaminated and converted to acetate.

Non-Stickland reactions sometimes rely on interspecies hydrogen transfers between syntrophic microorganisms and H_2 utilizers. According to the second law of thermodynamics, a reaction can only be feasible when the Gibbs energy change of the reaction under the standard condition is negative. Sometimes positive values can be achieved for theoretical non-Stickland reactions of certain amino acids such as glutamate and leucine (McInerney, 1988), as shown in Table 2.1. However, it was found that such reactions can be carried out when the partial pressure of product H_2 is decreased by the H_2 utilizers like methanogens (Nagase and Matsuo, 1982; Örlygsson, Houwen, and Svensson, 1994).

Table 2.1 Gibb's energy change for the degradation of glutamate and leucine (McInerney, 1988)

Reaction	ΔG° (kJ/reaction)
$glutamate^- + H_2O \rightarrow \alpha\text{-ketoglutarate}^{2-} + NH_4^+ + H_2$	+59.9
$leucine + 3H_2O \rightarrow isovalerate^- + HCO_3^- + NH_4^+ + H^+ + 2H_2$	+4.2

In an anaerobic treatment system of protein-rich wastewater, the amino acids are more likely to be fermented via Stickland reaction between coupled amino acids than non-Stickland reaction due to the diversity of amino acids constituting proteins (Tang et al., 2005). However, the experimental results from a study on casein degradation stoichiometry showed that the non-Stickland reaction that relied on the hydrogen utilizing microorganisms was more preferred than the Stickland reaction (Ramsay and Pullammanappallil, 2001). Hence, this indicates that the pathway of amino acid fermentation is highly related to the microorganisms present. In other words, the type of microorganisms in the protein-rich wastewater treatment system plays an important role in the degradation pathway of proteins.

2.3. Protein-degrading microorganisms

The ability of the microbial community to convert proteins into simpler molecules determines the protein conversion efficiency, which is highly related to the population of particular protein-degrading species. Some isolated protein degraders show versatile features in substrate utilization and enzyme production.

The hydrolysis of proteins in anaerobic digestion is carried out by a specialized group of proteolytic bacteria (McInerney, 1988). The isolated species of proteolytic bacteria from anaerobic digestion sludge, are from genera such as *Clostridium*, *Peptococcus*, *Bifidobacterium, Staphylococcus, Eubacterium*, *Bacteriodes, Butyrivibrio, Fusobacterium, Selenomonas*, and *Coprothermobacter* (Gagliano et al., 2015; Hassan and Nelson, 2012; McInerney, 1988; Siebert and Toerien, 1969).

The fermentation of amino acids is mediated by species from genera such as *Acidaminobacter, Acidaminococcus*, *Clostridium, Campylobacter, Eubacterium*, *Peptostreptococcus*, *Cloacibacillus, and PeptostreptocPCRoccus* (Barker, 1961; Ganesan et al., 2008; Ramsay and Pullammanappallil, 2001; Zindel et al., 1988).

Bacteria from the genus *Clostridium* are the most prevalent protein degrading microorganism found in anaerobic digestion(Siebert and Toerien, 1969), which are responsible for both protein hydrolysis and amino acid fermentation. These bacteria do not necessarily metabolize the same substrate and produce different types of enzymes. Species such as *Clostridium bifermentans* and *Clostridium sporogenes* are both saccharolytic and proteolytic. Species such as *Clostridium ghoni* and *Clostridium subterminale* can only produce proteolytic enzymes. Some species are neither proteolytic nor saccharolytic but can ferment specific amino acids as substrates (Ramsay and

Pullammanappallil, 2001). More information on the metabolism of isolated protein-degrading microorganisms was listed in Appendix A.

In the anaerobic treatment system of protein-rich wastewater, diverse microorganisms are present as mixed-culture. The ability of a mixed-culture to convert various substrates highly depends on the abundance of the particular species present, which can be modified by acclimation. The acclimation of the mixed-culture to a specific substrate can bring changes to the relative microbial population of the mother culture, thus results in a different substrate utilization pattern after acclimation (Gavala and Lyberatos, 2001). Previous studies show that the acclimation step is necessary for protein degradation in the process of anaerobic digestion. Breure *et al.* (1986) reported that in a substrate-limiting CSTR system when biomass was first fed with glucose and switched to gelatin after the steady-state, the biomass was washed out. When the biomass was first fed by glucose and gelatin was added as a second substrate, 30% gelatin was hydrolyzed. When the biomass was firstly acclimated to gelatin and then fed with glucose as a second substrate, 95% of the gelatin was hydrolyzed. Moreover, gelatin-adapted biomass can degrade glucose instantaneously. Perle, Kimchie, and Shelef (1995) also found that the acclimated culture has higher proteolytic activity than the non-acclimated culture when casein fed as the substrate to the culture.

2.4. Operational conditions of the acid-phase

Most studies on protein degradation in the acid-phase were initiated in terms of engineering practice, of which topics mainly focused on the operational conditions. The effects of temperature, pH, hydraulic retention time (HRT), and the nutrients in the feed have been widely investigated and evaluated.

Temperature

An anaerobic process is either conducted under a mesophilic condition (20-45 ℃) or a thermophilic condition (55-65 ℃). A thermophilic condition can accelerate the protein degradation, and a mesophilic condition is more favorable for the stability of the microbial community (Guerrero et al., 1999; Liu et al., 2002; Yu et al., 2002; Yu and Fang, 2003). Guerrero et al. (1999) found a higher efficiency of acidification and protein removal at 55℃ than at 37℃ in the acid phase of fish meal wastewater treatment. But it was also found that high temperature gave rise to the dissociation of ammonium and the release of the free ammonia was detrimental to the methane phase. Yu and Fang (2003) investigated the acidogenesis of synthetic gelatin-containing wastewater over the temperature range of 20℃ to 55℃ in an upflow anaerobic reactor. Experimental results showed that the degree of model protein gelatin degradation increased from 56.4% at 20℃ to 72.6% at 55℃. And the specific degradation rate also increased with temperature, from 0.370 g/gVSS d at 20℃ to 0.443 g/gVSS d at 55℃. Yu et al. (2002) compared the acidification efficiency of synthetic dairy wastewater under a mesophilic (37 ℃) condition and a thermophilic (55℃) condition. It was found that under the same organic loading rate of 4 g COD L-1 d-1, there is no significant difference in the degree of

milk protein degradation for both conditions while the specific degradation rate in the thermophilic condition was greater than the rate in the mesophilic condition. Moreover, a study on the microbial community dynamics during the start-up of acid-phase of dairy wastewater treatment showed that the microbial community change was more significant and rapid in the thermophilic condition than in the mesophilic condition (Liu et al., 2002). It was also pointed out that condition of higher temperature requires heating energy, of which economics need to be considered for the treatment system in practice.

pH

Protein degradation is sensitive to pH variation. The optimum pH range reported for the overall protein degradation in the acid-phase is around 6 to 6.5 although protein hydrolysis favors higher pH at 7 (Breure and van Andel, 1984; Yu and Fang, 2002, 2003). Breure and van Andel (1984) investigated the influence of pH on hydrolysis and acidogenic fermentation of gelatin in carbon-substrate limited chemostat culture. The experimental results showed that the optimum pH for gelatin hydrolysis was at pH 7, while for acidogenesis was at pH 6.3. Yu and Fang (2002) investigated the effect of pH in the range of 4-6.5 on the acidification of synthetic dairy wastewater. The optimum pH for milk protein acidification was found to be 6.5, at which maximum protein degradation extent was reached. Yu and Fang (2003) also explored the effect of pH on gelatin degradation in an upflow anaerobic reactor with both experimental method and model simulation. Based on the experimental results, the optimum pH for hydrolysis of gelatin was found to be at 7, and for acidogenesis was 6.5. The optimum pH calculated by the model was slightly lower than the experimental one, which was at 6 for acidogenesis. What's more, the three aforementioned studies all showed that different pH results in different volatile fatty acids (VFAs) as products of protein acidification. Lower pH range (i.e. pH<5) encourages the production of propionate, whereas the higher pH range (i.e. pH>6) favors the production of acetate and butyrate (Yu and Fang, 2002, 2003).

VFAs, together with the other two main products: ammonium and bicarbonate, play an important role in the acid-phase pH (Hassan and Nelson, 2012). Without pH control, the pH of the acid-phase system is highly related to the interaction among these three buffers and the nature of wastewater (Alexiou et al., 1994). To stabilize the pH of the acid-phase system, the addition of a base such as NaOH and $NaHCO_3$ can be considered (Charalambous et al., 2020).

Hydraulic retention time (HRT)

The optimum hydraulic retention time needed for protein acidification varied from study to study since different types of feed flow and different operational conditions were applied to different acid-phase bioreactors. Breure and van Andel (1984) found that the maximal dilution rate allowing steady-state growth of the microbial culture was 0.23 h^{-1} when gelatin was fed as the only carbon source to a chemostat culture. Since a dilution rate is defined as the inverse of hydraulic retention time (HRT), this means the shortest HRT needed for gelatin degradation in this study is around 4.4 hours. By using a gelatin

solution in the same level of carbon content in the previous study, Breure et al. (1985) found that the extent of acidification of gelatin in an upflow reactor was weakened by decreasing HRT. Experimental results showed that the extent of gelatin acidification was decreased from 97% to 71% when HRT was decreased from 5 h to 0.5 h and the composition of the gelatin fermentation products was independent of the HRT. Guerrero et al. (1999) investigated the optimum HRT for acidification of fish meal wastewater in continuous stirred tanks by trying HRT in a range of 6 h to 48 h. The maximum protein removal was achieved as 80% at 55℃ and HRT of 24 h. But the maximum protein removal rate took place at HRT of 6 h. Fang and Yu (2000) operated an upflow reactor at six HRTs ranging from 4 h to 24 h to explore the effect of HRT on the acid-phase of dairy wastewater in a condition of pH 5.5 and 37 ℃. The COD concentration of the dairy wastewater used for this study was 4000 mg/L, in which proteins accounted for 24%. Results showed that the extent of protein acidification substantially increased with HRT from 57% at 4 h to 86% at 24 h. But when the HRT applied longer than 16 h, the increase of the acidification degree with time became slight. What's more, among the three major constituents of dairy wastewater, carbohydrates were found to have higher conversion efficiency than proteins and lipids at all HRTs.

Nutrients in the feed

Protein-rich wastewater is complex in the composition, of which components can be nutrients for microorganism growth. For example, dairy wastewater contains proteins, carbohydrates, lipids, phosphorus, and trace elements, which are the source of macro- and micro-nutrients for microorganisms (Demirel et al., 2005). The effects of the protein itself, carbohydrates, lipids, and trace metals on anaerobic protein degradation in the acid-phase have been investigated in previous studies.

Protein itself has been reported to be one of the limiting factors for protein degradation. The more soluble proteins are, the easier proteins can be degraded (McInerney, 1988; Yu and Fang, 2002). Fang and Yu (2002) also studied the influence of protein concentration on protein degradation by applying gelatin as model protein under different concentrations. Their results showed that the extent of gelatin degradation decreased with the increase of gelatin concentration in the influent.

Carbohydrates are reported to be preferentially degraded over proteins in the anaerobic process of protein-rich wastewater (Hassan and Nelson, 2012; Yu and Fang, 2001). Breure et al., (1986b) observed that the presence of glucose could progressively retard the degree of gelatin degradation with the increase of glucose concentrations in the feed and this inhibition effect was not caused by the production of VFAs. Even when biomass was pre-acclimated to gelatin, gelatin fermentation was still inhibited by the addition of glucose as a second substrate (Breure et al., 1986a). Tommaso et al. (2003) observed that the overall degradation extent of bovine serum albumin (BSA) was not significantly affected by starch and glucose as additional substrates, but the initial degradation rate of

BSA can be slowed down. Unfortunately, as stated in Chapter 1, the mechanism behind the effect of carbohydrates on protein degradation in these studies is not clarified.

Lipids are found to have inhibitory effects on the overall biomass activity, also leading to a negative effect on protein degradation (Perle et al., 1995). It is argued that the adsorption of lipid to the surface of the biomass can result in the limitation of the transport of soluble substrates to the cell and thus the conversion rate of other substrates is reduced (Sayed et al., 1988; Vidal et al., 2000).

In engineering practice, carbohydrates, proteins, lipids, together with other organic compounds present in the wastewater, are termed as chemical oxygen demand (COD). The COD volumetric loading rate is an important operational parameter for anaerobic digestion design (van Lier et al., 2008), which should be wisely chosen for protein-rich wastewater treatment when considering the aforementioned inhibitory effects.

Trace metals, Co and Ni, are found to affect the pathways of protein degradation (Tang et al., 2005). Tang et al. (2005) compared the microbial community structure for BSA degradation under the condition with and without trace metal addition. Results suggested that the addition of Ni and Co favored the growth of archaeal microorganisms, which resulted in different pathways of protein degradation as well as degradation products. Other elements such as P, Na, K, Ca, Mg, Mn, Cu, Zn, and Fe, that are also reported to be present in the protein-rich wastewater (Demirel et al., 2005), are identified as nutrients for anaerobic microorganisms when the concentration of each element is in a range that is unlikely to affect adversely to the microbial growth (Hendriks et al., 2018).

2.5. Acid-phase bioreactor configuration

Different configurations of reactors have been developed and applied to the anaerobic digestion of protein-rich wastewater in both laboratory-scale and full-scale. However, for acid-phase only, there are fewer types of bioreactors have been reported.

CSTR (continuous-flow stirred tank reactor) and upflow reactors, which are easier to operate, are commonly used in laboratory-scale studies on the acid-phase of protein-rich wastewater digestion (Demirel et al., 2005; Ince, 1998; Yu et al., 2002; Yu and Fang, 2003). Other bioreactor configurations such as anaerobic membrane bioreactor (AnMBR) and packed-bed bioreactor, although are not common, have also been used (Lee et al., 2001; Tommaso et al., 2003). Lee et al. (2001) tried submerged membranes in the acid phase of anaerobic digestion of piggery wastewater. Experimental results showed that the application of membrane could increase the sludge retention time of acidogens, which enhanced the acidification and resulted in a BOD removal above 90% and COD removal of 80% for the overall anaerobic treatment system ended with methane-phase. Tommaso et al. (2003) successfully used a horizontal-flow anaerobic immobilized biomass(HAIB) reactor as a plug-flow reactor to prove the negative influence of carbohydrates and lipids on protein degradation. The problem with HAIB in that study

was that the reactor failed to expel the biogas promptly and caused the accumulation of biogas in the reactor.

In full-scale applications, continuously stirred tanks are the most reported reactors for the acid-phase (Demirel et al., 2005; van Lier et al., 2008). High-rate anaerobic bioreactors such as anaerobic filters, expanded granular sludge beds (EGSB), and upflow anaerobic sludge blankets (UASB), which can uncouple the biomass retention and liquid retention, have been widely used for anaerobic digestion of protein-rich wastewater (Karadag et al., 2015; van Lier et al., 2008; Vidal et al., 2000). But most high-rate bioreactors are applied either as a two-phase reactor or methane-phase reactor for the whole system, not for acid-phase.

There is a controversy over the reactor design that whether the acid-phase is necessary to be separated from the methane-phase in the anaerobic digestion process. Some researchers argued that a separate system is more favorable for the conversion of organic materials and biogas production due to the separation of fermentative microorganisms and methanogens, which are different in their growth requirements and growth kinetics (Demirel and Yenigün, 2002; Jeyaseelan and Matsuo, 1995). Some researchers reported that not complete acidification but partial acidification in a separate acid-phase is beneficial to the overall COD removal for protein-rich wastewater treatment (Göblös et al., 2008; Ince, 1998; Yang et al., 2003). With the wide application of granule sludge bed reactors in full-scale, it is argued that a one-stage process that brings acid-phase and methane-phase together favors the formation of granule sludge (van Lier et al., 2008). Besides, it is also argued that the break-down of proteins leads to the production of ammonia, which is detrimental to the biogas production in the methane-phase (Yenigün and Demirel, 2013). Considering that the largest extent of protein conversion and biogas production are both desired in anaerobic digestion of protein-rich wastewater, more microbial information about protein degradation in acid-phase is in need to help answer whether a separate acid-phase is necessary for the treatment of this type of wastewater.

2.6. Techniques for microbial community composition analysis

In this section, several techniques that have been developed and used in the microbial community analysis in wastewater treatment are introduced. Among all available techniques, the high-throughput molecular technique of 16S rRNA gene-based amplicon sequencing is the mainstream approach to characterize microbial communities from the wastewater treatment system. Two important parameters, identity, and abundance of the microorganisms can provide qualitative and quantitative information of the microbial community, respectively.

Early efforts to define microbial community structure are culture-dependent methods, whereby valuable information to characterize isolated strains is provided. The culture-dependent method is also called enrichment culture, which involves successive exposure

and dilutions in a selective growth media and can lead to the isolation of a single strain. However, the culture-based technique has the following limitations: first, it requires pre-existing knowledge of the nutritional and environmental conditions that can enrich for the target microorganisms; second, it overlooks the microorganisms that cannot be cultured by the growth media and thus fails to accurately reflect the actual microbial community structure. Third, it requires laborious experimental work (Rittmann and McCarty, 2001; Theron and Cloete, 2000).

Subsequently, staining methods such as non-specific fluorescent dye technique and fluorescent antibody technique have been developed, which allow the direct identification and enumeration of individual bacteria in the environmental samples with microscopy. However, these methods also have drawbacks. First of all, they require information acquired from a pure-culture of the target microorganism. More importantly, due to the limitation of the resolution, the microscopy may overlook microorganisms that are hard to be recognized with light microscopy and thus fail to reflect the diversity of the microbial community (Madigan et al., 2012; Theron and Cloete, 2000).

The development of molecular biology tools has provided a chance to overcome the shortcomings of the aforementioned methods and identify the microbial community structure by directly targeting the genetic information of microorganisms. The most common approach is to target the small-subunit ribosomal RNA (SSU rRNA, also known as 16S rRNA) or the gene codes for it (Boon et al., 2002; Gadow et al., 2013). The use of fluorescence in situ hybridization (FISH) or dot blotting with 16S rRNA-directed, tailor-made oligonucleotide probes can identify the presence and estimate the relative abundance of the microorganisms of interest in the samples (Liu et al., 2002; Tang et al., 2005). These methods are only suitable for microorganisms that have been isolated and sequenced. Similarly, the 16S rRNA gene is also available for identifying and quantifying microorganisms. The major steps are DNA extraction, polymerase chain reaction (PCR) amplification, denaturing gradient gel electrophoresis (DGGE), cloning, DNA sequencing, and analysis (Madigan et al., 2012). DNA extraction can be carried out by available commercial kits (Parameswaran et al., 2010; Shin et al., 2010a). PCR amplification needs primers to target the microbial group. A primer is a short single-stranded nucleic acid utilized by all living organisms in the initiation of DNA synthesis, it can be chosen as domain-level such as Bacteria or specific for a strain (Rittmann and McCarty, 2001). DGGE can visualize the target gene in DNA bands (called fingerprints) whether or not the target microorganisms have been isolated and sequenced (Rittmann and McCarty, 2001). Via the fingerprint, the identity and number of the phylotypes in the sample can be analyzed (Boon et al., 2002). Molecular cloning can sort out the phylotypes of interest before sequencing (Madigan et al., 2012). The sequences can be compared to the reference sequence database, based on which a phylogenetic tree can be constructed (Tang et al., 2005). The quantitative aspects of the microbial community can be determined by operational taxonomic units (OTU) assignment based on the sequencing of 16S rRNA

clone library (Rivière et al., 2009; Tuan et al., 2014). What's more, the quantification of the specific microorganism can be carried out by real-time quantitative polymerase chain reaction (qPCR) (Shin et al., 2010b; Tang et al., 2005). Although these molecular techniques have provided invaluable information, they are low-throughput and have limitations in determining rare species and discriminating closely related members of certain taxa, and thus fall short to perform thorough taxonomic profiling (Hao et al., 2014).

In recent years, high-throughput molecular techniques such as DNA chips, meta-omics, and amplicon sequencing, are gaining interest in the analysis of microbial community (Franzosa et al., 2015; Madigan et al., 2012). DNA chips are one of the microarray methods integrated with specific rRNA probes or rRNA gene-targeted oligonucleotide probes together. Thousands of taxa have been identified with a phylochip (a phylogenetic microarray) in a single sample from the wastewater treatment reactor (Xia et al., 2010). The meta-omics refer to metagenomics, metatranscriptomics, metaproteomics, and metabolomics, which enable not only the phylogenetic composition but also the functional potential of the microbial community (Vanwonterghem et al., 2014). The metagenomics technique, whereby all the DNA or expressed genes (mRNA) from a certain community can be sequenced, has been successfully used for community structure analysis of complex environmental samples from anaerobic digestion (Guo et al., 2015; Kovács et al., 2013). Amplicon sequencing is regarded as one of metagenomics to perform phylogenetic or functional profiling even though only one or a few microbial genes are considered (Vanwonterghem et al., 2014). The DNA chips and meta-omics require more skills in molecular biology and bioinformatics. From the standpoint of environmental engineers, 16S rRNA gene-based amplicon sequencing is recommended in the wastewater treatment field (Loosdrecht et al., 2016).

As a whole, the high-throughput molecular techniques of 16S rRNA gene-based amplicon sequencing is the mainstream approach for wastewater microbiology. The microscopy-based methods can be used for imaging the community to obtain morphologic information. Phylogenetic and metabolism information of isolated microorganisms acquired by previous culture-dependent techniques can be a good supplementary to characterize the species of interest.

Chapter 3. Research scope

3.1. Research objective

Based on the background information illustrated in Chapter 1 and the state of the knowledge illustrated in Chapter 2, the **research objective** of this MSc study is:
to investigate the relationship between microbial composition and the negative effect of carbohydrates on anaerobic protein degradation in the acid-phase.

3.2. Research questions and hypothesis

Based on the research objective, the **main research question** is:
How is microbial composition related to the negative effect of carbohydrates on anaerobic protein degradation in the acid-phase?

From literature study, it has been known that the microorganisms in the anaerobic wastewater treatment system are present as mixed-culture. It also has been known that protein degraders do not necessarily utilize protein as the only carbon source for metabolism. Therefore, in this research, we assume the microbial populations responsible for the degradation of proteins and carbohydrates in the acid-phase of anaerobic digestion can be categorized into two groups. One group is generalist, which can utilize not only proteins but also carbohydrates for metabolism. The other group is specialist, which can utilize proteins only or carbohydrates only for metabolism.

From a competitive point of view, the change of the main populations from specialists to generalists due to the substrate shift from proteins to mixtures of proteins and carbohydrates might lead to the negative effect of carbohydrates on anaerobic protein degradation in the acid-phase.

Therefore, the **sub-questions** and **hypotheses** can be derived as follows:
Sub-question 1: What are the predominant populations of the mixed-culture in an anaerobic acid-phase reactor fed with proteins as the sole carbon source?
Hypothesis 1: Protein specialists (i.e. microorganisms that can grow only on proteins) outgrow other microbial populations and become predominant in the mixed-culture of an anaerobic acid-phase reactor fed with proteins as the sole carbon source.
Sub-question 2: What happens to the predominant populations of the mixed-culture in an anaerobic acid-phase reactor when carbohydrates present as an additional carbon source?
Hypothesis 2: The generalists (i.e. microorganisms that can both grow on proteins and carbohydrates) outgrow the specialists and become predominant populations when carbohydrates present as an additional carbon source.
Sub-question 3: How does the predominant populations of the mixed-culture in an anaerobic acid-phase reactor utilize the carbon substrates when carbohydrates present as an additional carbon source?
Hypothesis 3: The predominant generalists preferentially utilize carbohydrates over proteins.

Chapter 4. Material and methods

4.1. Experiment design

This study was carried out by comparative experiments involving two bioreactors (named R1 and R2) fed with different carbon substrates in two experimental stages, as shown in Figure 4.1. In experimental stage I, R1 was fed with proteins as the sole carbon source and R2 was fed with carbohydrates as the sole carbon source. When both R1 and R2 displayed stable performance (i.e. acidification degree varied within a limited range of 10%), the carbon source was changed from a single substrate to a mixture of proteins and carbohydrates, signifying the start of experimental stage II. The performance of two reactors, in two distinct experimental stages, were evaluated in the VFA product spectrum, acidification degree, deamination degree. The biomass in two reactors was sampled and analyzed in the aspects of microbial composition and protease activity.

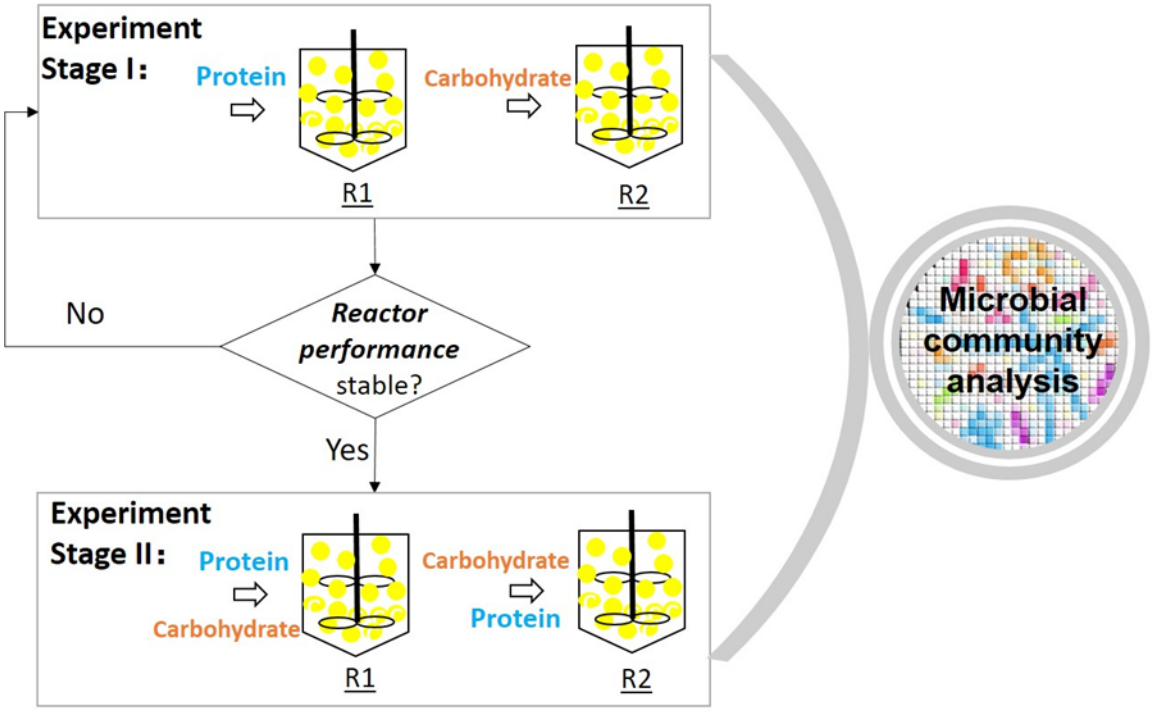

Figure 4.1. Experiment design

4.2. Materials

4.2.1. Inoculum

The anaerobic mixed-culture used as inoculum was a flocculent sludge solution taken from a dairy wastewater treatment plant from Poland. The average content of volatile suspending solids (VSS) of this sludge is 14 g per kg wet weight.

4.2.2. Feedstock

In this study, casein (CAS 9000-71-9, Fisher Scientific) and lactose (CAS 5989-81-1, Sigma Aldrich) was chosen as the model protein and model carbohydrate, of which chemical structure is shown in Figure 4.2. Three formulas of feedstock were prepared for feeding the bioreactors in two different experiment stages (feedstock A and B for stage I, feedstock C for stage II), as shown in Table 4.1, Table 4.2, and Table 4.3, respectively. Feedstock A had casein as the only carbon source. Feedstock B contains lactose as the only carbon source. Feedstock C contained both casein and lactose as the carbon sources. The contents of carbon source in terms of COD concentration in the aforementioned three formulas of feedstock was 6 g COD/L. The concentrations of ammonium chloride in the three formulas were 450 mg/L, 3298 mg/L, and 1873 mg/L, respectively, to maintain a COD: N ratio of 7:1. Sodium bicarbonate was added as a buffer for the pH control in the bioreactors. Besides, to make sure the metabolism of microorganisms was unlimited by nutrients, other macro-and micro-nutrients such as P, K, Na, Ca, Mg, and Fe were added, of which concentration following the protocol of growth media for anaerobic fermentation process proposed by Hendriks et al. (2018). Besides, a concentrated micronutrient solution, Vithane (Biothane, Netherland), was also added as the source of elements such as Co, Ni, Zn, Cu, Mn, Mo, Se, B, and S.

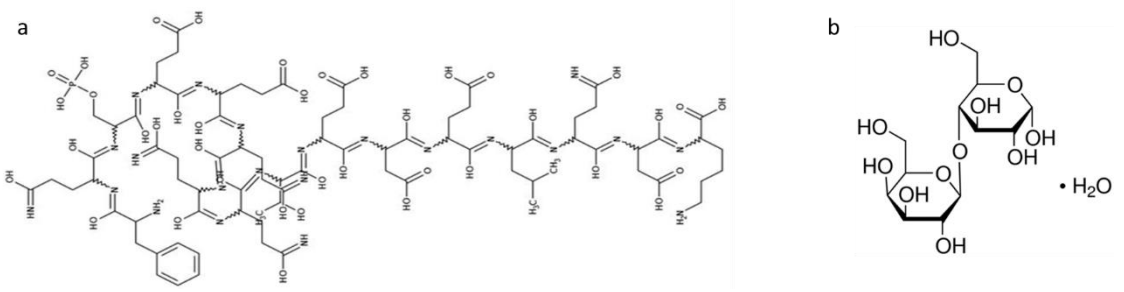

Figure 4.2. Model protein - casein (a) and model carbohydrate - lactose (b)

Table 4.1. Composition of feedstock A

Nutrient	Formula	Conc. [mg/L]	Purpose	Remarks
Casein	$C_{81}N_{22}H_{125}O_{39}P$	4989	Source of C, N, P	6 g/L COD, 745 mg TN/L
Ammonium Chloride	NH_4Cl	450	Source of N	118 mg TN/L
Monosodium Phosphate	$NaH_2PO_4{\cdot}H_2O$	130	Source of P	
Sodium Chloride	NaCl	28	Source of Na	
Potassium Chloride	KCl	86	Source of K	
Calcium Chloride	$CaCl_2{\cdot}2H_2O$	15	Source of Ca	
Magnesium Chloride	$MgCl_2{\cdot}6H_2O$	4.06	Source of Mg	
Iron(II) Sulfate	$FeSO_4{\cdot}7H_2O$	4.929	Source of Fe	
Sodium Bicarbonate	$NaHCO_3$	500	Buffer	
Vithane (Biothane, Netherlands)	-	1.5	Source of Co, Ni, Zn, Cu, Mn, Mo, Se, B, S	

Table 4.2. Composition of feedstock B

Nutrient	Formula	Conc. [mg/L]	Purpose	Remarks
α-Lactose monohydrate	$C_{12}H_{22}O_{11}\cdot H_2O$	5630	Source of C	6 g/L COD
Ammonium Chloride	NH_4Cl	3298	Source of N	863 mg TN/L
Monosodium Phosphate	$NaH_2PO_4\cdot H_2O$	130	Source of P	
Sodium Chloride	NaCl	28	Source of Na	
Potassium Chloride	KCl	86	Source of K	
Calcium Chloride	$CaCl_2\cdot 2H_2O$	15	Source of Ca	
Magnesium Chloride	$MgCl_2\cdot 6H_2O$	4.06	Source of Mg	
Iron(II) Sulfate	$FeSO_4\cdot 7H_2O$	4.929	Source of Fe	
Sodium Bicarbonate	$NaHCO_3$	2000	Buffer	
Vithane (Biothane,Netherland)	-	1.5	Source of Co, Ni, Zn, Cu, Mn, Mo, Se, B, S	

Table 4.3. Composition of feedstock C

Macronutrient	Formula	Conc. [mg/L]	Purpose	Remarks
Casein	$C_{81}N_{22}H_{125}O_{39}P$	2495	Source of C, N, P	3 g/L COD, 373 mg TN/L
α-Lactose monohydrate	$C_{12}H_{22}O_{11}\cdot H_2O$	2815	Source of C	3 g/L COD
Ammonium Chloride	NH_4Cl	1873	Source of N	490 mg TN/L
Monosodium Phosphate	$NaH_2PO_4\cdot H_2O$	130	Source of P	
Sodium Chloride	NaCl	28	Source of Na	
Potassium Chloride	KCl	86	Source of K	
Calcium Chloride	$CaCl_2\cdot 2H_2O$	15	Source of Ca	
Magnesium Chloride	$MgCl_2\cdot 6H_2O$	4.06	Source of Mg	
Iron(II) Sulfate	$FeSO_4\cdot 7H_2O$	4.929	Source of Fe	
Sodium Bicarbonate	$NaHCO_3$	1750	Buffer	
Vithane (Biothane, Netherland)	-	1.5	Source of Co, Ni, Zn, Cu, Mn, Mo, Se, B, S	

4.3. Methods

4.3.1. Experimental set-up

Two parallel experimental set-ups were operated for comparison, as shown in Figure 4.3. Each set-up comprised a bioreactor, a feedstock tank, a drainage tank, two peristaltic pumps, and a base dosing unit. Both bioreactors had a total working volume of 3.5 L and a working volume of 2L, water-jacketed, and was equipped with a mixer and probes for pH measurement. Each bioreactor was connected to a feedstock tank, a drainage tank, and peristaltic pumps used for feeding and extracting. A gas bag with a volume of 2 liters was connected to the bioreactor for biogas collection. The feedstock tanks were stored in the fridge where the temperature was controlled under 4 °C to minimize the microbial activity. The pH in the bioreactors was maintained by the base dosing units, each of which comprised of a base tank and a base dosing pump. The base dosing pumps were connected to a pH controller (Hach sc 200, USA) for the automatic addition of a base solution to the bioreactors.

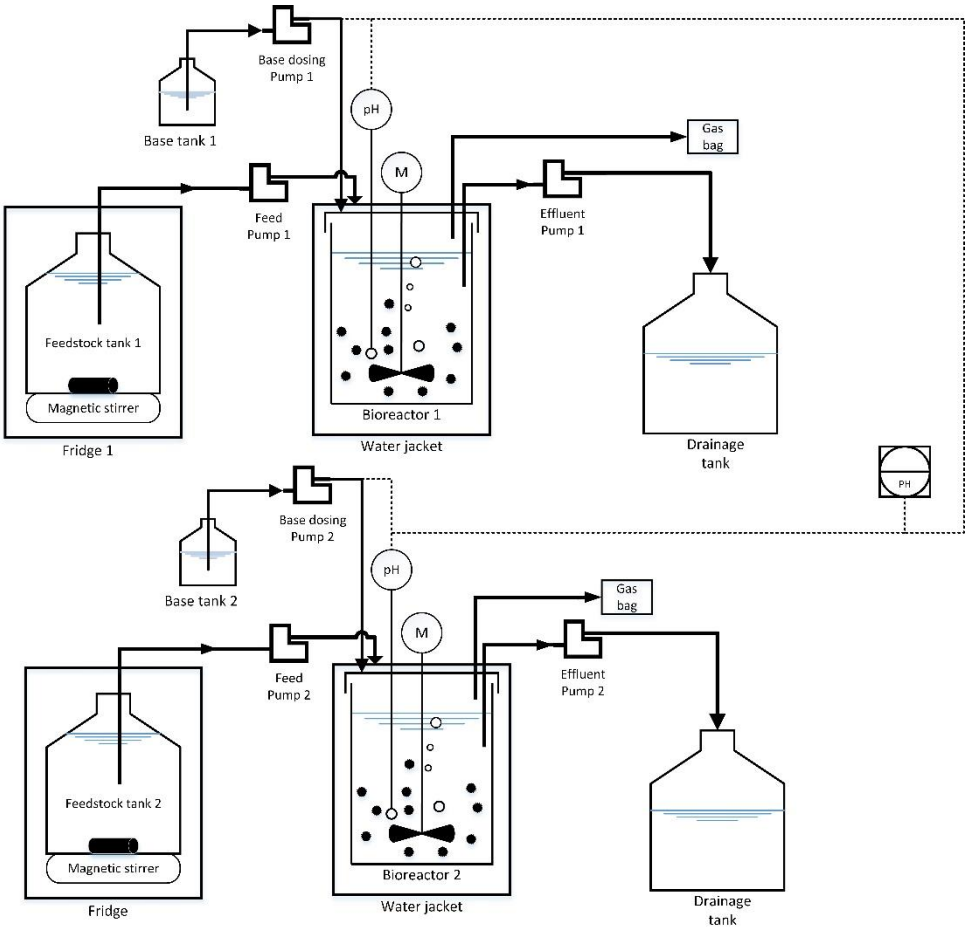

Figure 4.3. Scheme of the experimental set-up

4.3.2. Start-up and operation

As a start-up, approximately 140 mL sludge and 1860 mL feedstock were added to each bioreactor, resulting in an initial biomass concentration of 1g VSS/L. Both reactors were sparged with nitrogen gas to displace air for an anaerobic condition. In the first 5 days, the reactors were continuously mixing but no effluent was extracted, thus achieving the initial acclimation of the inoculum.

After 5 days, the operation of reactors was switched to pulse feeding mode. The pulse feeding mode was performed by periodically feeding the bioreactors with a cycle time of 7.5 minutes, 192 cycles per day. During the first 0.1 minutes of each cycle, the reactors were fed and effluent was extracted at a flow rate of 100 mL/min, followed by a 7.4-minute reaction (i.e., no feeding or extraction). The parameters of the operational conditions were summarized in Table 4.4.

Table 4.4. Operational conditions of bioreactors

Feeding mode	Pulse (0.1min feeding/7.5min cycle)
Bioreactor configuration	Continuously stirred tank
Mechanical stirring rate	50 rpm
Liquid volume in the reactor	2 L
Temperature	Ambient (25±3℃)
pH	6±0.2
HRT	24 h
Average feed flow rate	2 L/day
Pulse feed flow rate	100 mL/min
OLR	6 g COD/(L· day)

The bioreactor configuration chosen was a continuously stirred tank, where inflow and outflow were controlled under the same flow rate to obtain a stable liquid volume of 2 L. Both reactors were well-mixed by mechanical stirring at a stirring rate of 50 rpm. The HRT was controlled at 24 hours for both reactors, which resulted in an average feed flow of about 2L/day and an organic volumetric loading rate (OLR) of 6 g COD/(L·day).

The pH of both bioreactors was maintained at pH 6±0.2 by automatic titration with 0.5 M NaOH solution. The temperature was controlled at 25±3°C by recirculating water from thermocirculator (Thermo Haake DC 10) through the water jacket.

Both R1 and R2 were operated for 131 days, of which 84 days for stage I and 47 days for stage II. In experiment stage I, R1 was fed by feedstock A (casein only as carbon substrate) and R2 was fed by feedstock B (lactose only as carbon substrate). In experiment stage II, both reactors were fed by feedstock C. The operational schemes were displayed in Appendix D.

4.3.3. Physicochemical analysis

COD

TCOD and SCOD of feed and effluent samples were analyzed by using Hach Lange COD kits (LCK014, 1000-10000 mg/L), Hach Lange thermoreactor (Hach, model: LT 200), and Hach Lange spectrometer (Hach, model: DR 3900). For SCOD, samples were centrifuged at 13300 rpm for 5 minutes and filtered through a 0.45 μm filter (Agilent Technologies) before measurement. The procedures of the measurements were carried out by following the manufacturer's instructions.

Nitrogen

The nitrogen related parameters (TKN, SKN, and TAN) were measured by the Kjeldahl method (APHA, 2017). For SKN and TAN, samples were centrifuged at 13300 rpm for 5 minutes and filtered through a 0.45 μm filter (Agilent Technologies) before measurement.

Solids

Solids (TSS and VSS) of feed and effluent samples were measured by following the standard method (APHA, 2017).

Volatile fatty acids(C2-C6) and alcohols

Each sample was filtered with a 0.45 µm filter (Agilent Technologies) before measurement, and 0.75 mL filtrate was diluted with 0.75 mL internal standard solution in a 1.5 mL vial. The internal standard solution was pentanol at a concentration of 300 mg/L. 10 µL formic acid (98%) was also added to the vial to acidify the samples. The prepared samples then were analyzed with a gas chromatography (GC, model: 7890A, Agilent Technologies), equipped with a flame ionization detector (FID) using a capillary HP-FFAP column (Agilent 19091F-112, 25m x 320µm x 0.5µm). The temperature of the injector and detector were set at 80 °C and 240 °C, respectively. Helium was used as the carrier gas at a flow rate of 67 mL/min with a split ratio of 25:1. The temperature of the GC oven was set as follows: starting at 80 °C and increasing to 180 °C in 10.5 min.

Gas composition

The composition of the gas produced from reactors was analyzed by a gas chromatograph (GC, model: 7890A, Agilent Technologies) equipped with a thermal conductivity detector (TCD) using HP-PLOT Molsieve column (Agilent 19095P-MS6, 30m × 0.53mm ID). The temperature of the inlet and the detector were both set at 200 °C. Helium was used as the carrier gas at a flow rate of 23 mL/min. The temperature of the GC oven was set as follows: 1) starting at 45°C and holding for 6 min; 2) increasing to 100 °C at 25°C/min.

4.3.4. Microbial analysis

Protease activity

The protease activity was analyzed by using Pierce™ Fluorescent Protease Activity kit (Thermo Scientific, IL, USA), with contents of FTC-Casein, TPCK Trypsin, and BupH™ Tris-buffered saline. Considering the potential interference of the solids, both mixed liquor samples and supernatant samples (centrifuged at 13300 rpm) were prepared. 2 mL sample was prepared and diluted for 4 times before measurement. 100 µL diluted sample was mixed with 100 µL FTC-Casein working reagent (10 µg/mL FTC-Casein in 25mM Tris, 0.15M NaCl, pH 7.2 assay buffer) in an assay plate (Pierce White Opaque 96-Well Plates). The assay plate was incubated for 2 h at room temperature, away from light. During the incubation, the fluorescence was read by a fluorimeter (FLUOstar, galaxy) with a fluorescein filter set of 485 nm excitation wavelength and 520 nm emission wavelength. Each assay was repeated three times, based on which the mean, as well as the standard deviation, were calculated. The assay of each sample was calculated as BAEE[1] units per mL, calibrated by the fluorescence of reference protease Typrsin

[1] One BAEE unit will produce a ΔA_{253nm} of 0.001 per minute with BAEE as substrate at pH 7.6 at 25℃ in a reaction volume of 3.2 ml.

concentration in BAEE units per mL (14000 BAEE units/L equivalent to 50 mg/L Trypsin). A calibration curve involving a linear relation between fluorescence and Typrsin's BAEE unit can be found in Appendix C.

Morphology

The biomass samples were observed by a light microscope (Olympus, model: BX51 TRF, Japan) of which lens with magnification ranging from 4x to 100x. The images of the biomass were captured by a digital camera (Olympus, model: DP73, Japan), which can reveal the physical structure, size, and shape of the microorganisms.

DNA extraction

Biomass samples were obtained after centrifuging the mixed liquor samples at 13300 rpm for 5 min and discarding the supernatant and stored at −20°C before DNA extraction. The genomic DNA of each biomass sample was extracted by using the commercial DNA extraction kit (mpbio laboratories, USA) following the manufacture's instruction. Extracted DNA samples were checked in quantity with the Qubit dsDNA HS Assay Kit and the Qubit 3 Fluorimeter (Thermo Fisher Scientific, U.S.). The samples of which concentration larger than 20 ng/μL were stored at – 80°C until further analysis.

16S rDNA amplicon sequencing

The extracted DNA samples were packed in dry ice and sent for 16S rDNA amplicon sequencing at a commercial company (Novogene, UK). The primer chosen for amplification was the universal primer set 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) -806R (5′-GGACTACHVGGGTWTCTAAT-3′), targeting the V4 region of the 16S rRNA gene. All polymerase chain reactions (PCR) were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed with 1X loading buffer (contained SYB green) in the same volume for electrophoresis operation on 2% agarose gel. Samples detected with the bright main strip between 400bp-450bp during electrophoresis operation were taken as qualified for further sequencing. The qualified PCR products were further mixed at equal density ratios and were purified with the Qiagen Gel Extraction Kit (Qiagen, Germany) before sequencing. Sequencing libraries were generated by using NEBNext® UltraTM DNA Library Prep Kit for Illumina, quantified by Qubit and quantitative-PCR, and analyzed on an Illumina pair-end platform.

OTU identification and taxonomic annotation

The paired-end raw sequencing data were quality filtered and merged to obtain clean data and further performed with chimera removal to obtain effective data for operational taxonomic units (OTUs) clustering, which was performed by Uparse software (Uparse v7.0.1001). Sequences with a similarity ≥97% were assigned to the same OTUs. Representative sequence for each OTU was screened for species annotation by using Mothur software against the SSUrRNA database of the SILVA Database. The top 10 taxa in the different taxonomic ranks were selected to calculate the relative abundance. The similarity among all the samples was analyzed by a hierarchical clustering method called

the unweighted pair-group method with arithmetic mean (UPGMA). Samples with high similarity were clustered together and displayed in a cluster tree.

4.3.5. Calculations

Acidification degree

The acidification degree, indicating the conversion of carbon substrate into VFAs(C2-C6) on a COD basis, was calculated from the concentration of total COD in the feed and VFA in the effluent, on the COD basis, as shown in equation 3.1 below:

$$acidification\ degree(\%) = \frac{\sum COD\ of\ VFAs\ in\ the\ effluent\ (mg/L)}{total\ COD\ in\ the\ feed\ (mg/L)} \cdot 100 \qquad \text{(eq.3.1)}$$

The conversion factors (gCOD/gVFA) for calculating the COD of VFAs are listed in Appendix B.

Deamination degree

The deamination degree, more specifically, the conversion of protein nitrogen into ammonium nitrogen, was calculated from the concentration of TAN in the feed and effluent, as shown in equation 3.2 below:

$$deamination\ degree(\%) = \frac{TAN\ in\ the\ effluent(mg/L) - TAN\ in\ the\ feed(mg/L)}{TKN\ in\ the\ \text{feed}\ (mg/L) - TAN\ in\ the\ feed(mg/L)} \cdot 100 \qquad \text{(eq.3.2)}$$

Chapter 5. Results

5.1. VFA product spectrum

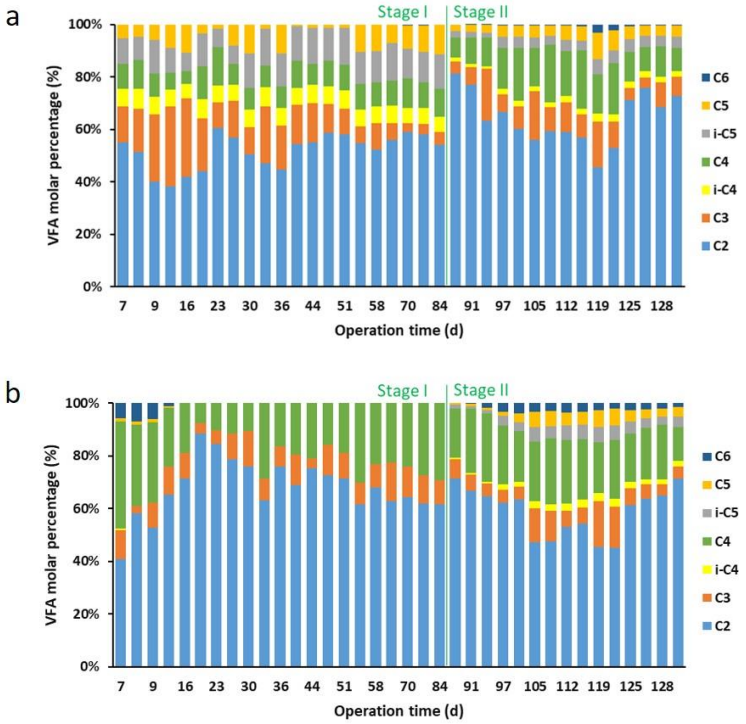

Figure 5.1. VFA product spectrum (in molar percentage) of R1(a) and R2 (b); The green line set at day 86 is to make a distinction between stage I and stage II; in stage I, R1 was fed with proteins and R2 was fed with carbohydrates; in stage II, both reactors were fed with a mixture of proteins and carbohydrates.

As can be seen from Figure 5.1a, the VFA spectrum of R1 during stage I displayed some fluctuations in the molar percentage of each VFA product during the first 51 days, after which, a relatively stable VFA distribution was observed till the end of stage I. The main VFA products, identified from day 64 to the end of stage I and in the order of their molar fraction ranging from the high to the low, were acetic acid (57%), iso-valeric acid (12%), n-butyric acid (10%), n-valeric acid (9%), iso-butyric acid (6%), and propionate (5%). Caproic acid was a minor product, which was only detected in two runs and was with a molar percentage of less than 1% when detected.

As shown in Figure 5.1b, R2 displayed two distinct profiles in the VFA composition during stage I. More VFA types were detected during the first 14 days, whereafter only acetic acid, propionate, and n-butyric acid was identified. A relatively stable VFA distribution was observed from day 64 to day 84 when the most abundant acid was acetic acid (63%) and its relative standard deviation of the molar fraction was less than 4%. From day 64 to day 84, n-butyric acid and propionate were identified as the second and third abundant acids with an average molar percentage of 26% and 11%, respectively.

As for stage II, a big change in the VFA spectrum was observed in R2, where more types of VFA product, ranging from C2 to C6, were found in R2 throughout stage II. Although the VFA product type in R1 from stage II was almost as same as in stage I, small changes in the molar percentage of each VFA in R1 were observed. A relatively stable VFA distribution was observed from day 125 in stage II when the average molar percentage values of acetic acid as dominant VFA in both reactors were over 65%, varied with a relative standard deviation less than 7%. The second and third abundant VFA in both reactors was found to be n-butyric acid and propionate. The other VFAs such as iso-butyric acid, n-valeric acid, iso-valeric acid, and caproic acid was also detected in both reactors, each with a molar percentage less than 5%.

5.2. Deamination degree

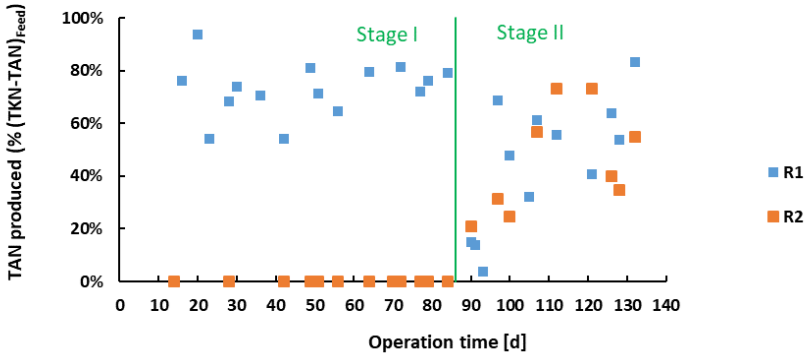

Figure 5.2. Deamination degree in R1 and R2; The green line set at day 86 is to make a distinction between stage I and stage II; in stage I, R1 was fed with proteins and R2 was fed with carbohydrates; in stage II, both reactors were fed with a mixture of proteins and carbohydrates.

In stage I, no protein but only ammonium was added as a nitrogen source to R2, and accordingly, no deamination occurred in R2. For R1, proteins were fed as the sole carbon substrate, and ammonium was added as a nutrient source. As shown in Figure 5.2, during the first 64 days, R1 displayed some fluctuations in deamination degree, whereafter a stable trend was observed. The average deamination degree from day 70 to day 84 was calculated as 77%, with a relative standard deviation of less than 5%.

In stage II, when proteins and carbohydrates were both fed to two reactors, an overall growing trend of deamination degree in R2 was observed in Figure 5.2, with deamination degree ranging from 0% to 73%. Meanwhile, the deamination degree of R1 was observed to have a steep drop from 77% to 15% at the beginning of stage II. Subsequently, a general growing trend with small fluctuations was observed till the end. The deamination degree of R2 in stage II ranged from 4% to 83%.

5.3. Acidification degree

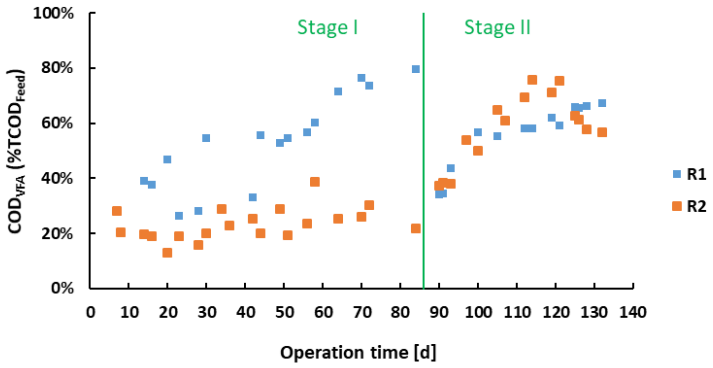

Figure 5.3. Acidification degree of R1 and R2; The green line set at day 86 is to make a distinction between stage I and stage II; in stage I, R1 was fed with proteins, and R2 was fed with carbohydrates; in stage II, both reactors were fed with a mixture of proteins and carbohydrates.

Looking at the results of stage I showed in Figure 5.3, fluctuations in the degree of acidification were observed during the first 64 days. From day 64 to day 84, the acidification in R1 was observed to be relatively stable, with an average acidification degree of 75% and a relative standard deviation of less than 5%. By contrast, R2 displayed fewer fluctuations than R1 throughout stage I. From day 64 to day 84, the acidification in R2 stabilized at around 26% and the relative standard deviation was less than 15%.

Regarding the results of stage II shown in Figure 5.3, an initial drop from 75% to 34% in the acidification of R1 was observed from the beginning, followed by a growing trend till leveling off at about 66% after 40 days. For R2, the acidification degree considerably rose from 37% to 76% during the first 36 days of stage II and dropped to 63% at day 125, after which, the acidification in R2 displayed relatively stable with an average acidification degree of 60% and a relative standard deviation less than 5%.

5.4. Protease activity

At the end of experimental stage I (day 84), the protease activity of the mixed liquor and supernatant samples from both reactors were analyzed. As shown in Figure 5.4a, no protease activity was detected from R2. In contrast, high protease activity was observed in R1, where the equivalent protease concentration referred to as BAEE units per mL increased from 3 to 26 BAEE unit/mL within 80 min for the mixed liquor sample. The supernatant sample displayed a similar growing trend, of which equivalent protease concentration increased from 0 BAEE unit/mL to 19 BAEE unit/mL on average.

Figures 5.4b and Figure 5.4c displayed the protease activity of both reactors from stage II (day 108 and day 128). R1 and R2 were both found to have protease activity in stage II

but at a low level. For example, on day 108, the protease activity of the mixed liquor sample from R2, of which equivalent protease concentration, increased from 5 BAEE unit/mL to 8 BAEE unit/mL within 80 min. On day 128, the protease activity in the mixed liquor sample from R1, of which equivalent protease concentration, also increased from 5 BAEE unit/mL to 8 BAEE unit/mL within 80 min. Overall, the difference between the measurements of the mixed liquor samples and supernatant samples from stage II, either from R1 or from R2, was observed to be slight and less than 3 BAEE unit/mL.

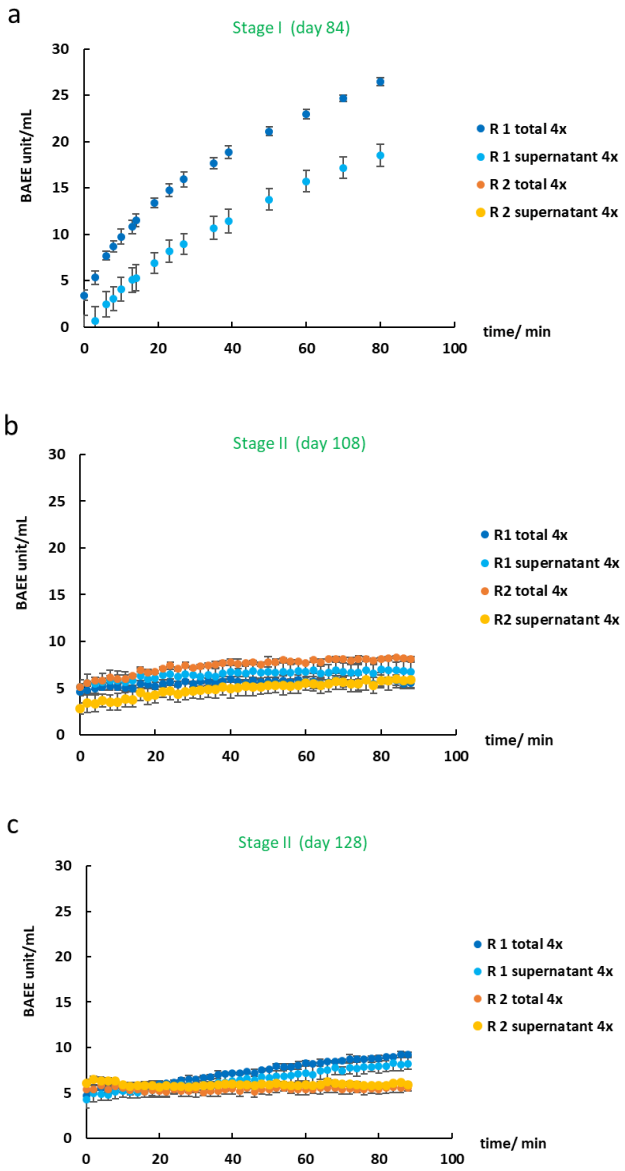

Figure 5.4. Protease activity of R1 and R2: ''R1 total 4x''- mixed liquor sample from R1, diluted 4 times; ''R1 supernatant 4x''- supernatant sample from R1, diluted 4 times; ''R2 total 4x''- mixed liquor sample from R2, diluted 4 times; ''R2 supernatant 4x''- supernatant sample from R2, diluted 4 times; a: Stage I, day 84; b: Stage II, day 108; c: Stage II, day 128; in stage I, R1 was fed with proteins and R2 was fed with carbohydrates; in stage II both reactors were fed with a mixture of proteins and carbohydrates.

5.5. Microbial community composition

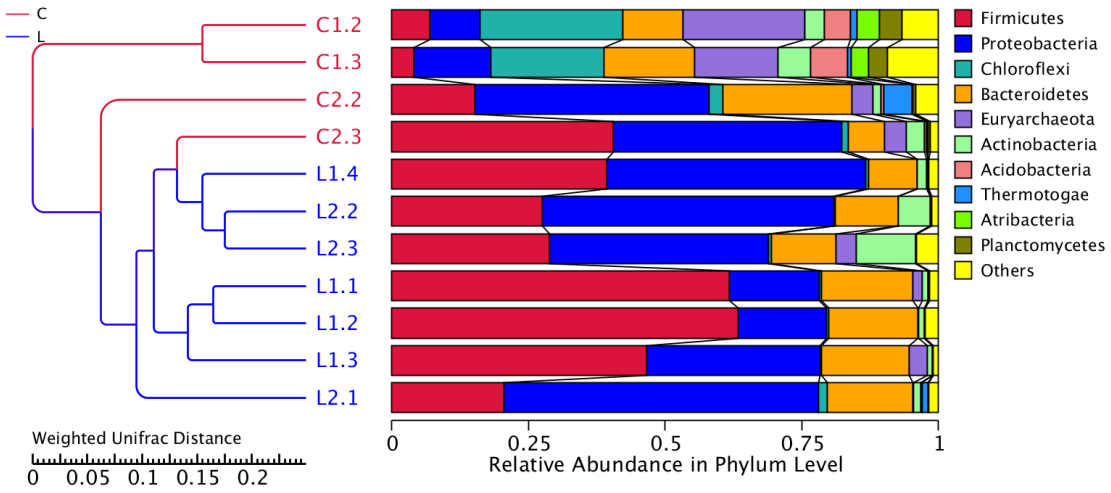

Figure 5.5. UPGMA cluster tree (left) and relative abundance of the top 10 phyla (right): C represents reactor R1, L represents reactor R2; C1.2, suspending biomass, stage I (day 62); C1.3, suspending biomass, stage I (day 84); C2.2, biofilm, stage II (day 132); C2.3, suspending biomass, stage II (day 132); L1.1, suspending biomass, stage I (day 8); L1.2, suspending biomass, stage I (day 62); L1.3, biofilm, stage I (day 84); L1.4, suspending biomass, stage I (day 84); L2.1, suspending biomass, stage II (day 112); L2.2, biofilm, stage II (day 132); L2.3, suspending biomass, stage II (day 132); in stage I, R1 was fed with proteins and R2 was fed with carbohydrates; in stage II, both reactors were fed with a mixture of proteins and carbohydrates; The top 10 phyla were displayed in the different color bar, the rest were grouped into the ''others'' and displayed in the yellow bar.

The UPGMA cluster tree on the left of Figure 5.5 illustrated the similarity among all the samples. The shorter the distance between the two samples, the higher the similarity in the microbial composition. As shown in Figure 5.5 (left), protein-feeding samples (C1.2 and C1.3) displayed a longer distance to the co-fermentation (protein-and-carbohydrate-feeding) samples (C2.2, C2.3, L2,1, L2.2, L2.3) than carbohydrate-feeding samples (L1.1, L1.2, L1.3, L1.4). Thus, compared with protein-feeding, the microbial composition related to carbohydrate-feeding is more similar to the microbial composition regarding co-fermentation.

Figure 5.5 (right) illustrates the relative abundance of the top 10 phyla in each sample. The most abundant populations from the top 10 phyla can be identified in the highest resolution by checking the taxonomic annotation displayed in KRONA charts. KRONA is a web page-based interactive display platform for sequencing results. The snapshots of KRONA charts regarding all the samples can be found in Appendix K.

Microbial populations from phylum *Chloroflexi* and *Euryarchaeota* displayed unique dominance in protein-feeding samples (sample C1.2 and C1.3). Under a higher taxa resolution, it was found that sequences of phylum *Chloroflexi* in samples C1.2 and C1.3 were mainly affiliated to family *Anaerolineaceae,* assigned by more than 70% *Chloroflexi* sequences. Among affiliates of the family *Anaerolineaceae,* more than 50% were assigned

to the genus *Longilinea*. As for phylum *Euryarchaeota*, more than 85% of sequences of this phylum in sample C1.2 and C1.3 were assigned to species *Methanosaeta thermophila PT.*

Bacteria from phylum *Proteobacteria* were predominant in samples regarding carbohydrate-feeding and co-fermentation (sample C2.2, C2.3, L1.3, L1.4, L2.1, L2.2, and L2.3), with more than 30% relative abundance of OTUs assigned. Sequences of phylum *Proteobacteria* in these samples were not assigned to the same affiliates. More than 60% *Proteobacteria* sequences of C2.2 were assigned to the genus *Stenotrophomonas.* Approximately 70% *Proteobacteria* sequences of C2.3 were affiliated to the family *Burkholderiaceae*, of which more than 90% unable to be further assigned to known genera. About 67% *Proteobacteria* sequences of L2.1 were assigned to the genus *Pseudomonas*. *Proteobacteria* sequences of L1.3, L1.4, L2.2, and L2.3 were highly affiliated to the family *Enterobacteriaceae.* The corresponding fractions of *Proteobacteria* sequences assigned to this family were orderly 59%, 71%, 91%, and 91%. Unfortunately, this family also could not be further assigned to any known genera for the aforementioned samples.

Microorganisms from phylum *Firmicutes* were also predominant in samples regarding carbohydrate-feeding and co-fermentation (sample C2.3, L1.1, L1.2, L1.3, L1.4, L2.1, L2.2, and L2.3), with more than 20% relative abundance of OTUs assigned. The percentage of *Firmicutes* sequences assigned to family *Clostridiaceae 1* for these samples were orderly 71% *,* 64%, 39%, 24%, 37%, 53%, 52%, and 39%. These samples also have a substantial abundance of members from family *Ruminococcaceae,* with *Firmicutes* sequences assigned in the percentage of 10%, 20%, 20%, 48%, 18%, 18%, 17%, and 14%, respectively. Sample L1.2, L1.3, and L1.4 were also abundant in species *Lactococcus lactis*, from genus *Lactococcus* of the family *Streptococcaceae*. The corresponding percentages of *Firmicutes* sequences assigned to this genus were 23%, 12%, and 21%, respectively.

Bacteria from phylum *Bacteroidetes* were present as a top-three population in all the samples. The relative abundance of this phylum for these samples were all above 10% except for sample C2.3 and L1.4. The dominating populations were mainly assigned to order *Bacteroidales*, but their genus affiliates were not the same. About 47% and 60% *Bacteroidetes* sequences of sample C1.2 and C1.3 were assigned to genus *Proteiniphilum*. Around 57% of the *Bacteroidetes* sequences of sample C2.2 was assigned to genus *Acetobacteroides*. Approximately 32% and 34% of *Bacteroidetes* sequences of sample L1.1 were affiliated to genus *Elizabethkingia* and genus *Bacteroides.* Sample L1.2, L1.3, and L2.1 were abundant in genus *Bacteroides* with more than 50% *Bacteroidetes* sequences assigned. *Bacteroidetes* sequences of sample L2.2 and L2.3 were both affiliated to genus *Bacteroidetes* and *Prevotella 9*, each genus with more than 25% *Bacteroidetes* sequences assigned.

Chapter 6. Discussions

6.1. The negative effect of carbohydrates on anaerobic protein degradation

This study confirms the conclusions of previous studies (Breure et al., 1986a, 1986b; Tommaso et al., 2003; Yu and Fang, 2001) that carbohydrates do have a negative effect on protein degradation. The indicator of deamination degree in this study can directly reflect the extent of protein degradation in the acid-phase. After being fed with proteins for 84 days, the average deamination degree in the protein-feeding reactor was achieved as 77%. This value was considerably dropped to 15% when carbohydrates present as an additional carbon source. Although an overall growing trend was observed subsequently, the deamination degree throughout the co-fermentation period was constantly fluctuating and most values were no larger than 77%. Also, when the carbon source was changed from proteins to a mixture of proteins and carbohydrates, it was observed that the protease activity of the mixed-culture adapted to protein feeding was significantly reduced. Lower protease activity indicates the lower level of proteolytic ability of the mixed-culture. Thus, the results above clearly show that carbohydrates can negatively affect anaerobic protein degradation in the acid-phase.

6.2. Generalists rather than specialists are predominant in protein fermentation

Before experiments, it was hypothesized that protein specialists (i.e. microorganisms that can grow only on proteins) might outgrow other microbial populations and become dominating in the mixed-culture of an anaerobic acid-phase reactor fed with proteins as the sole carbon source. However, experiment results disprove this hypothesis.

Based on the results of microbial composition analysis, the predominant bacteria in the anaerobic acid-phase reactor fed with proteins as the sole carbon source was found to be populations from phylum *Bacteroidetes* and phylum *Chloroflexi*. The main affiliates of these two phyla were assigned to genus *Proteiniphilum* and genus *Longilinea*, respectively. Members of these two genera were reported as generalists which can utilize proteins as well as carbohydrates for microbial growth in the pure-culture studies (Hahnke et al., 2016; Yamada et al., 2007). This indicates that the mixed-culture in an anaerobic acid-phase reactor fed with proteins are unlikely to be dominated by protein specialists.

The VFA product spectrum of co-fermentation of carbohydrates and proteins showed that proteins and carbohydrates, more or less, were degraded by protein-adapted culture. The VFA products in the protein-adapted reactor, ranging from high to low molar fraction, were acetic acid, iso-valeric acid, n-butyric acid, n-valeric acid, iso-butyric acid, and propionate. Meanwhile, the VFA products in the carbohydrate-adapted reactor, ranging from high to low molar fraction, were acetic acid, n-butyric acid, and propionate. These VFA spectrums were similar to the results reported in previous studies (Bevilacqua et al., 2020; Kissalita et al., 1989) where casein and lactose were also used as model

protein and model carbohydrate. When mixtures of proteins and carbohydrates began to be fed, the main VFA products, ranging from high to low molar fraction, became acetic acid, n-butyric acid, propionate, n-valeric acid, iso-valeric acid, and iso-butyric acid. This VFA product spectrum was in accordance with the findings of previous studies (Demirel and Yenigun, 2004; Yu et al., 2002; Yu and Fang, 2001) on acidogenic fermentation of dairy wastewater, where casein and lactose were both present as carbon substrates. The top-three VFA products were likely to be the outcomes of lactose fermentation and the rest VFA products were likely to be the consequence of casein fermentation. Therefore, the VFA product spectrum of the co-fermentation seemed to be the summation of the VFA product spectrums obtained when a single carbon source was fed. This indicates that protein-adapted culture can also ferment carbohydrates.

The results of acidification degree and deamination degree imply that microorganisms in protein-adapted culture can ferment carbohydrates instantly. After the addition of carbohydrates, the deamination degree in the protein-adapted reactor considerably dropped from 77% to 15% while the acidification degree decreased from 75% to 34%. Thus, at the beginning of the co-fermentation, 34% of the total carbon source (50% were from proteins and 50% were from carbohydrates) were degraded into VFA products while proteins were only degraded by 15%. This difference indicates that acidification at the beginning of the co-fermentation is mainly contributed by carbohydrate fermentation instead of protein fermentation. This finding was similar to the results from studies did by Breure et al. (1986b, 1986a), who also found that gelatin-adapted mixed-culture was retarded in gelatin fermentation while was able to ferment glucose instantly when glucose added as a second carbon substrate.

Therefore, generalists that can degrade proteins as well as carbohydrates, rather than specialists that can only degrade proteins, are more likely to be dominating in the protein-adapted culture.

6.3. Generalists prefer carbohydrates are predominant in co-fermentation

The reactor performance in VFA product spectrum, acidification degree, and deamination degree throughout the co-fermentation period further support that generalists are predominant in the mixed-culture fed with carbohydrates and proteins. Similar to the VFA product spectrum at the beginning of co-fermentation, the VFA product spectrum in the late period still looked like a summation of the VFA product spectrums obtained when a single carbon substrate was fed. Besides, general growth trends were both observed in the acidification degree and deamination degree of two reactors. These results indicate that carbohydrates and protein, more or less, were degraded by the mixed-culture of co-fermentation, which can be attributed to the dominance of generalists.

However, some generalists seem to have a preference for carbohydrates over proteins. Based on the results of the similarity analysis of the microbial composition, it was found

that the predominant bacteria in the co-fermentation cultures have more populations in common with the carbohydrate-adapted culture than the protein-adapted culture. These cultures were both dominated by microorganisms from phylum *Proteobacteria* and *Firmicutes*, with a total relative abundance of over 70%. Their mutual populations were mainly from phylum *Firmicutes*, of which affiliates were assigned to the family *Clostridiaceae 1*, *Ruminococcaceae*, and *Lachnospiraceae*. Results from protease activity showed that none of the microbial populations in carbohydrate-adapted culture had the proteolytic ability. Furthermore, the mixed-culture throughout the co-fermentation period was at a lower level of proteolytic ability compared to the protein-adapted culture. Therefore, it was likely that those mutual populations could lose the proteolytic ability, and preferentially ferment carbohydrates were predominant in the co-fermentation mixed-culture. This may explain the phenomenon of the negative effect of carbohydrates on anaerobic protein degradation in the acid-phase. From the thermodynamic point of view, carbohydrates can be preferentially degraded by anaerobic mixed-culture because more energy can be harvested from carbohydrate degradation than protein degradation (Regueira et al., 2020). The preference of microorganisms to ferment carbohydrates might cause the loss of proteolytic ability by repressing the synthesis of the proteolytic enzyme (Breure et al., 1986b; Siegal, 2015).

The loss of the proteolytic ability of the predominant microorganisms in the co-fermentation mixed-culture likely leads to the retard of protein degradation in the hydrolysis step. However, a previous study reported that protein hydrolysis was unlimited by co-fermentation and the hydrolysis kinetics under co-fermentation remained the same as carbohydrates or proteins fed as the only carbon substrate (Gavala and Lyberatos, 2001). A recent study did by Bevilacqua et al. (2020) demonstrated that preferential fermentation of amino acids by anaerobic mixed-culture could also account for incomplete protein degradation. Since different experimental conditions are employed for different studies, it is hard to reach an agreement that whether the retard of anaerobic protein degradation by the presence of carbohydrates is mainly in the hydrolysis step or amino acid fermentation step. Thus, further research on the limiting step is still in need to have a better understanding of incomplete protein degradation and its relation to the metabolism function of microorganisms.

In addition, the functions carried out by versatile microorganisms are not always the same under different circumstances (Rittmann and McCarty, 2001). To confirm that the dominance of generalists preferentially ferment carbohydrates lead to the negative effect of carbohydrates on anaerobic protein degradation, functional analysis of the microbial populations in the mixed-culture regarding single substrate feeding and co-fermentation should be conducted. Multiple ‘omic’ data analyses such as transcriptomics, proteomics, and metabolomics can be applied to better profile the metabolism function of microorganisms in-situ (Franzosa et al., 2015).

Chapter 7. Conclusions and recommendation

7.1. Conclusions

The main objective of this research is to explore the relationship between microbial composition and the negative effect of carbohydrates on anaerobic protein degradation in the acid-phase. Based on the obtained results, the key information can be concluded:

- Microbial composition study offers a better understanding of the negative effect of carbohydrates on anaerobic protein degradation in the acid-phase.
- Different predominant microbial populations are found in the mixed-cultures regarding protein fermentation and mixed-culture regarding co-fermentation of proteins and carbohydrates.
- Generalists (i.e. microorganisms that can ferment both proteins and carbohydrates), mainly from phylum *Bacteroidetes* and phylum *Chloroflexi,* are the predominant populations of the mixed-culture in an anaerobic acid-phase reactor fed with proteins as the sole carbon source.
- Generalists, mainly from phylum *Proteobacteria* and *Firmicutes*, are the predominant populations of the mixed-culture in an anaerobic acid-phase reactor fed with mixtures of proteins and carbohydrates.
- The dominance of generalists that can preferentially utilize carbohydrates over proteins likely leads to the incomplete degradation of proteins when carbohydrates are present as an additional carbon source to the mixed-culture in an anaerobic acid-phase reactor.

7.2. Recommendation

To fully understand the mechanism behind the negative effect of carbohydrates on protein degradation in the acid-phase of anaerobic digestion of protein-rich wastewater, some recommendations are given for future researches on this topic:

- **Functional analysis should be employed to further confirm the preferential metabolism of carbohydrates over proteins carried out by the predominant microorganisms in the mixed-culture of an anaerobic co-fermentation reactor.** In this study, the metabolism of carbohydrates and proteins carried out by predominant microbial populations are inferred by the reactor performance and the pure-culture studies on known isolated microorganisms. The actual functions of protein and carbohydrate metabolism carried out by dominating microbial populations in-situ need to be further verified by functional analysis. With the development of molecular techniques, multiple 'omic' data analyses such as transcriptomics, proteomics, and metabolomics can be applied to better profile the metabolism functions of microorganisms in-situ (Franzosa et al., 2015).

- The limiting step of incomplete protein degradation caused by the presence of carbohydrates is still unclear.
  As stated in section 6.3, the preferential fermentation of carbohydrates over proteins can cause the loss of the proteolytic ability of generalists, which may lead to the inhibition of the protein hydrolysis step. Other studies reported that amino acid fermentation was the limiting step and resulted in an incomplete protein fermentation. More researches on the limiting step should be carried out to have a better understanding of incomplete protein degradation and its relation to the metabolism function of microorganisms.
- The use of real protein-rich wastewater should be considered.
  In this study, the feedstock for the anaerobic reactor running was synthetic wastewater. Proteins and carbohydrates, each accounted for 50% COD of 6000 mg/L COD in the feedstock. However, the COD ratio of proteins and carbohydrates in the real protein-rich wastewater is unlikely to equal 1:1 and hard to be constant all the time (Ahmad et al., 2019; Bustillo-Lecompte and Mehrvar, 2015; Carvalho et al., 2013; Demirel et al., 2005). To further confirm the preferential metabolism of carbohydrates by predominant generalists in the mixed-culture for co-fermentation of proteins and carbohydrates, real protein-rich wastewater should also be tested.
- The bias from the inoculum should be considered.
  In this study, the inoculum was sampled from a dairy wastewater treatment plant. The dominating microbial populations are originated from the initial microbial populations in the mother culture, which might show different predominant populations if different inoculum is used. The results of microbial composition in this study should be compared with results from other microbial composition studies regarding the anaerobic degradation of protein-rich wastewater.
- Microbial composition studies under different operational conditions such as HRT and pH can be considered.
  Although the effects of operational conditions on anaerobic protein-degradation have been widely explored, their correlation with the microbial composition is not explored. By comparing the microbial compositions under different operational conditions such as HRT and pH, the bias from different operational conditions can be known.

Knowing that carbohydrates are preferentially fermented by the predominant microorganisms in the mixed-culture for co-fermentation of carbohydrates and proteins, the following suggestions regarding process design can be considered to achieve complete conversion of proteins in the anaerobic digestion process of protein-rich wastewater:

- A separate acid-phase reactor can be considered for the process design of anaerobic digestion of protein-rich wastewater.

For a two-phase process system, protein degradation can be fully converted into VFAs in an acid-phase reactor operated as a sequencing batch. For a one-stage process, a buffer tank that is commonly used for the storage of raw wastewater can function as an acid-phase reactor to achieve complete protein degradation. Considering the release of ammonium from protein degradation might be detrimental to the biogas production in the methane-phase (Yenigün and Demirel, 2013), recovery of ammonium from the effluent of the acid-phase reactor can be considered to improve the performance of the methane-phase. Available technologies are simultaneous ammonia recovery with calcium removal by biogas recirculation, gas-membrane absorption, struvite precipitation (Gu et al., 2019; Huang et al., 2015; Shi et al., 2019).

Chapter 8. Reference

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Chapter 9. Supplementary information

Appendix A. Classification of some amino acid fermenters

Table 9.1. Classification of amino acid fermenters

Classification	Species	Enzyme production	Amino acids utilized	Reaction characteristics
I	*C. bifermentans*	proteo/saccharolytic	proline, serine, arginine, glycine, leucine, isoleucine, valine, ornithine, lysine, alanine, threonine, phenylalanine, tyrosine, tryptophan, and glutamate	organisms that carry out Stickland reaction; proline utilized by by all species; δ-aminovalerate α-aminobutyrate and γ-aminobutyrate produced.
	C. sordellii	proteo/saccharolytic		
	C. botulinum types A, B, F	proteo/saccharolytic		
	C. caloritolerans	–		
	C. sporogenes	proteo/saccharolytic		
	C.cochlearium – one strain.	specialist		
	C. difficile	saccharolytic		
	C. putrificum	proteo/saccharolytic		
	C. sticklandii	specialist		
	C. ghoni	proteolytic		
	C. mangenotii	proteolytic		
	C. scatologenes	saccharolytic		
	C. lituseburense	proteo/saccharolytic		
	C. aerofoetidum	–		
	C. butyricum	saccharolytic		
	C. caproicum	–		
	C. carnofoetidum	–		
	C. indolicum	–		
	C. mitelmanii	–		
	C. saprotoxicum	–		
	C. valerianicum	–		
II	*C. botulinum types C*	proteo/saccharolytic	glycine, arginie, histidine and lysine	glycine used by all species; δ-aminovalerate not produced.
	C. histolyticum	proteolytic		
	C. cochlearium – one strain	specialist		
	C. subterminale	proteolytic		
	C. botulinum types G	–		
	P. anaerobius	–		
	P. variabilis	–		
	P. micros	–		
III	*C.cochlearium – one strain.*	Specialist	glutamate, serine, histidine, arginine, aspartate, threonine, tyrosine, tryptophan and cysteine	δ-aminovalerate not produced; histidine, serine and glutamate used by all species.
	C. tetani	Proteolytic		
	C. tetanomorphum	Saccharolytic		
	C. lentoputrescens	–		
	C. limosum	proteolytic		
	C. malenomenatum	specialist		
	C. microsporum	–		
	C. perfringens	proteo/saccharolytic		
	C. butyricum	saccharolytic		
	P. asaccharolyticus	–		
	P. prevotii	–		
	P. activus	–		
IV	*C. putrefaciens*	proteolytic	serine and threonine	δ-aminovalerate not produced
V	*C. propionicum*	specialist	alanine, serine, threonine, cysteine, and methionine	δ-aminovalerate not produced

Source: Ramsay and Pullammanappallil (2001); *C–Clostridium*; *P – Peptostreptococcus*; Specialists neither proteolytic nor saccharolytic species but specialized organisms that use only one or a few substrates.

Appendix B. Conversion factors to calculate COD of VFAs

Table 9.2. Conversion factors (gCOD/gVFA) for VFA in terms of COD

VFA	COD conversion factor (gCOD/gVFA)
Acetic acid	1.066
Propionic acid	1.512
Iso-butyric acid	1.816
Butyric acid	1.816
Valeric acid	2.036
Iso-valeric acid	2.036
Caproic acid	2.204

Appendix C. Protease standard curve

Table 9.3. Reference protease Trypsin concentration and corresponding BAEE unit vs fluorescence response (corrected by the blank)

BAEE units	Trypsin Conc.	Standard
unit/mL	µg/mL	Response
0	0	0
0.35	1.25	110
0.7	2.5	393
1.4	5	1086
2.8	10	2052
3.5	12.5	2787
5.6	20	4113
7	25	4669
8.4	30	4509
11.2	40	6682

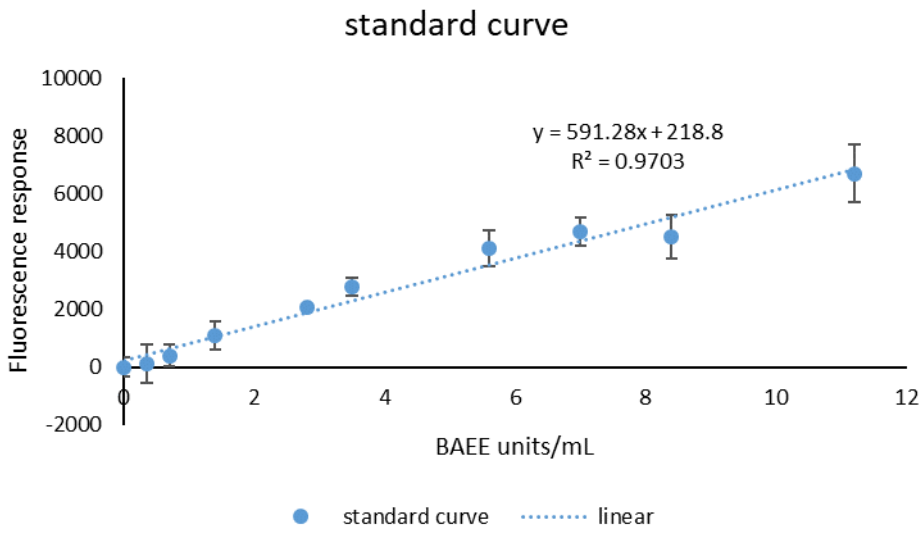

Figure 9.1. Standard curve of protease activity

Appendix D. Operational schemes

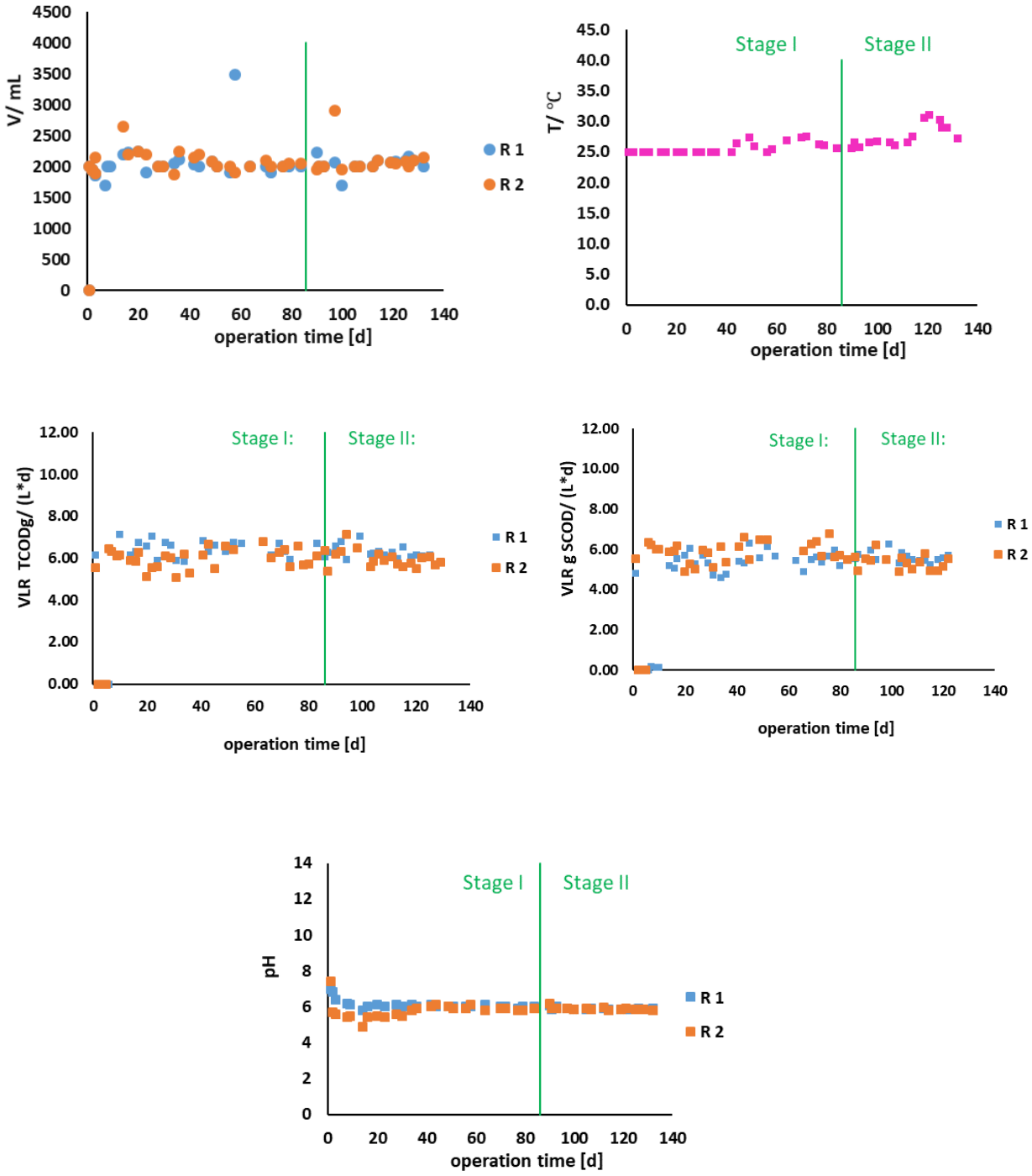

Figure 9.2. Operational scheme of R1 and R2; The green line set at day 86 is to make a distinction between stage I and stage II; in stage I, R1 was fed with proteins, and R2 was fed with carbohydrates; in stage II, both reactors were fed with a mixture of proteins and carbohydrates.

Appendix E. Morphology

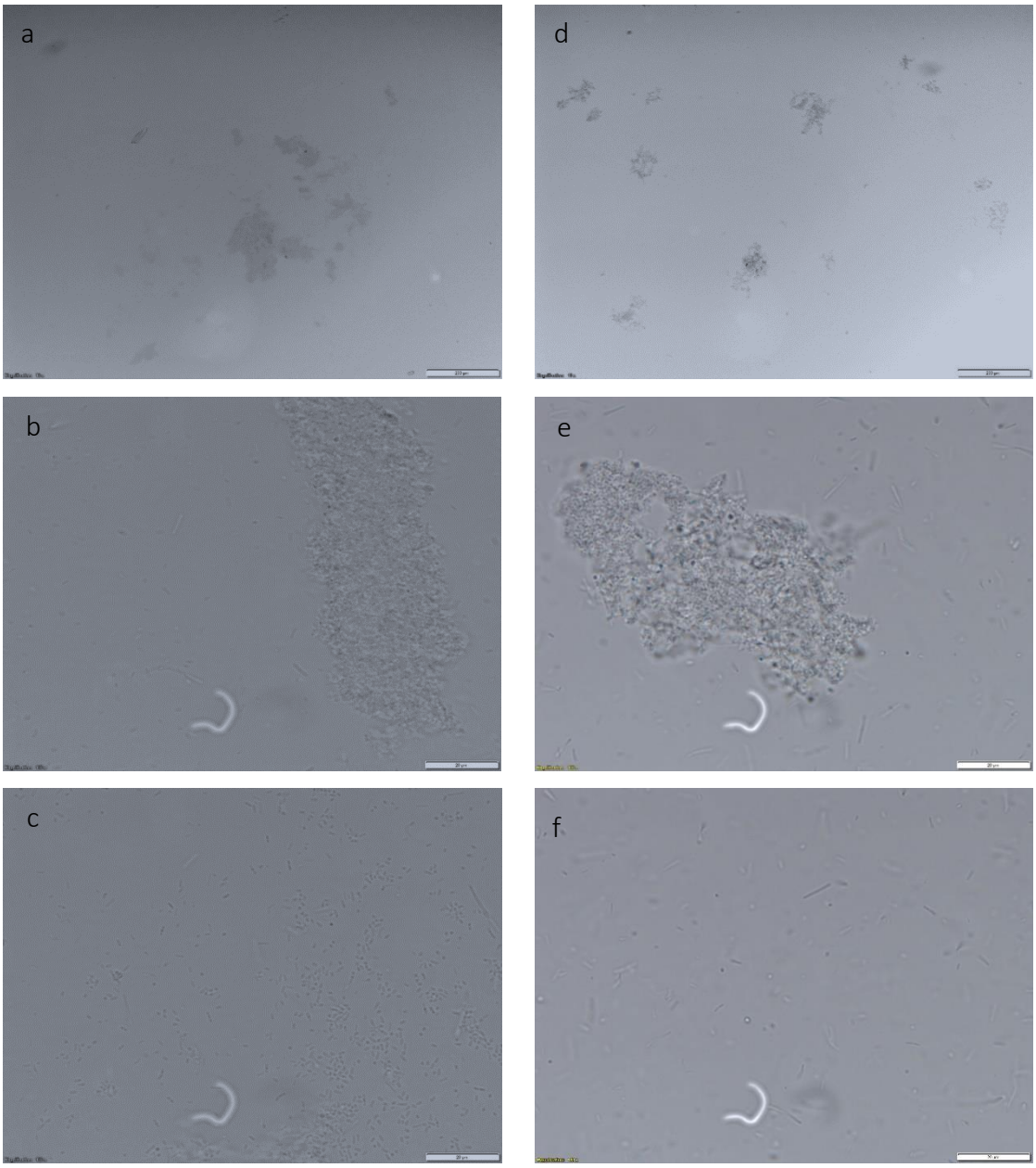

Figure 9.3. Microscopic images of biomass from R1 (Stage I): day 35, flocs (10x), with 200µm scale bar (a); day 35, a compact floc (100x), with 20µm scale bar (b); day 35, rod-shaped free-living cells and small clusters (100x), with 20µm scale bar (c); day 76, flocs (10x), with 200µm scale bar (d); day 76, a floc (100x), with 20µm scale bar (e); day 76, rod-shaped free living cells (100x), with 20µm scale bar (f);

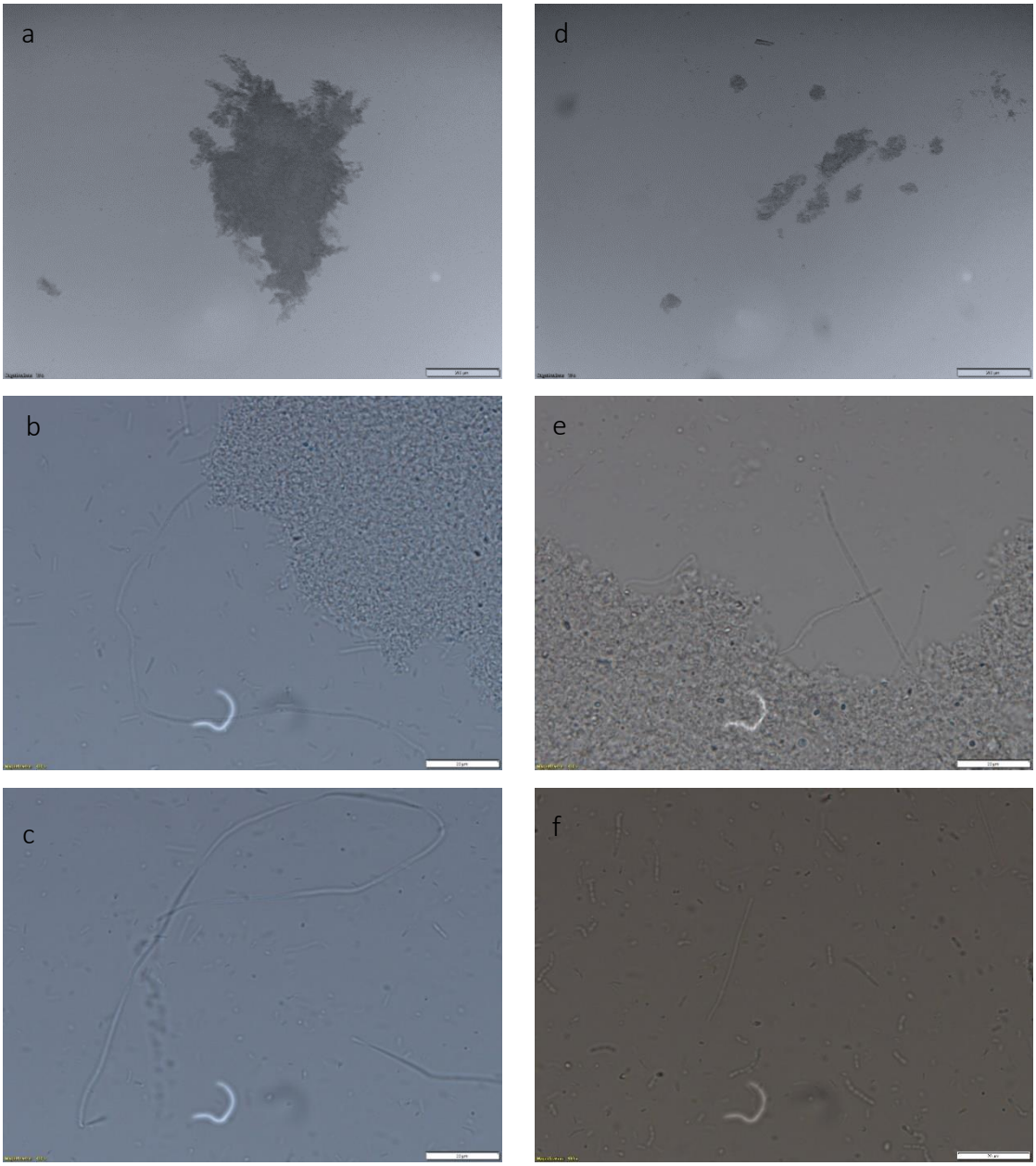

Figure 9.4. Microscopic images of biomass from R1 (Stage II): day 97, flocs (10x), with 200µm scale bar (a); day 97, a compact floc(100x), a filament (100x), and rod-shaped free-living cells (100x), with 20µm scale bar (b); day 97, rod-shaped free-living cells and a coiled filament (100x), with 20µm scale bar(c); day 125, floc (10x), with 200µm scale bar (d); day 125, a compact floc and straight filaments (100x), with 20µm scale bar (e); day 125, rod-shaped free-living cells and rod-shaped cells clustered in chain (100x), with 20µm scale bar (f);

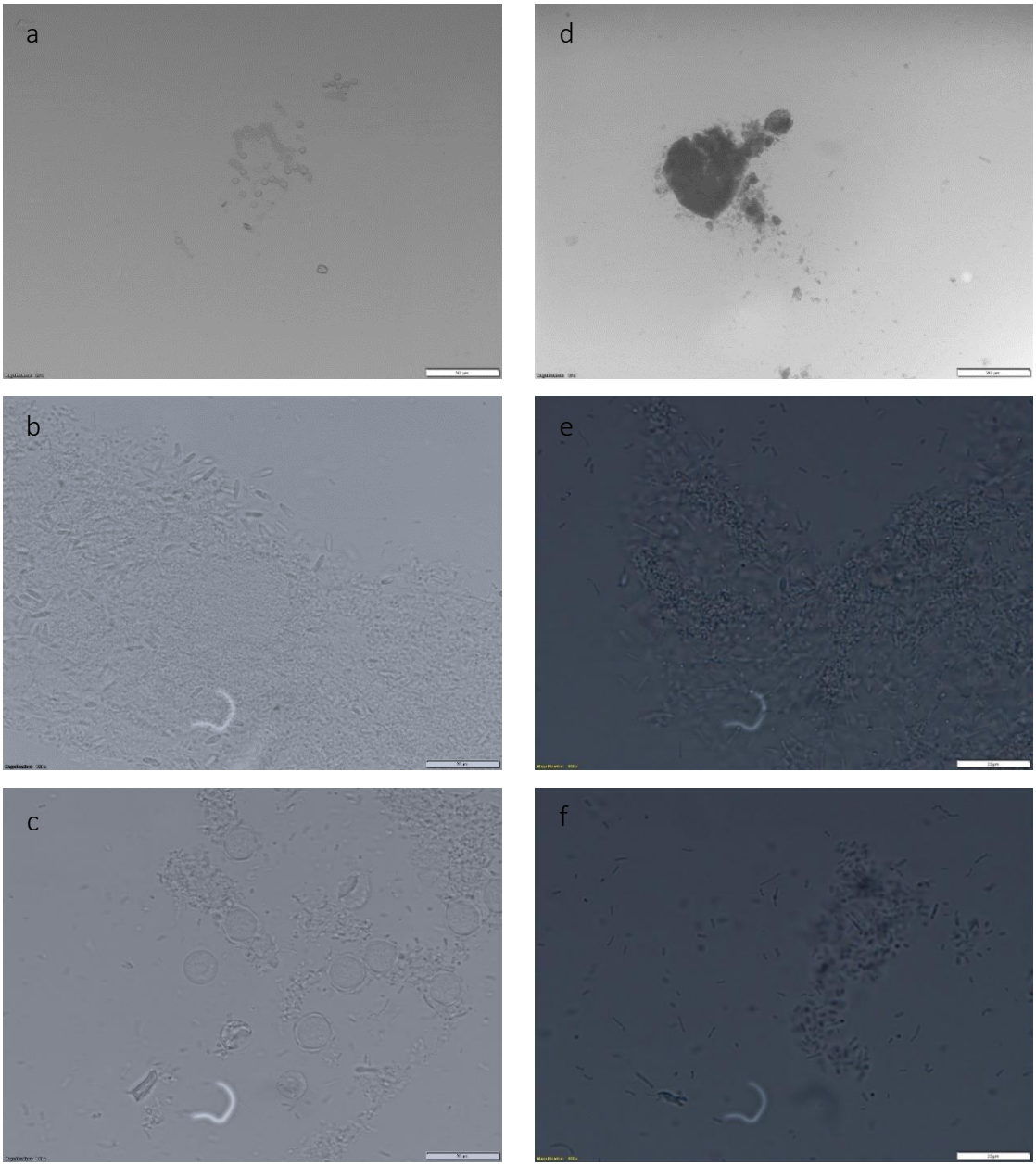

Figure 9.5. Microscopic images of biomass from R2 (Stage I): day 35, flocs and bubbles (20x), with 100µm scale bar (a); day 35, a big floc and rod-shaped free-living cells (100x), with 20µm scale bar (b); day 35, small flocs, bubbles, and rod-shaped free-living cells (100x), with 20µm scale bar (c); day 76, flocs (10x), with 200µm scale bar (d); day 76, a big compact floc and rod-shaped free-living cells (100x), with 20µm scale bar (e); day 76, a small floc and rod-shaped free-living cells (100x), with 20µm scale bar (f);

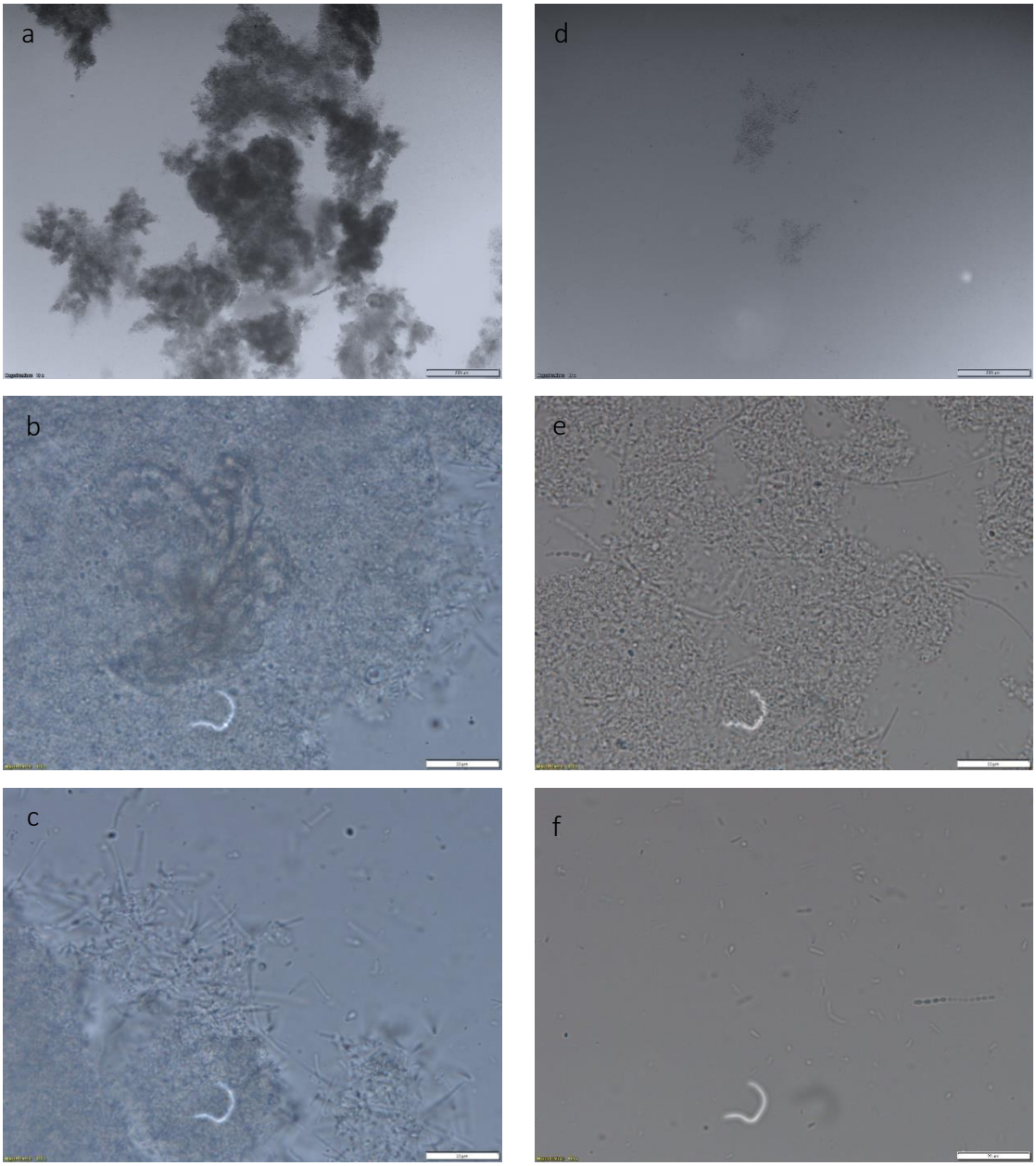

Figure 9.6. Microscopic images of biomass from R2 (Stage II): day 97, flocs (10x), with 200µm scale bar (a); day 97, a big floc containing with unknown matters (100x), with 20µm scale bar (b); day 97, the fringe of a floc and rod-shaped free living cells (100x), with 20µm scale bar(c); day 125, flocs (10x), with 200µm scale bar (d); day 125, an open floc and filaments (100x), with 20µm scale bar (e); day 125, rod-shaped free-living cells and rod-shaped cells clustered in chain (100x), with 20µm scale bar (f);

Appendix F. Alcohol production

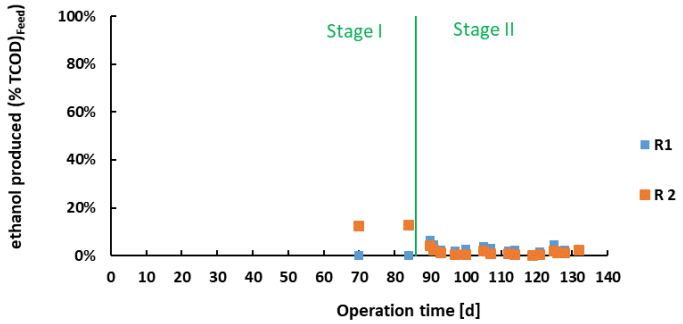

Figure 9.7. Ethanol production of R1 and R2; The green line set at day 86 is to make a distinction between stage I and stage II; in stage I, R1 was fed with proteins, and R2 was fed with carbohydrates; in stage II, both reactors were fed with a mixture of proteins and carbohydrates.

Appendix G. Gas production

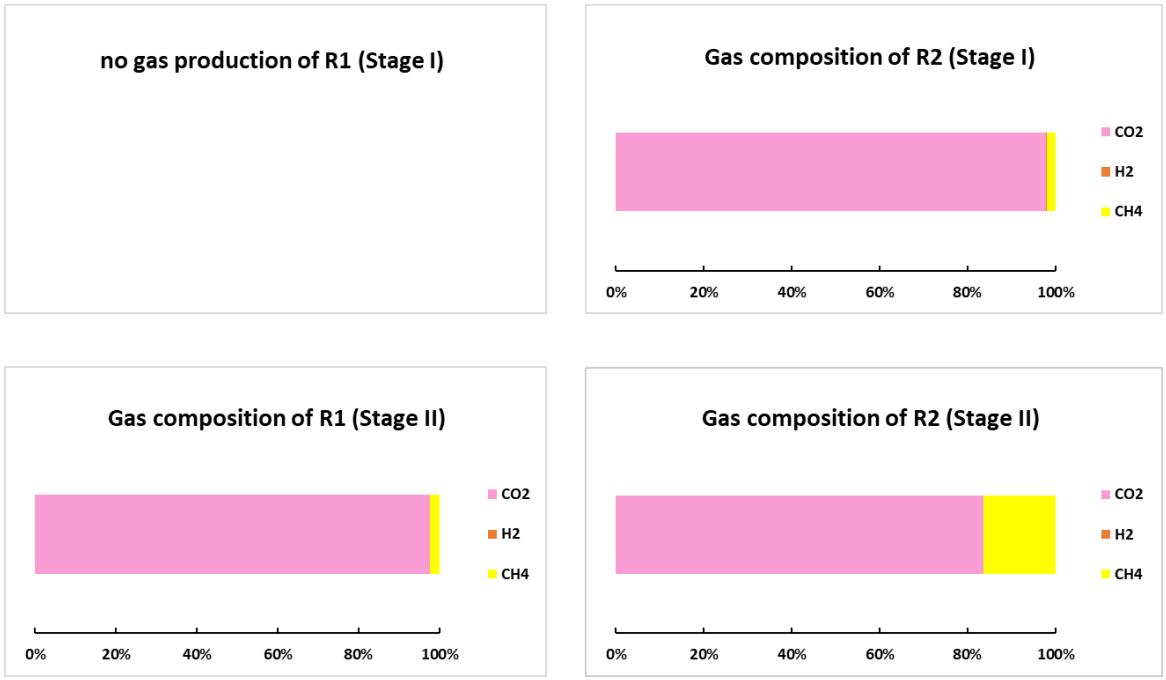

Figure 9.8. Gas production in stage I and stage II; in stage I, R1 was fed with proteins, and R2 was fed with carbohydrates; in stage II, both reactors were fed with a mixture of proteins and carbohydrates.

Appendix H. TSS and VSS

Table 9.4. TSS and VSS of each reactor measured at the end of each experiment stage

Reactor	Stage I		Stage II	
	TSS [mg/L]	VSS [mg/L]	TSS [mg/L]	VSS [mg/L]
R1	1.687	1.596	1.516	1.323
R2	1.041	0.956	1.580	1.461

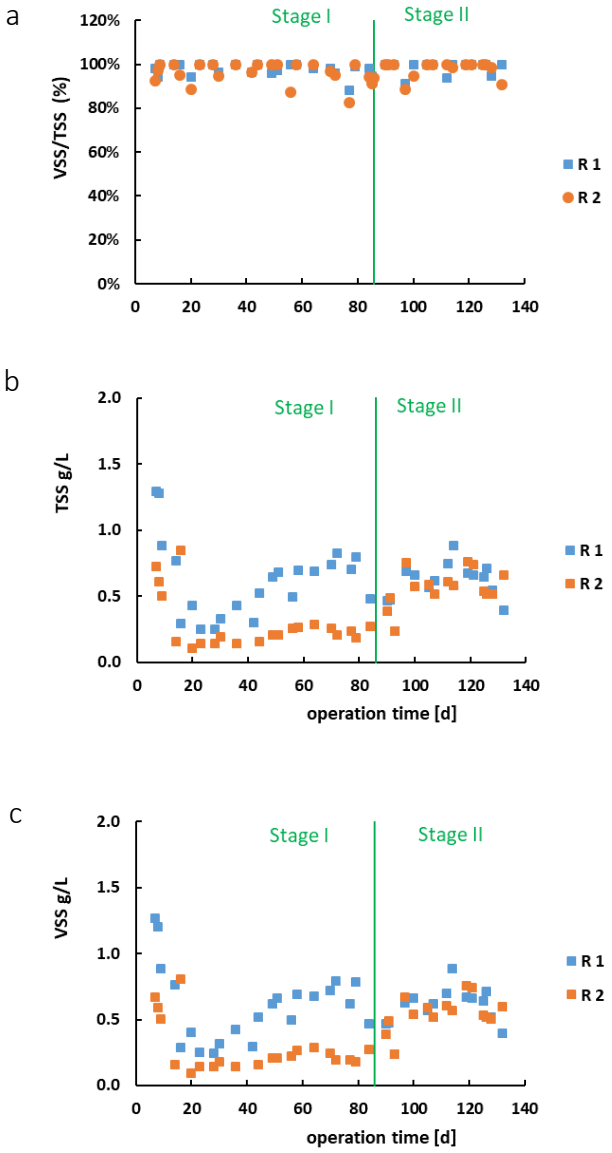

Figure 9.9. VSS/TSS (a), TSS concentration (b), and VSS concentration (c) of mixed liquor in R1 and R2; The green line set at day 86 is to make a distinction between stage I and stage II; in stage I, R1 was fed with proteins, and R2 was fed with carbohydrates; in stage II both reactors were fed with a mixture of proteins and carbohydrates.

Appendix I. COD balance and N balance

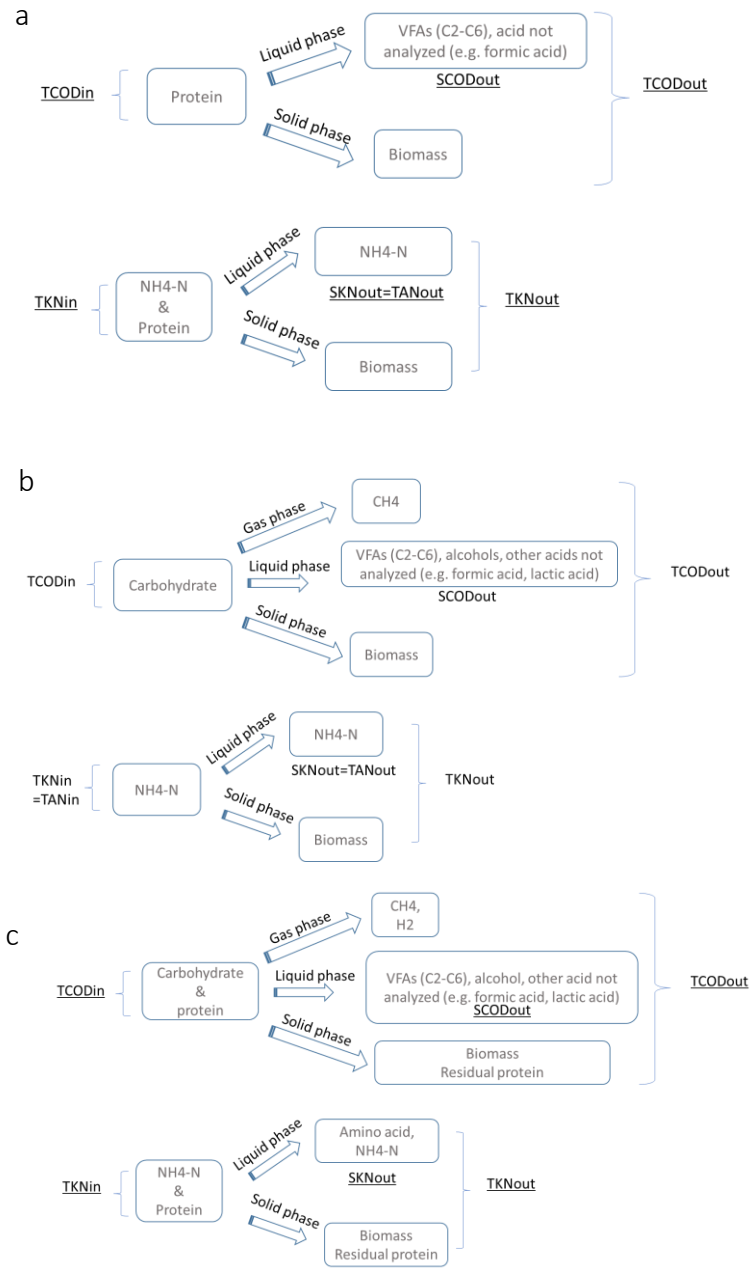

Figure 9.10. Estimation of COD and TN balance of R1 in stage I (a), R2 in stage I (b), R1 & R2 in stage II (c); in stage I, R1 was fed with proteins and R2 was fed with carbohydrates; in stage II both reactors were fed with a mixture of proteins and carbohydrates.

Appendix J. 16S rRNA gene sequencing results from Novogene

Table 9.5 Sludge samples information for 16s RNA gene sequencing

Reactor	Sampling time	stage I (day 8)	stage I (day 62)	stage I (day 84)		stage II (day 112)	stage II (day 132)	stage II (day 132)
R1	Sample name	C1.1	C1.2	C1.3		C2.1	C2.2	C2.3
	sampling location	quality failed	mixed liquor	mixed liquor		quality failed	reactor wall	mixed liquor
R2	Sample name	L1.1	L1.2	L1.3	L1.4	L2.1	L2.2	L2.3
	sampling location	mixed liquor	mixed liquor	reactor wall	mixed liquor	mixed liquor	reactor wall	mixed liquor

Table 9.6. QC stat of qualified samples

Sample Name	Raw PE(#)	Raw Tags(#)	Clean Tags(#)	Effective Tags(#)	Base(nt)	AvgLen(nt)	Q20	Q30	GC%	Effective%
C1.3	142,771	138,345	136,088	119,319	30,217,617	253	99.27	97.81	55.63	83.57
C1.2	112,123	109,529	107,840	95,659	24,234,494	253	99.34	97.98	56.08	85.32
C2.2	136,456	133,337	131,293	111,525	28,208,799	253	99.34	98.03	54.19	81.73
C2.3	95,808	93,584	92,197	54,307	13,739,142	253	99.43	98.24	53.29	56.68
L1.1	140,647	136,529	133,948	108,559	27,458,557	253	99.42	98.20	51.30	77.19
L1.2	135,612	131,756	129,568	106,447	26,906,012	253	99.36	98.05	51.59	78.49
L1.3	143,002	139,518	137,055	113,600	28,729,200	253	99.42	98.22	52.83	79.44
L1.4	128,846	125,179	123,390	102,291	25,863,921	253	99.42	98.23	53.77	79.39
L2.1	136,077	131,806	129,182	111,312	28,165,009	253	99.22	97.78	52.28	81.80
L2.2	140,209	136,954	134,851	121,188	30,654,907	253	99.44	98.28	54.67	86.43
L2.3	121,542	118,412	116,622	101,504	25,674,787	253	99.41	98.18	54.24	83.51

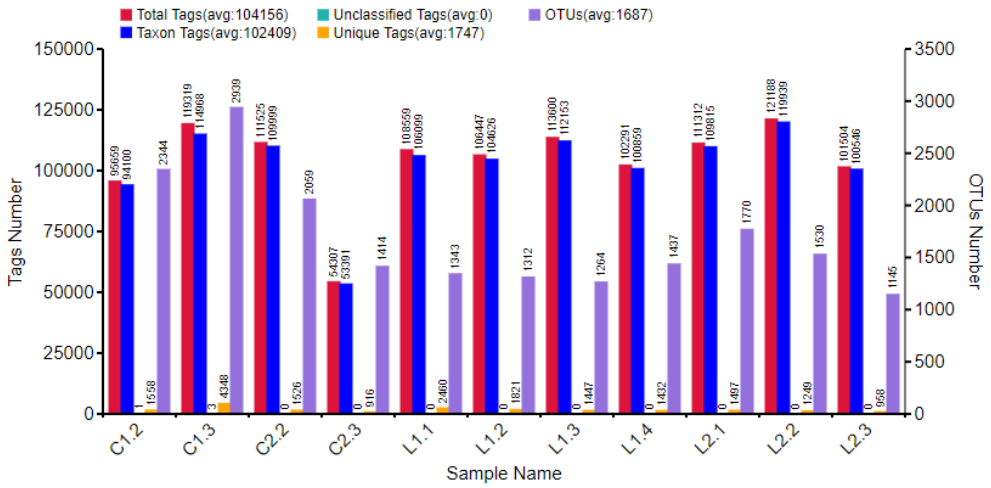

Figure 9.11. Taxo number of each sample

Appendix K. Snapshots of KRONA charts

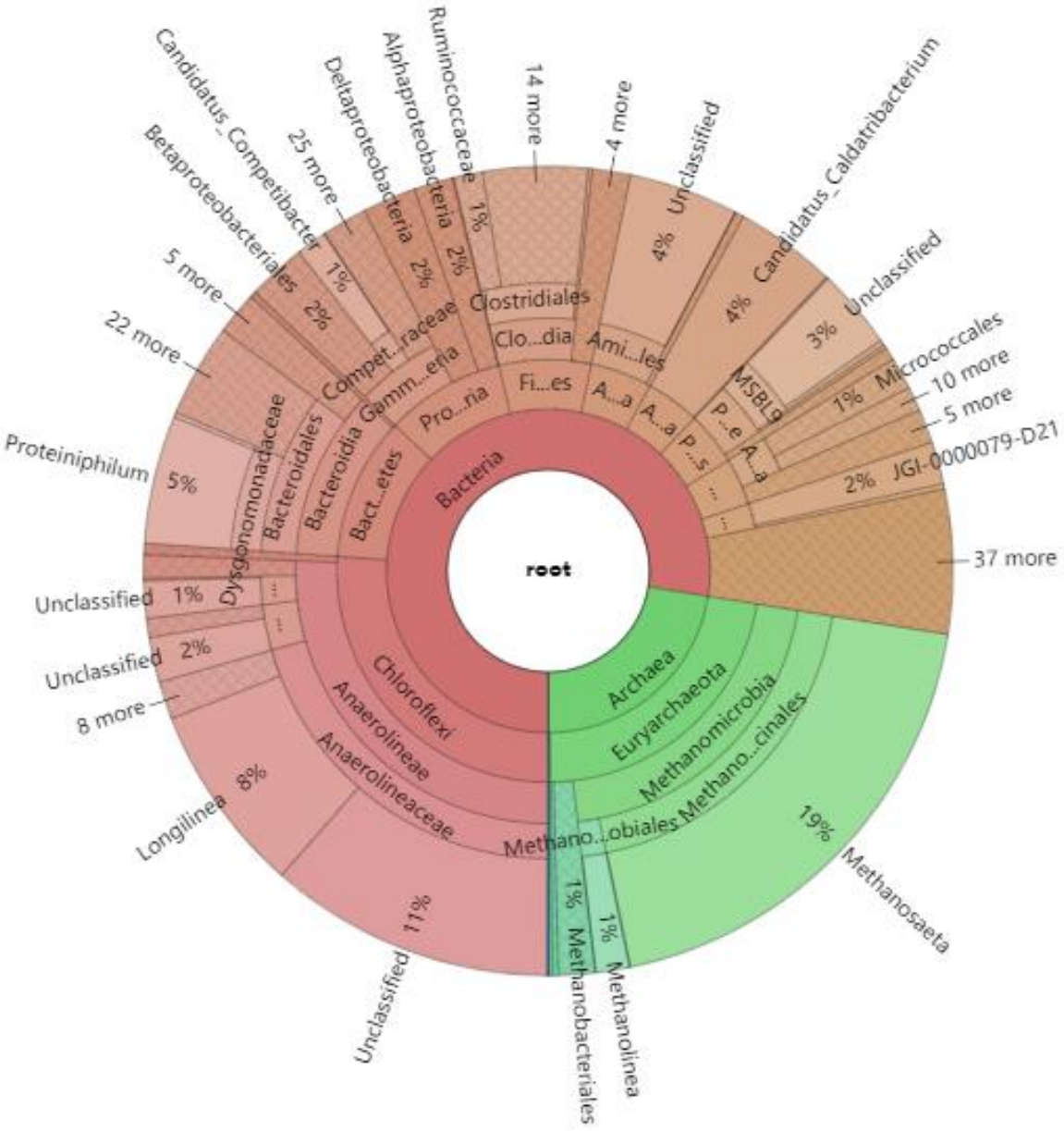

Figure 9.12. KRONA chart of sample C1.2

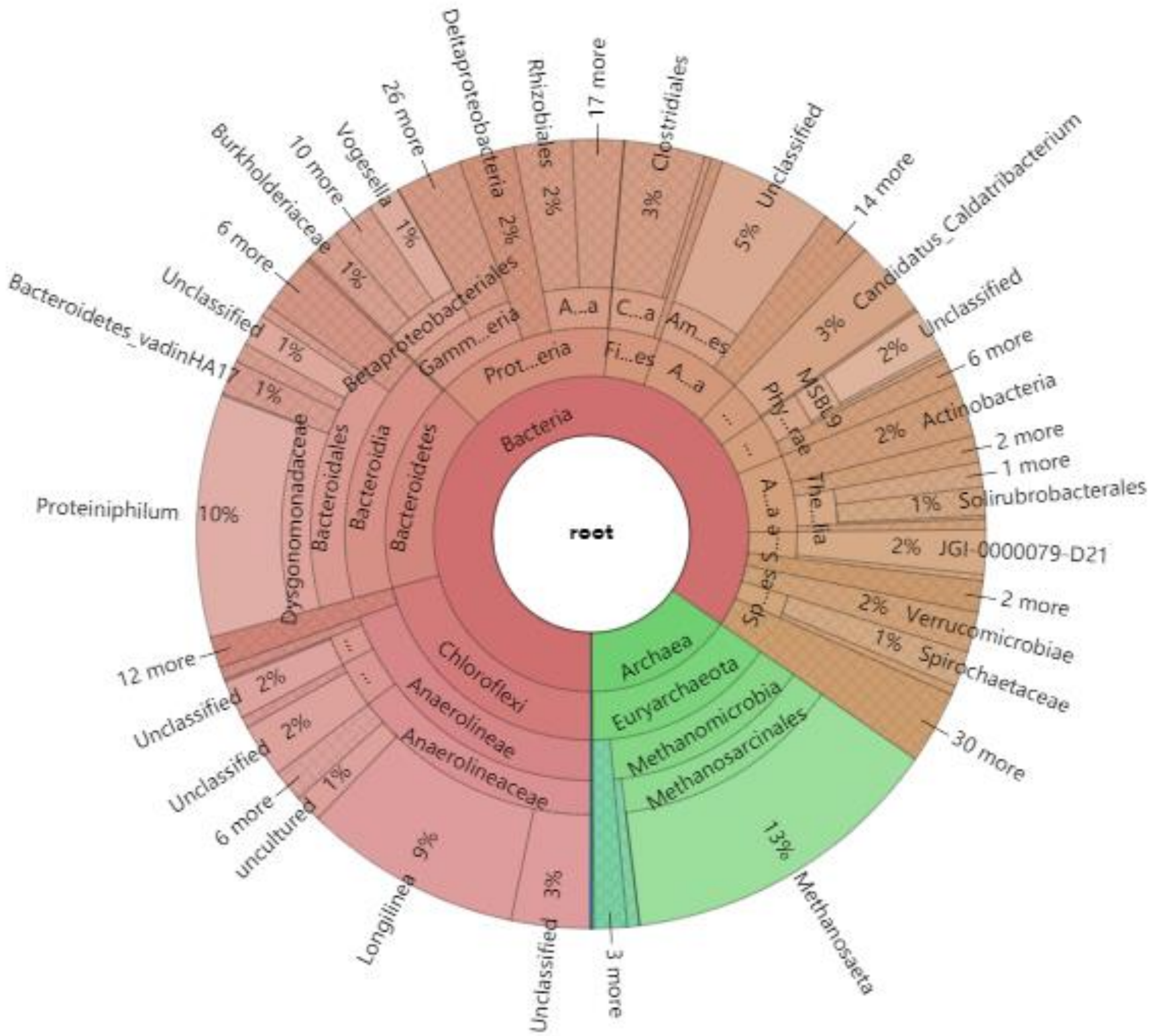

Figure 9.13. KRONA chart of sample C1.3

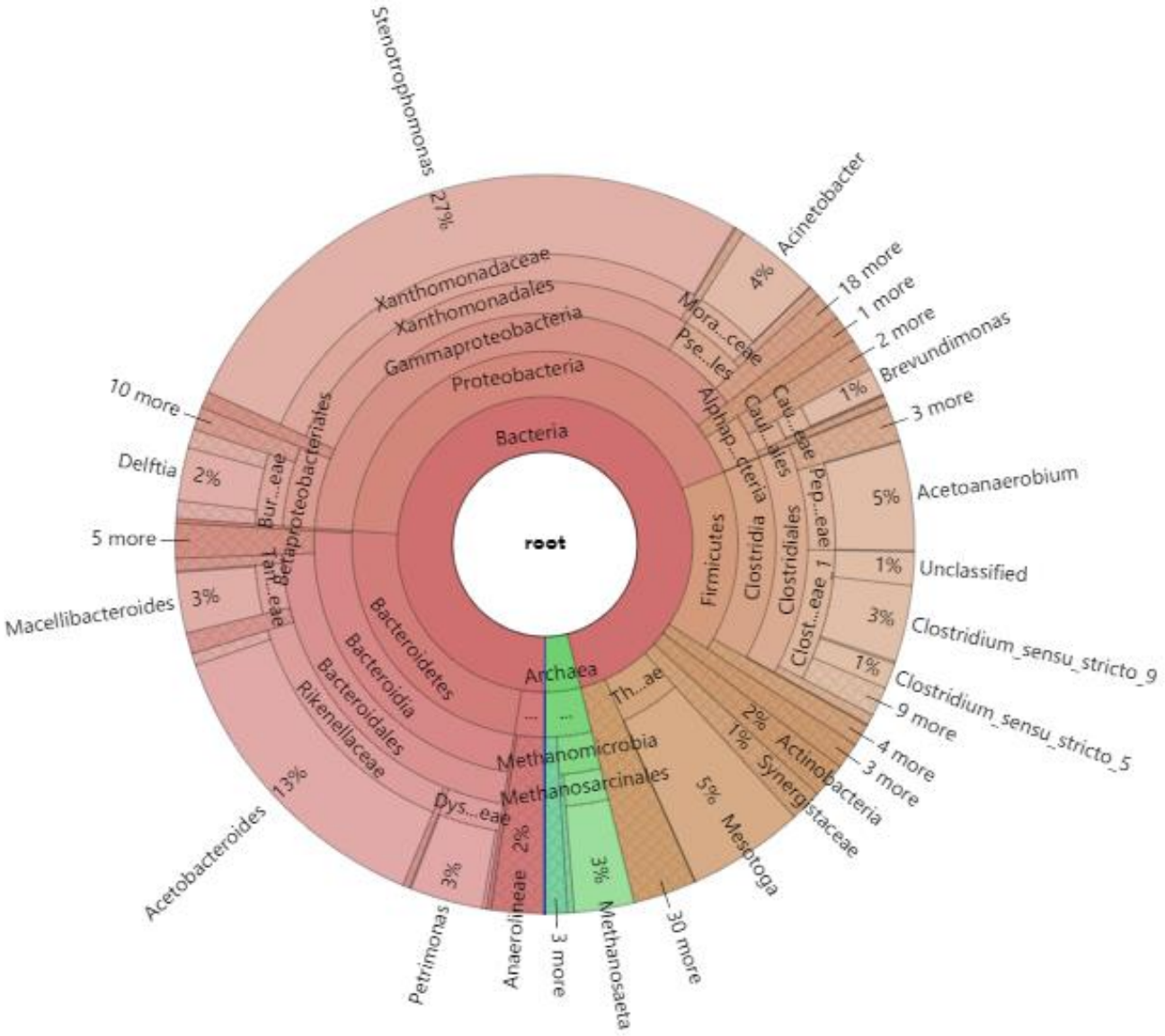

Figure 9.14. KRONA chart of sample C2.2

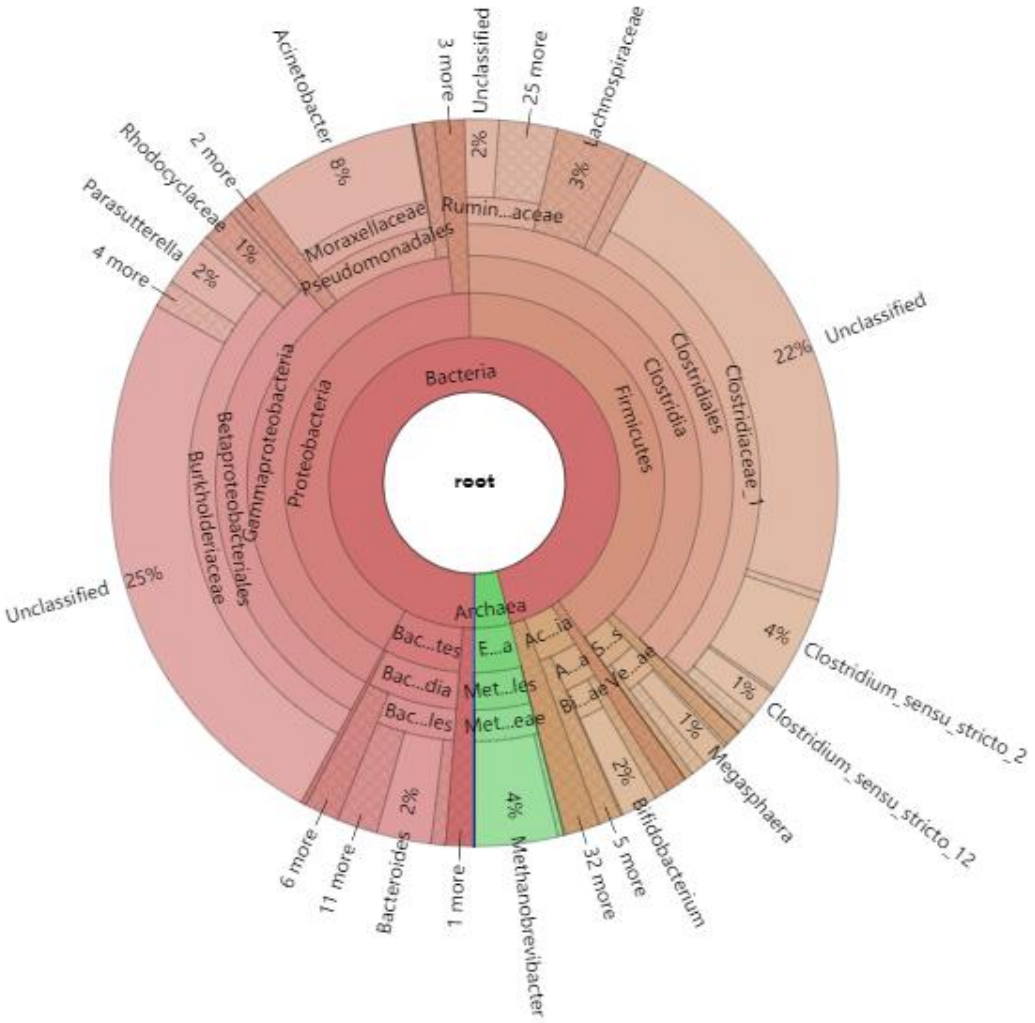

Figure 9.15. KRONA chart of sample C2.3

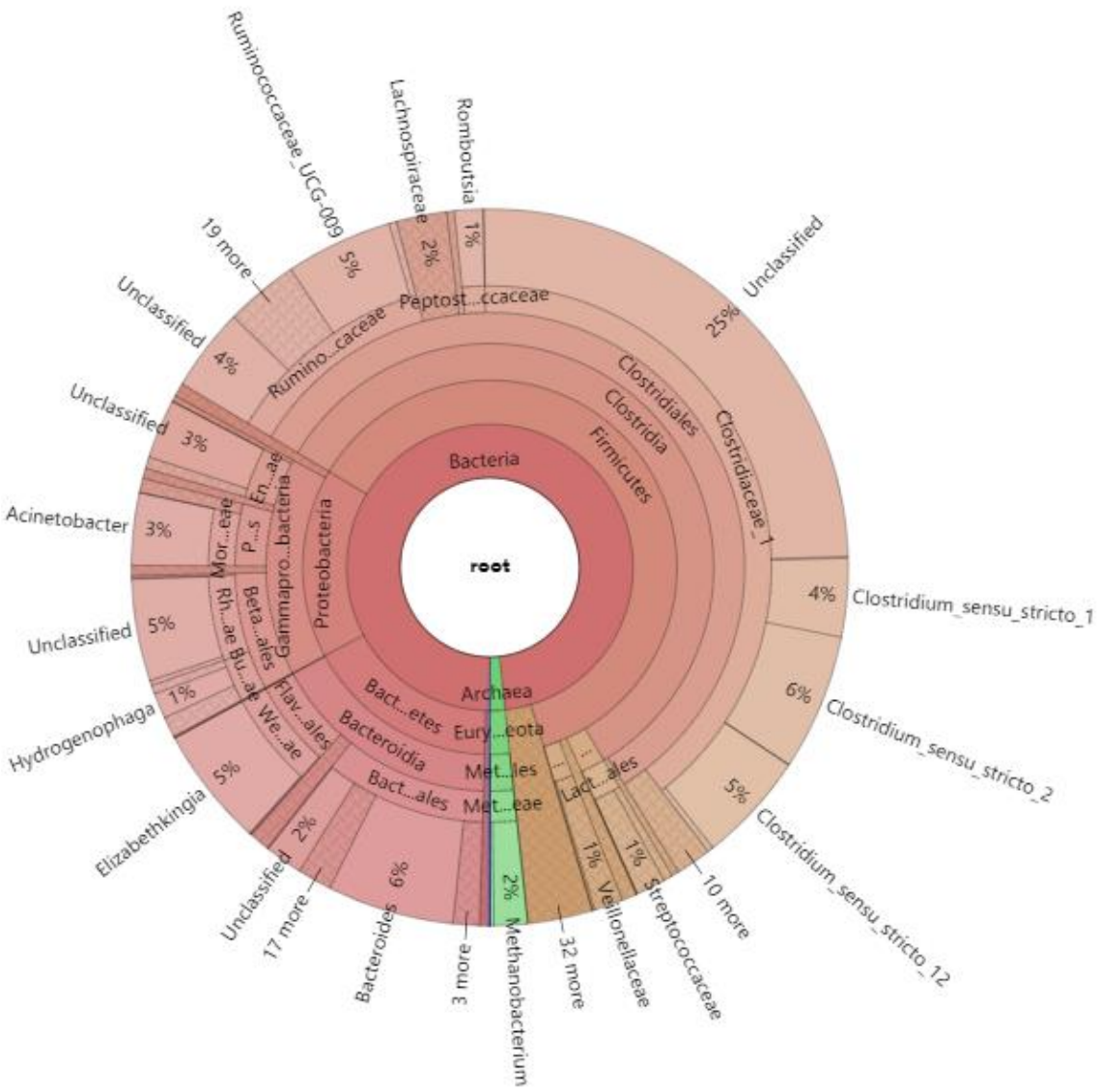

Figure 9.16. KRONA chart of sample L1.1

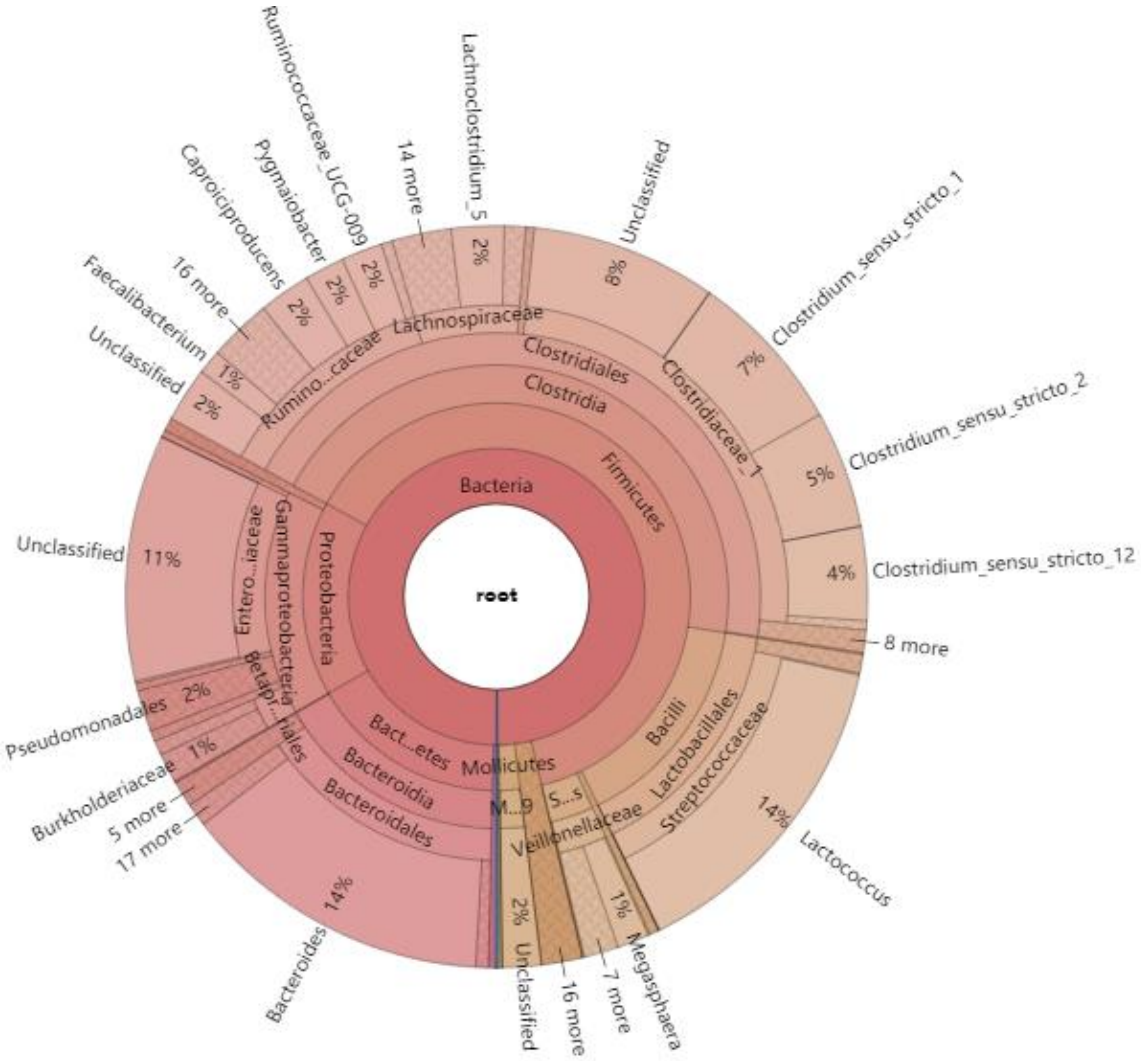

Figure 9.17. KRONA chart of sample L1.2

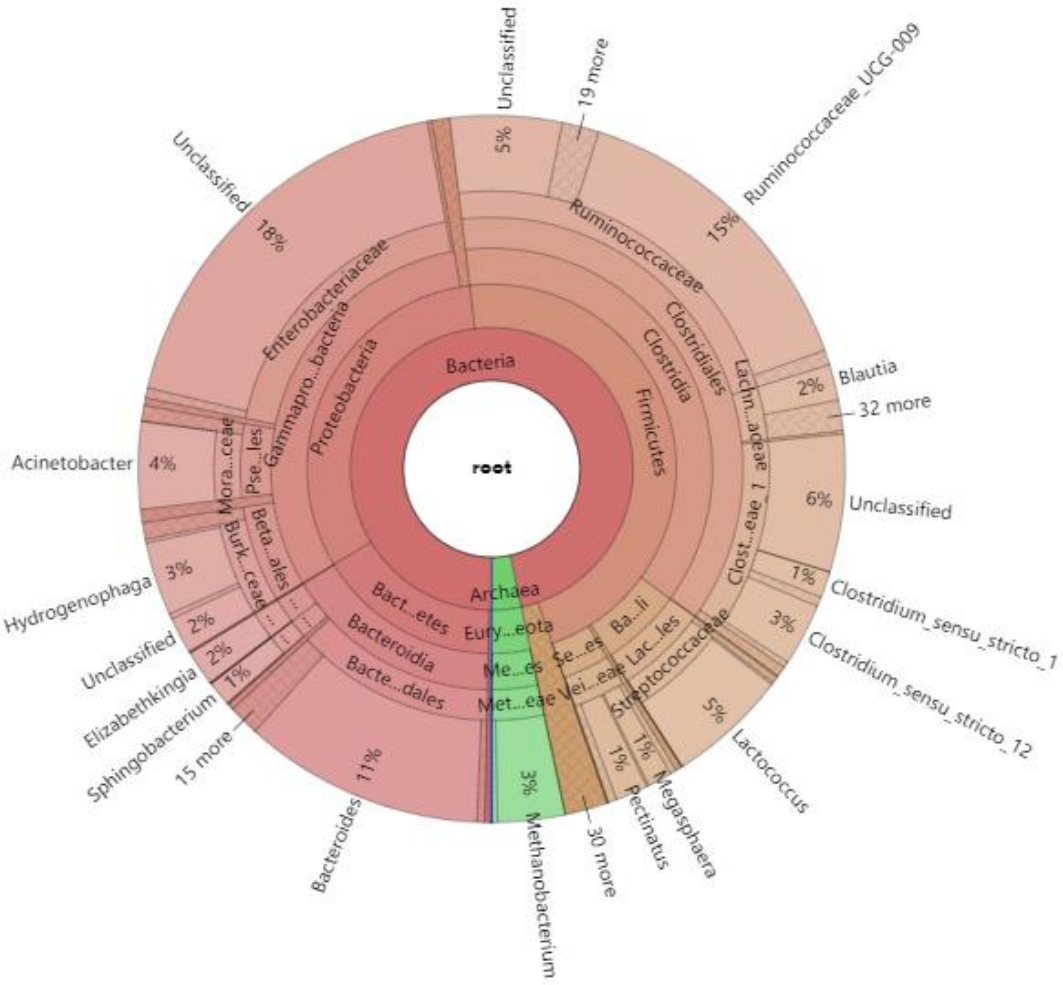

Figure 9.18. KRONA chart of sample L1.3

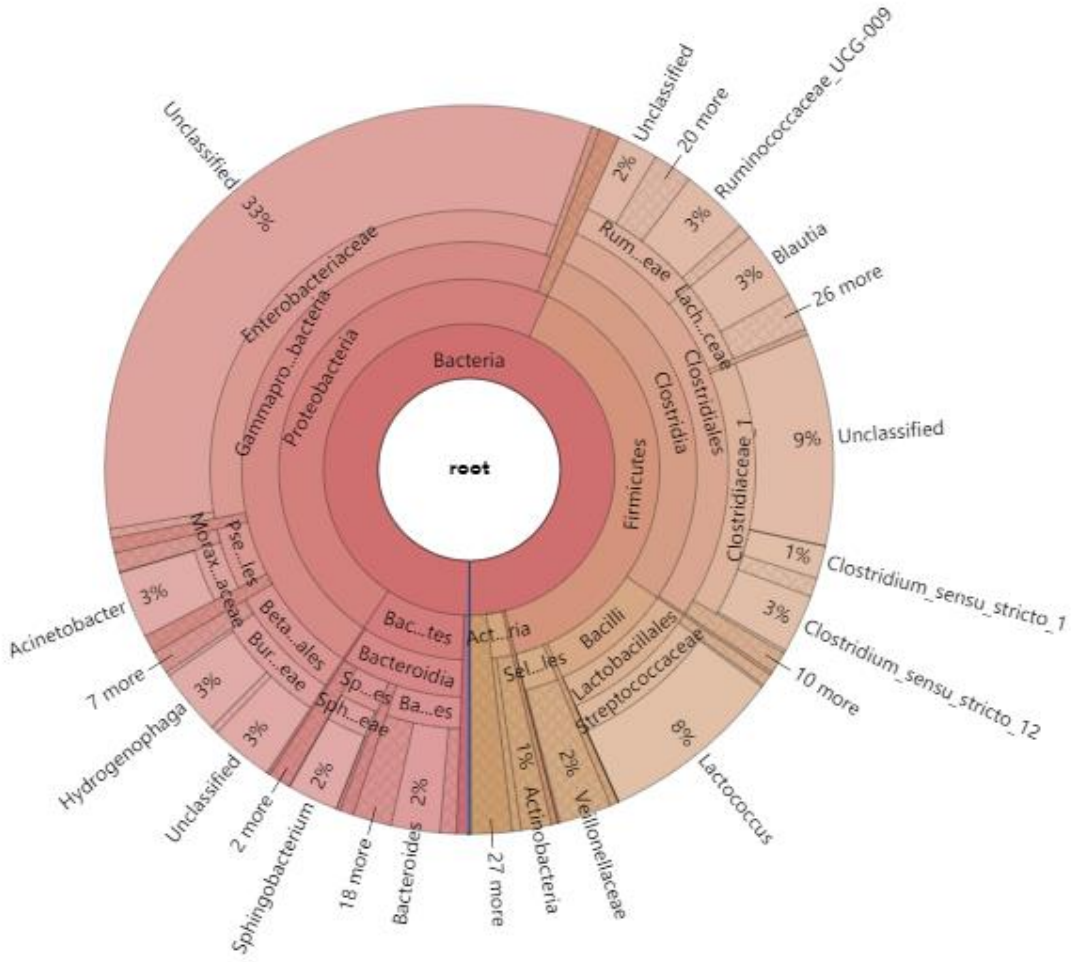

Figure 9.19. KRONA chart of sample L1.4

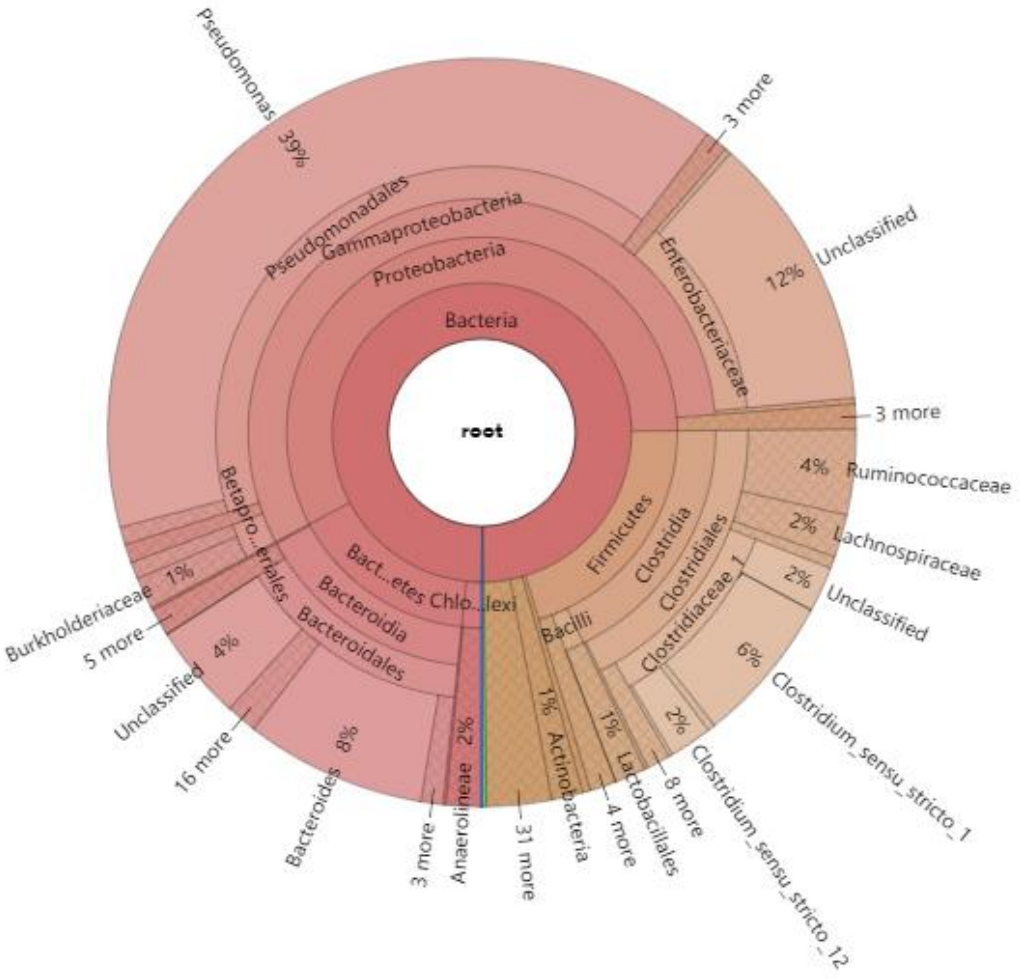

Figure 9.20. KRONA chart of sample L2.1

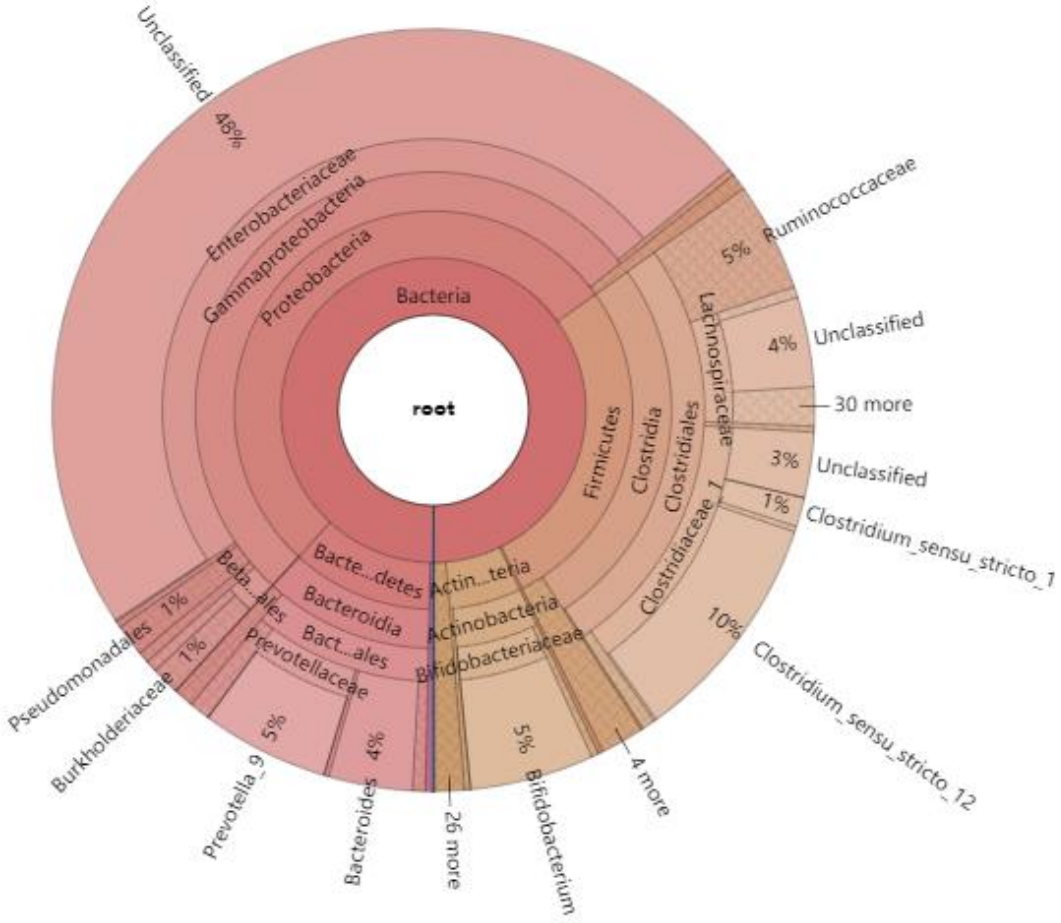

Figure 9.21. KRONA chart of sample L2.2

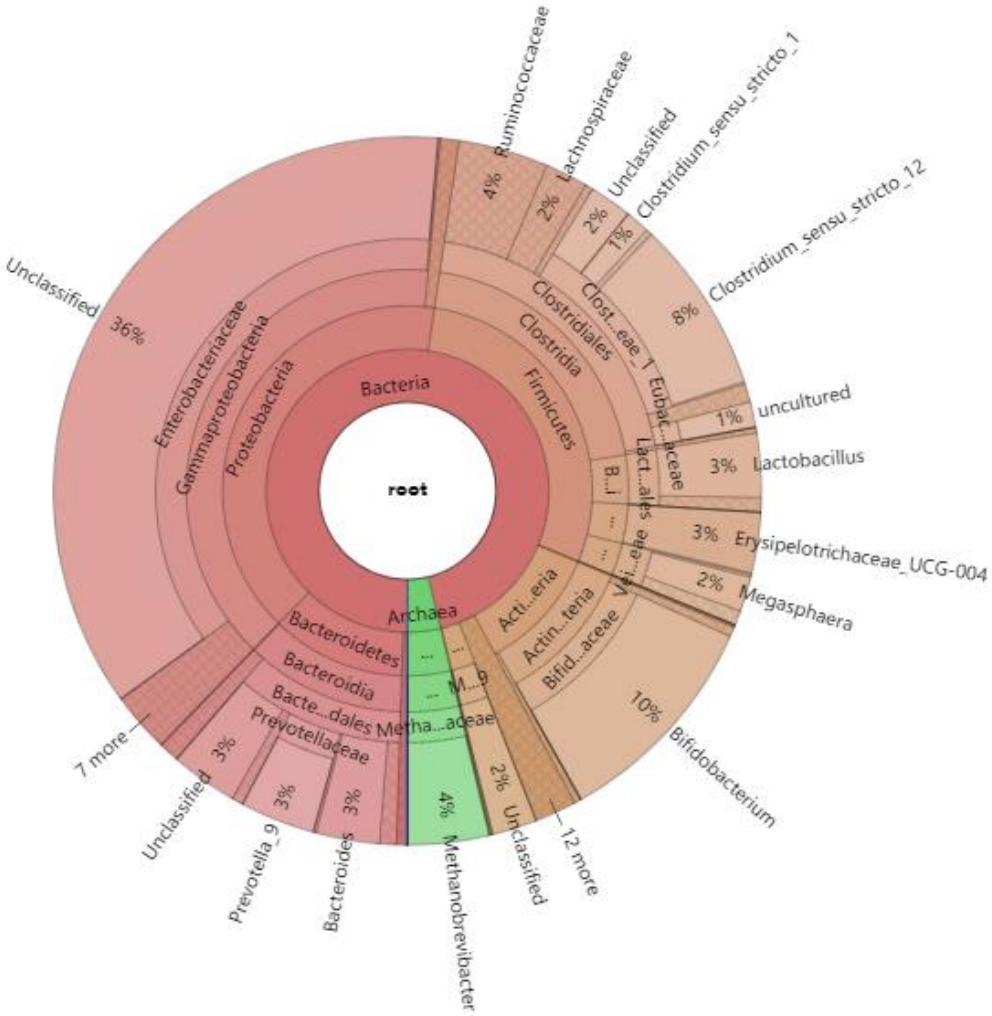

Figure 9.22. KRONA chart of sample L2.3