VOLATILE FATTY ACIDS AND METHANE PRODUCTION FROM PROTEIN

Thu Hang Duong

Propositions

1. Use of ammonium and methane as sole indicators for anaerobic protein hydrolysis leads to underestimation of the hydrolysis rate.
   (this thesis)

2. Biomass retention times determine whether VFA production from (dissolved) proteins at neutral pH is limited by hydrolysis or acidogenesis.
   (this thesis)

3. Scientists learn more from failure than success in research.

4. Scientific research without networking reduces output.

5. One has many places to go but only home to return.

6. A comment is a gift. *(inspired by Ad van Dommelen)*

Propositions belonging to the thesis, entitled

"Volatile fatty acids and methane production from protein"

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Volatile fatty acids and methane production from protein

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Volatile fatty acids and methane production from protein

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Thesis

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For my beloved family,

ABSTRACT

Many industrial wastes and wastewaters, e.g. dairy, beverage, slaughterhouse wastewater, and food processing waste streams contain appreciable quantities of protein. Anaerobic treatment is appropriate for wastewaters and wastes rich in organics to achieve pollution control and resource recovery, e.g. energy rich methane or important chemical platforms such as volatile fatty acids (VFA) or added-valuable amino acids. However, the understanding of the hydrolysis, especially of proteins, is still limited, resulting in non-optimized anaerobic systems dealing with these protein rich wastewaters. Therefore, this thesis aimed to investigate anaerobic conversion of proteins, focussing on the hydrolysis process to accomplish sufficient protein degradation for VFA and methane production. Batch and completely-stirred tank reactor (CSTR) experiments were carried out to study the effect of different environmental and microbial factors on hydrolysis and further degradation of gelatine, a model protein, at mesophilic conditions. Results show that in contrast to earlier suggestions in literature, carbohydrates did not directly affect the protein hydrolysis rates either under methanogenic or non-methanogenic conditions at neutral pH. However, the high VFA concentrations strongly inhibited the protein hydrolysis rate in the batch experiments. Methanogenesis did not stimulate the rate of hydrolysis and acidification of protein at pH 7. Yet, protein hydrolysis was inhibited at pH 5. The hydrolysis rate constant for protein at pH 5 (0.05 L g^{-1} VSS day^{-1}) was much lower than at pH 7 (0.62 L g^{-1} VSS day^{-1}). Even long-term exposure (480 days) of the microbial population to pH 5 did not result in an enhanced hydrolysis of dissolved protein. Hydrolysis always is the rate-limiting step of protein degradation at pH 5 between a solid retention time (SRT) of 12 and 30 days. At pH 7, protein degradation was limited by hydrolysis at SRTs >8 days or acidogenesis at SRTs $\leq$ 8 days. The pH also determined the VFA product spectra. Different mathematical models for kinetics of hydrolysis were tested for the steady states of the CSTR pH 7 during 600 days. The modelling results indicated that the high concentrations of amino acids may inhibit hydrolysis of proteins. The findings in this study give direction how to solve problems associated with insufficient protein degradation and how to design and operate anaerobic treatment processes for protein rich wastewaters. For methane production, the reactor design should be based on methanogenesis being the slowest process if the wastewater is mainly comprised of dissolved proteins. Regarding the VFA production, the optimum volumetric VFA productivity was 2.3 g COD_{VFA} L^{-1} day^{-1} at SRT 10 days and pH 7. Recommendations for improving the VFA productivity include design of anaerobic granular-based reactors to attain high biomass concentrations, operation at an optimum pH between pH 5 and pH 7 to avoid methanogenesis and active separation of VFA to avoid the inhibitory effect of the VFA on the protein hydrolysis.

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Chapter 1. General Introduction

1.1 Sources of protein rich waste and wastewater

Proteins are present in many kinds of waste and wastewater including sewage, sewage sludges, aquaculture and livestock, and food-related waste streams (Sayed et al., 1984, Behling et al., 1997, Palenzuela, 1999, Demirel et al., 2005, Hwang et al., 2008, Feng et al., 2009, Hassan and Nelson, 2012, Palatnik et al., 2015, Shin et al., 2015, Arslan et al., 2016). Especially, the food industry is one of the most important contributors to world economic growth and is associated with various environmental issues including resource and energy use, and waste and wastewater generation (RedCorn et al., 2018, FAO, 2020). Figure 1.1 shows the production rates of major food constituents in the world in 2020 (FAO, 2020). Asia is responsible for a large part (42%) of the food produced worldwide, and the fish, meat and dairy industry make up a substantial part (45%) of the total produce. Worldwide projections indicate a further increase of the demand for (and thus production of) food by 45% when the global population is reaching nine billion by 2050, and an increase of the water and energy demand for food processing by 30% and 45%, respectively by 2050 (FAO, 2017). Approximately one quarter of food is lost during production and processing in waste and wastewater (Kummu et al., 2012, Caldeira et al., 2019), equivalent to about 1.3 billion tons of food waste annually. The food sector is thus among the largest producers of wastewater and waste, which has significant impact on global environmental issues, i.e. global warming and climate change, and sustainable development (Olmez, 2013). For example, the European food and beverage industry shares 8-15% of total industrial water use, and is responsible for 5.3% of the industrial total energy use, 18% of the greenhouse gas emissions and 90 million tonnes of food waste disposed annually (EC, 2018).

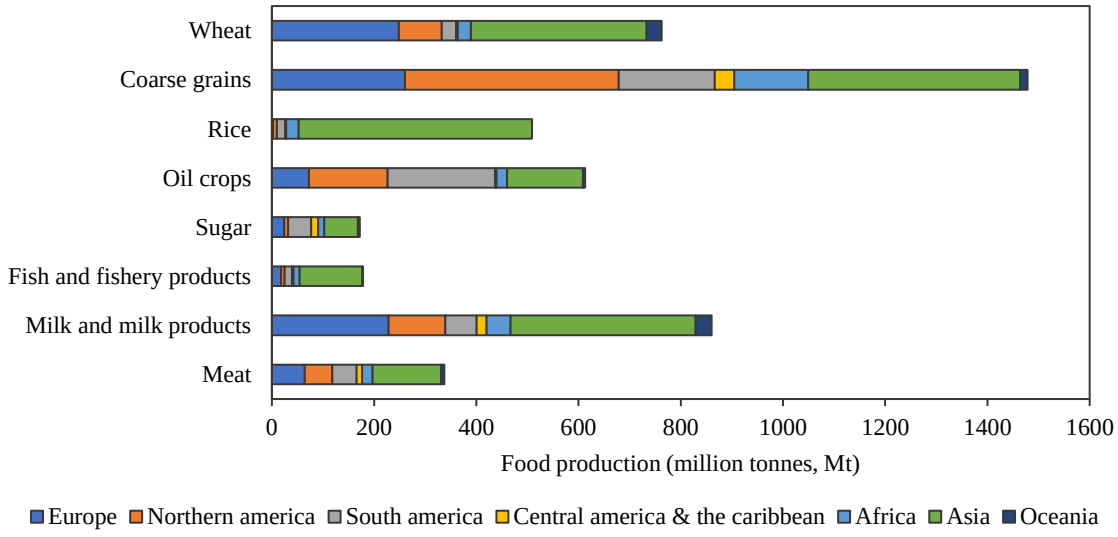

Figure 1.1. Global food production in 2020 (FAO, 2020).

Water mass balances show that about 70% of the water used by the food industry ends up as wastewater (Olmez, 2013, Valta et al., 2013), thus large amounts of food processing wastewater are generated. E.g., cheese manufacturing demands 1.1-3.6 m^3 water per m^3 of milk processed; production of potato chips consumes approximately 5 m^3 of water for each ton of raw potatoes; slaughterhouse water usage shows great variations depending on the animal and slaughter processing types, e.g. 1.5-10 m^3 for pigs, 2.5-40 m^3 for cattle and 6-30 m^3 for poultry per ton of meat (Valta et al., 2013).

Wastewater and waste generated by the food industry typically have high concentrations of organics, and thus high Biochemical and Chemical Oxygen Demand (BOD or COD) (Sayed et al., 1984, Behling et al., 1997, Palenzuela, 1999, Demirel et al., 2005, Hassan and Nelson, 2012). Protein is one of major organic constituents, accounting for 20-75% of the COD content of wastewater generated by the slaughterhouse, meat and fish-, dairy and cheese-processing industry (see Table 1.1).

Table 1.1. Protein and other organics in food industrial wastewaters

Effluent	pH	Total COD ($g\ L^{-1}$)	Protein ($g\ L^{-1}$)	Carbohydrate ($g\ L^{-1}$)	Lipid ($g\ L^{-1}$)	% Protein in COD	Reference
Slaughterhouse	6.6-6.9	7.8-15.9	4.2-10.0	-	-	70	(Sayed, 1987)
	4.9-8.1	0.5-16.0	0.3-5.3	-	-	40-75	(Bustillo-Lecompte and Mehrvar, 2017)
Meat processing	-	111±10.2	51.5±4.6	1.8±0.1	13.3	56	(Arslan et al., 2012)
Fish processing:							
Finfishes	4.2-7.9	55.2-55.4	23.0-28.8	1.9	4.4-21.6	42-52	(Palenzuela, 1999)
Invertebrates	4.2-7.9	18.5	4.1	13.7	0.7	22	(Palenzuela, 1999)
Dairy:							
Cow fluid milk	3-11	1.0-2.4	0.2-0.6	0.3-0.9	0.1-0.5	22-24	(Demirel et al., 2005)
Skimmed-milk	5-9.5	1.2	0.3	0.5	0.1	29	(Vidal et al., 2000)
Cheese (whey)	4.0-5.9	71.4	9.1	44.4	-	15	(Yang et al., 2003a)
	4.3-8.7	5.4-77.3	2.3-33.6	-	0.4-5.7	52	(Kalyuzhnyi et al., 1997)
Potato processing	-	26.4±0.7	5.9±0.2	22±16	0.2	27	(Arslan et al., 2012)
Food waste-recycling[a]	4.0±0.3	149±31	25±7	21±12	31±14	20	(Shin et al., 2015)

Note: [a]: wastewater generated from recycling food wastes for fertilizer and animal food; -: not available.

In many cases industries can partially recover protein from their waste and wastewater as added-value ingredients for food and by-products. For instance, whey proteins from cheese production are reused or recovered as food supplement (e.g. dressing) (Palatnik et al., 2015); protein extracts in fish processing wastewaters can be used for production of fish sauce, culture media preparation and animal feed (Palenzuela, 1999); animal blood collected separately from the slaughterhouse wastewater (Valta et al., 2013), can be used as food supplement, etc. However, depending on local conditions and availability of certain techniques in many food industries, still considerable amounts of proteinaceous compounds, even after recovery of some protein constituents are present in waste and wastewater.

Food industrial wastewater in Vietnam

The food industry contributes 15% to the GDP and 40% to the export in Vietnam in 2017, which is the largest share of all industrial value produced by the domestic and foreign-invested sector (EC, 2020). Main products include meat (about 4.8 Mt) and fish and seafood (8.4 Mt) products in 2018 (FAO, 2020). Vietnam ranked the 13th and 4th top production countries in meat and fish globally (FAO, 2020). The dairy industry has rapidly developed in production with an annual growth rate of about 13% since 2013, reaching 3 billion litres of raw milk in 2018 (EC, 2020). The processing of fish and meat results in protein containing wastewaters (WB, 2017). Moreover, the dairy industry involves processing raw milk into various dairy products such as milk, butter, cheese, yogurt, etc., generating large amounts of wastewater and by-products, including whey. Since a limited amount of whey is treated or used for recovery of resources at dairies in Vietnam (personal communication with a dairy company in Vietnam, 2017), this industry still disposes a significant fraction of the whey as wastewater. Consequently, there is special concern about the pollution load discharged into the environment by the food industry.

Pollution abatement and potential for resource recovery from protein rich waste and wastewaters

Discharge of wastewaters to surface waters has severe environmental and ecological impact, including eutrophication and dissolved oxygen depletion. To prevent such problems, food wastewaters commonly are treated by either aerobic processes, like activated sludge (Abdulgader et al., 2007) or anaerobic processes (van Lier et al., 2015, RedCorn et al., 2018, Nayak and Bhushan, 2019, van Lier et al., 2020). However, the costs and energy consumption

of the aerobic processes are high and the organic compounds are destroyed by mineralization into CO_2. In contrast to the aerobic treatment, during anaerobic treatment, organic pollutants are converted into methane (biogas) or short-chain (volatile) fatty acids (VFAs). Anaerobic digestion has been widely used for the treatment of organic wastewater and wastes and has the advantage that it combines pollution control with energy recovery (as biogas) or recovery of valuable metabolites such as hydrolysates and organic acids (Zeeman et al., 2008, van Lier et al., 2015, RedCorn et al., 2018, Nayak and Bhushan, 2019). In addition, high-rate anaerobic treatment has lower waste sludge production, requires a much smaller foot-print, which specially in industrial areas with limited space is a big advantage.

Protein rich waste streams have a high COD content. The COD, when converted to methane under anaerobic conditions, may contribute significantly to energy recovery from such wastewaters. Alternatively, anaerobic conversion of protein-rich waste streams potentially enables the production of important intermediates, i.e. amino acids, peptides, and VFAs (Abuine et al., 2019, Bevilacqua et al., 2020a, Regueira et al., 2020b). VFAs are chemical building blocks for production of valuable compounds in the bio-based economy such as bioplastics, biopolymers in textiles and cleaning agents (Chang et al., 2010, Kleerebezem et al., 2015, Tamis et al., 2015, Arslan et al., 2016). Besides that, free amino acids and peptides pose many nutritional benefits and physiological properties suitable for application in food, pharmaceutical and health product industries (Yang et al., 2003b, Abuine et al., 2019) provided that the products comply with e.g. health regulations. Under anaerobic conditions, amino acids, e.g. valine, leucine and iso-leucine are substrates for branched carboxylic acids formation. Fermentation of protein-rich waste streams thus may generate a rich mix of branched fatty acids, which are attractive for chain elongation towards branched medium fatty acids (Leeuw et al., 2019).

The market of VFA was recorded at 15 million tonnes in 2018 with an annual growth rate of 15% (Arslan et al., 2016, Nayak and Bhushan, 2019, Torres León et al., 2021). So far most of the VFAs required for industrial application are produced via petro-chemical processes. However, increasing awareness of their environmental effects in terms of pollution and climate change, has led to renewed interest in alternative methods for VFAs production, i.e. viz anaerobic treatment of organic waste streams. Several countries such as Canada, USA, China and The Netherlands are ambitious to replace 25-30% of petro-chemicals with bio-based

alternatives by 2030 (IEA, 2020), which can promote biological production of these compounds from food waste and wastewater.

It is clear that compared to the aerobic treatment, the possible application of an anaerobic process for treatment of protein rich wastewaters, such as food processing waste streams, is highly interesting because of the large amount of recoverable methane or high added-valuable intermediates such as VFA from digestion of these compounds (RedCorn et al., 2018). The tropical ambient temperature in Vietnam especially favours anaerobic treatment of waste streams, which makes this process very relevant for sustainable development in Vietnam and other high temperature developing countries.

1.2 Anaerobic conversion of proteinaceous wastewaters

The anaerobic conversion of complex organic matter containing proteins, carbohydrates and lipids is a sequential process, including hydrolysis, acidogenesis, acetogenesis, and methanogenesis, as shown in Figure 1.2. Microorganisms involved in anaerobic digestion include three key trophic groups: the hydrolytic acidogenic bacteria, acetogenic bacteria and methanogenic archaea. The individual steps in the degradation of proteins in anaerobic wastewater treatment systems has not been studied to a large extend, especially when compared with degradation of carbohydrates and lipids. Much of our knowledge of anaerobic protein and amino acid degradation has also been obtained through research on the intestinal systems of ruminants.

In the hydrolytic stage proteolytic acidogenic bacteria excrete extracellular proteolytic enzymes (proteases) to convert proteins into peptides and amino acids. Anaerobic sludge contains large numbers of the proteolytic bacteria, most of the isolates belong to the genus Clostridium and related organisms (McInerney, 1988). Depending on the site of action, proteases are broadly classified into two major classes such as exopeptidases (which cleave the peptide bond from the amino or carboxy termini of the polypeptide chain) and endopeptidases (which cleave internal peptide bond of the polypeptide). Any variation in the environmental conditions such as change of temperature, pH, carbon and nitrogen sources, and concentration of proteins, may influence the metabolic activity of the bacteria including protease production and also protease activity (McInerney, 1988, Sandhya et al., 2008).

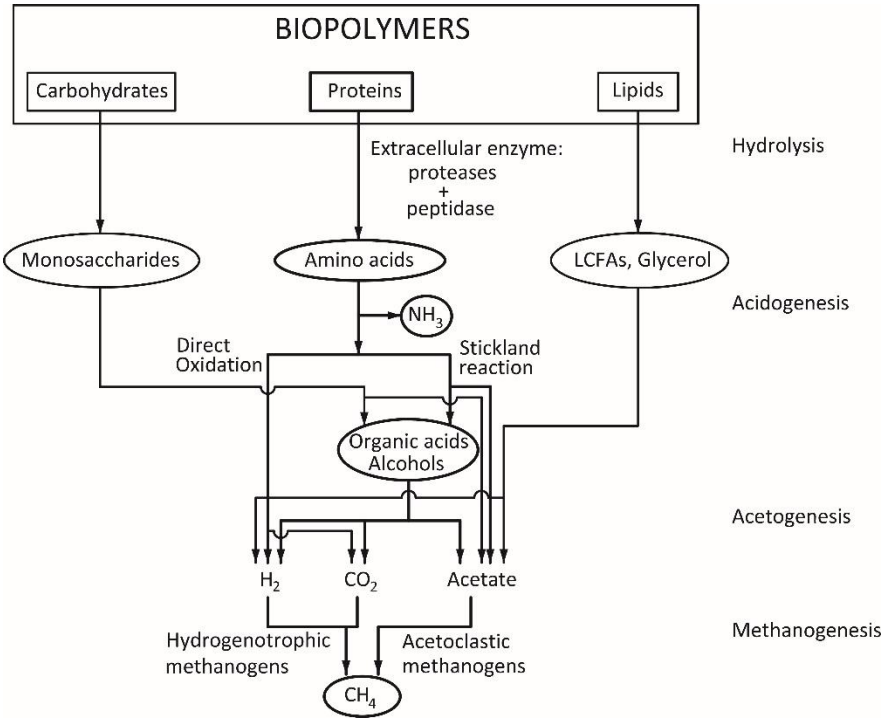

Figure 1.2. Scheme of anaerobic conversion of protein via four main consecutive steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (adapted from Barker (1981) and van Lier et al. (2020)).

During the acidogenesis, fermentative bacteria degrade the amino acids that are produced during hydrolysis further to volatile fatty acids (VFA) and alcohols, along with ammonia (NH_3), CO_2, sulphide (H_2S) and other by-products (depending on the nature of the amino acid). There are two major pathways in amino acid fermentation in anaerobic digesters: (i) single amino acids can be fermented via direct oxidation and (ii) pairs of amino acids can be degraded coupled via the Stickland reaction (McInerney 1988). Ramsay and Pullammanappallil (2001) studied the stoichiometry of the reactions involved in anaerobic protein degradation and concluded that fermentation of amino acids occurred predominantly via Stickland reactions. Amino acids can serve as electron donor (alanine, histamine or valine), electron acceptor (arginine, glycine and proline) or both (leucine, iso-leucine, phenylalanine, tryptophan and tyrosine) (McInerney, 1988, Plugge, 2001). However, the degradation model of Ramsay and Pullammanappallil (2001) excludes effects of environmental conditions such as pH and temperature, and the Stickland pathways also depend on the microorganisms present and the ratios of e-donor to e-acceptor, thus the amino acid composition in the different proteins (Plugge, 2001, Bevilacqua et al., 2020a, Regueira et al., 2020a). E.g. Plugge (2001) extensively studied syntrophic degradation of amino acids in thermophilic methanogenic granular sludge and indicated that the degradation of amino acids like glutamate is strongly affected by interspecies hydrogen transfer. Bacteria degrading amino acids in syntrophy with methanogens,

grow slower than dedicated amino acid fermenting bacteria. However, when growing syntrophically the bacterium and the methanogen converted a major part of amino acids, because methanogens can also take the role of the oxidant in the Stickland reaction (Plugge et al., 2001).

Subsequently, higher organic acids and alcohols produced by acidogenesis are further degraded to mainly acetic acid, CO_2 and hydrogen (H_2). The microorganisms participating in acetogenesis are obligate hydrogen producing acetogens and syntrophic acetogenic bacteria. Finally, during methanogenesis methane is produced through two ways: either by cleaving acetate into carbon dioxide and methane or by reduction of carbon dioxide with hydrogen (Appels et al., 2008). Hydrogenotrophic methanogenesis is essential because low hydrogen levels are required to allow, for thermodynamic reasons, direct amino acid oxidation and degradation of other VFAs i.e. butyrate and propionate to acetate (Plugge, 2001, Stams and Plugge, 2009). The presence of acetotrophic (also called acetoclastic) methanogens is equally important because accumulation of volatile fatty acids can decrease the pH and cause process failure. On the other hand, inhibition of methanogenesis may be advantageous if targeting to harvest the intermediates, i.e. VFA from protein rich wastewater.

1.3 Problems in anaerobic treatment of protein containing wastewater and waste

Anaerobic treatment is a proven technology for a variety of wastewaters since the late 1960s (van Lier et al., 2015). Low-rate reactors (e.g. completely stirred tank reactors (CSTRs)) and high-rate reactors (e.g. upflow anaerobic sludge bed (UASB) reactors) have been implemented for protein containing wastes and wastewaters (Sayed et al., 1984, Hassan and Nelson, 2012, Carvalho et al., 2013). Nevertheless, incomplete degradation of proteins, remains a problem (Bevilacqua et al., 2020b, Fra-Vázquez et al., 2020). Several researchers indicated that proteins are the main residual compounds after anaerobic treatment of wastewater (Torres, 1992, Hassan and Nelson, 2012, Khiewwijit et al., 2015b, Fra-Vázquez et al., 2020). Boe et al. (2012) and Kougias et al. (2013) reported a correlation between presence of proteins in wastewater and foam formation, even at low organic loading rates of anaerobic reactors but the effect may also be related to the presence of degradation products like amino acids, fermented organic acids and unhydrolyzed protein molecules (Kougias et al., 2013). Accumulated unhydrolyzed

proteins and their hydrophobic amino acids can also adsorb to anaerobic sludge flocs (Boe et al., 2012, Kougias et al., 2013), decreasing sludge settleability. In high rate anaerobic systems this may hinder the transport of soluble substrates to the active biomass. As a consequence foaming, biomass wash-out and a deteriorated effluent quality have been encountered during anaerobic treatment of protein-rich wastewaters (Fannin, 1987, Sayed et al., 1988, Van Andel and Breure, 1988, Perle et al., 1995, Gavala et al., 1999, Carvalho et al., 2013, Wagner et al., 2013). These problems hampered the application of anaerobic treatment for protein-rich wastewaters and narrowed the opportunity for producing valuable substances from food industry wastewaters. Improving protein degradation, i.e. the hydrolysis step and subsequently the conversion of amino acids to VFA and methane, will allow implementation of anaerobic treatment with all of its benefits for a variety of wastewaters that are relatively rich in proteins.

1.4 Hydrolysis may be the rate-limiting step in protein degradation

In general, hydrolysis of biopolymers is considered the rate limiting step in anaerobic treatment of complex biowastes. However, the hydrolytic process in the degradation of protein-rich waste streams is still poorly described. In many cases, kinetics for protein hydrolysis have been determined based on the acidification or methanization products, i.e. ammonium (NH_4-N) or methane (CH_4) (Mahmoud et al., 2004, Flotats et al., 2006, Lee et al., 2015). Hydrolysis reactions for proteins are generally expressed as first-order reactions with respect to the concentration of the biodegradable substrates and the rate constants for protein hydrolysis usually vary between 0.05 and 0.65 day^{-1} (see Table 1.2). The rate-limiting step in anaerobic degradation of particulate organic matter is normally considered to be the hydrolysis of solids (Vavilin et al., 2008), but with dissolved proteins this has not yet been studied in detail.

The variation in observed rates is due to differences in the nature (solid or soluble), particle size, structure and composition of the proteins, the cultures used, and environmental and process conditions, e.g. pH, temperature, mixing and retention times, presence of nutrients and other compounds in the complex waste and wastewater (see Table 1.2).

Table 1.2. Anaerobic first-order hydrolysis rate constants for proteins.

Substrates	k_h (day^{-1})	pH	T (°C)	Reference
Protein (particulate, in fish processing wastewater)	0.08[a]	5	30	(Palenzuela, 1999)
	0.11[a]	6		
	0.09-0.14[a]	7		
	0.11-0.18[a]	8		
Protein (particulate, in primary sludge)	0.12[b]	7	35	(Mahmoud et al., 2004)
Protein (particulate, in cattle offal)	0.28[a]	7	35	(Lee et al., 2015)
Protein (dissolved, meat peptone)	2.30[c]	7	37	(González et al., 2005)
Gelatine (dissolved)	0.65[a]	7	55	(Flotats et al., 2006)
Bovine serum albumin (particulate)	0.65[c]	7	35	(Elbeshbishy and Nakhla, 2012)
Gelatine (dissolved)	0.05[c]	5	35	this study
	0.48-0.62[c]	7	35	

Note: [a]: estimation based on ammonium (and total nitrogen) concentrations; [b]: based on methane formation concentration; [c]: based on protein concentration. Concentrations of seed sludge in previous studies: 1 g VS L^{-1} in Palenzuela (1999), 5 g COD L^{-1} in Mahmoud et al. (2004), 0.1 g VS L^{-1} in Lee et al. (2015); 3.89 g VSS L^{-1} in González et al. (2005), unknown in Flotats et al. (2006); 1.96 g VSS L^{-1} in Elbeshbishy and Nakhla (2012). Values of k_h normalized for seed sludge concentrations (g VSS L^{-1}) in this study.

1.5 Factors influencing hydrolysis of proteins

Different environmental factors, process conditions and also microbial interactions may affect protease production and catalytic activities, the availability and susceptibility of proteins to enzymatic attack and thus the hydrolysis rate of proteins during anaerobic treatment and subsequent formation of VFA and methane (Figure 1.3). These will be discussed in more detail in the following sections, while identifying knowledge gaps.

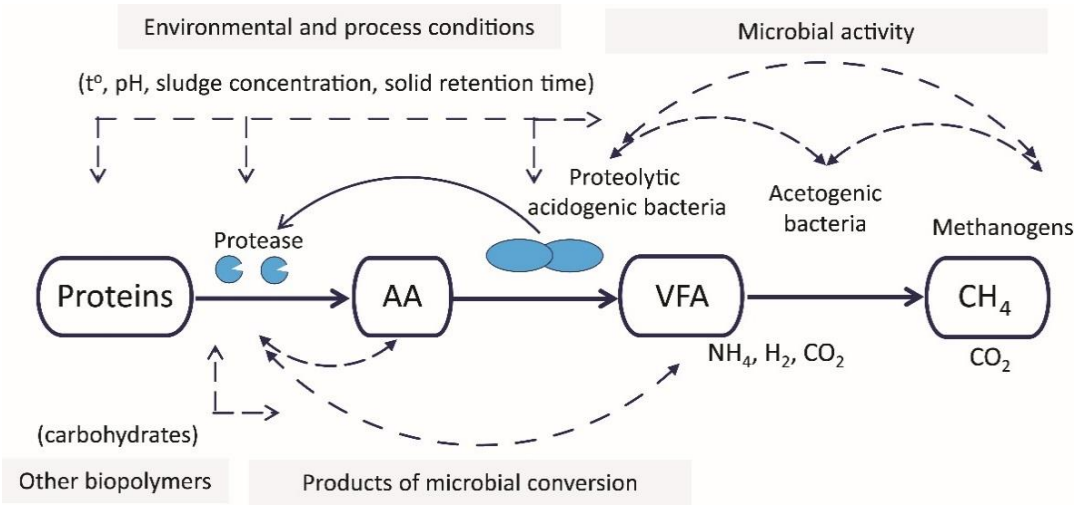

Figure 1.3. Overview of different environmental and microbial factors affecting protein hydrolysis

Note: solid lines indicate the degradation scheme; dashed lines indicate possible affecting factors. (AA: amino acids)

Environmental factors and process conditions

Temperature: Temperature affects the solubility of proteins, the growth rate of microorganisms and enzymatic activity (Sanders, 2001). O'Rourke (1968) observed a positive correlation between hydrolysis rate constants of particulate protein in primary sludge with temperature (15, 20, 25 and 35°C) at pH 7. Hydrolysis and acidogenesis of dissolved protein (gelatine) during anaerobic treatment in an upflow system at a hydraulic retention time (HRT) of 12 hours and pH 5.5 was reported to slightly increase with temperature in the range of 20°C to 55°C (Yu and Fang, 2003).

pH: Protein hydrolysis is strongly dependent on pH because of its effect on protein solubility, charge, molecular structure (primary - quaternary structure), enzymatic activity and the overall growth rate of the relevant microorganisms. For example, when the pH of dairy wastewater approaches the isoelectric point of casein (about 4.6), casein micelles start to grow, aggregate and form a dense coagulum, resulting in the susceptibility of the protein for enzymatic attack (Zeeman et al., 1997). Fra-Vázquez et al. (2020) reported limited protein conversion at pH range of 4.2 and 4.9 in cooked mussel processing wastewater treatment.

So far, knowledge about specialised microbes that can anaerobically grow on protein (and protein degraded products) at low pH is rare. Most hydrolytic acidogenic bacteria have an

optimum pH between 5 and 7 (Glenn, 1976, Azman, 2016). However, this is lower than the optimum pH range observed for proteases. E.g., hydrolysis of proteins was reported to be favoured at neutral or slightly alkaline pH (6-8) (Breure and Van Andel, 1984, Gallert and Winter, 1997, He et al., 2005). This was in agreement with Palenzuela (1999) who showed that protease activity in fish wastewater increased when the pH was increased from 4 to 9. Similar pH effects were observed during mesophilic co-fermentation of waste activated sludge and rice by Feng et al. (2009). The bacterial growth rate, protease activity and hydrolysis rates in the rumen were also found to increase when the initial pH was changed from 5 to 7 (Mouriño et al., 2001).

Sludge concentration: For dissolved protein such as gelatine, the mechanism of the enzymatic hydrolysis can be described as a random depolymerisation process and the hydrolysis rate is linearly related to the sludge concentration in batch experiments (Sanders, 2001). In contrast, Sanders (2001) observed that for particulate substrates the rate of hydrolysis of protein particles is restricted by the surface of the substrate and is not related to the sludge concentration.

Sludge retention time (SRT): Because acidogens are faster growing organisms than methanogens the SRT (often in combination with other environmental conditions) will determine the prevailing conditions, viz acidogenic or methanogenic. E.g. Miron et al. (2000) showed that during digestion of primary sludge in a CSTR operated at 25°C, acidogenic conditions and pH of 5-6 could be maintained at SRT<8 days, while methanogenic conditions and pH 7 prevail at higher SRTs; proteins in this type of sludge only were degraded at the latter conditions. Short SRTs are economically more favourable but often lead to process instability and biomass wash-out. An extended SRT, at a certain temperature, may be needed to obtain sufficient hydrolysis of proteins. However, under those conditions it may be difficult to recover VFAs, since growth of methanogens is hard to avoid at a long SRT (Khiewwijit et al., 2015b), and desired VFA may be degraded. An optimum SRT for protein hydrolysis and VFA production from protein rich wastewater has not been identified yet.

Microbiological activity and products of microbial conversion

Proteolytic acidogenic bacteria: The acidogens produce the necessary extracellular proteolytic enzymes and degrade the products of hydrolysis. The degradation of proteins in anaerobic

environments, i.e. anaerobic reactors is probably similar to that found in the rumen. In the rumen, the bacteria attach to the particulate proteins, followed by activity of cell-bound microbial protease (Brock et al., 1982).

Methanogenic activity: In connection to the favourable pH for protein hydrolysis discussed above, it is not known yet whether the absence of methanogenic activity or low pH conditions may inhibit protein hydrolysis. Palenzuela (1999) observed similar hydrolysis rates of particulate fish proteins, regardless of the presence of methanogenic seed sludge or a methanogenesis inhibitor (2-bromoethanesulfonate, BES) in CSTR systems operated at 30°C and a pH of 4 to 7. In contrast, Miron et al. (2000) found that protein hydrolysis in primary sludge did not occur at acidogenic conditions at low pH (5-6) in a CSTR at 25°C. As such, the role of methanogenic activity in the protein hydrolysis in anaerobic reactors is unclear.

Effect of protein hydrolysates (amino acids and peptides): Accumulation of amino acids from protein hydrolysis has been identified early as an inhibitor of the production of proteases and also to have a negative influence on the protease activity (Glenn, 1976). In addition, Palenzuela (1999) and Miron et al. (2000) suggested protein hydrolysis might be negatively affected by amino acid concentrations (higher than 0.2 g L^{-1}). In fact, amino acid and peptides concentrations were rarely measured in previous research dealing with protein-rich wastewater and wastes. More knowledge is available from protein hydrolysis in food research, in the human gut and in the rumen of livestock ruminants (Sales-Duval et al., 2002, Abdel Hamid et al., 2017, Deng, 2018). Non-polar (hydrophobic) amino acids from fish skin hydrolysates (gelatine) were demonstrated as enzyme inhibitors to several proteases, like e.g. ACE (angiotensin-I converting enzyme) (Bar-Even et al., 2011). Free amino acids from casein hydrolysates decreased proteolytic activity and protease formation of several ruminal bacteria (Sales-Duval et al., 2002). Thus, it is rational to speculate that accumulation of these protein hydrolysates may inhibit protein hydrolysis in degradation of protein containing (food) wastewater as well.

Effect of acidogenesis products (VFA and ammonia): Inconsistent information about the effect of VFA and ammonia on hydrolysis can be found in literature (Zeeman, 1991). VFA accumulation due to fermentation of easily biodegradable substrates, such as lactose in dairy wastewater, induces a pH decrease that can inhibit the hydrolysis rate of proteins. Veeken et al. (2000) found little effect of VFA concentrations up to 30 g COD L^{-1} on the hydrolysis of organic

solid waste at pH values of 5-7. In contrast, hydrolysis of dissolved peptone is strongly inhibited by acetic acid (0.25-2) g L^{-1} at pH in a range from 5-7 under mesophilic and saline conditions (González et al., 2005). Acidogens are less prone to inhibition by undissociated acetic, propionic or butyric acids than methanogens. Inhibitory thresholds towards acidogenic microbes were reported as i.e. 0.7 g L^{-1} for undissociated acetic acid (González et al., 2005), and 0.4 g L^{-1} for propionic acid and 0.3 g L^{-1} butyric acid (Xiao et al., 2016).

Ammonium and ammonia which are released during acidification of amino acids can act as a pH buffer. However, high concentrations of ammonia inhibit methanogenesis (Zeeman, 1991, Miron et al., 2000, Nakakubo et al., 2008), so if methanogenic activity would be important for protein hydrolysis, the effect of ammonia cannot be excluded. On the other hand, Palenzuela (1999) indicated ammonium of 0.6-1.5 g NH_4^+-N L^{-1} did not inhibit hydrolysis of protein in fish processing wastewaters at pH 6.7-7.0 and mesophilic conditions. Ammonia and ammonium concentrations are also influenced by pH and temperature conditions, thus their effect is associated with the specific environmental conditions. Moreover, the ammonium tolerance level of sludge is dependent on its adaptation (Fernandes et al., 2012).

Effect of ionic strength: Increased concentrations of dissociated acidified products contribute to the ionic strength in the reactors. The ionic strength may affect the activity of protease by changing the electron flows, stability and solubility of the enzyme as well as those of the protein structure (Xu et al., 2013, Carvalho et al., 2019).

Effect of other biopolymers

Effect of carbohydrates: Carbohydrates and proteins are dominant in most food related wastewater and waste, e.g. dairy, beverage and food-processing effluents (see Table 1.1). Some authors indicate that carbohydrates have an effect on anaerobic protein degradation via suppression of the synthesis of exopeptidases, a group of enzymes facilitating protein hydrolysis (Breure et al., 1986a, Breure et al., 1986b). Carbohydrates are reported to be preferentially degraded over protein (Breure et al., 1986a, Breure et al., 1986b). E.g., Yu and Fang (2001) observed that the protein concentration did not decrease until the carbohydrates were depleted (within two days) in a batch acidification experiment of mid- and high synthetic dairy wastewater (2-30 g COD L^{-1}, carbohydrate to protein ratio of 1.58) at pH 5.5 and 37°C. In contrast, Tommaso et al. (2003) observed that hydrolysis efficiency of bovine serum albumin

(BSA) was not negatively affected by the presence of carbohydrate in an experiment with a horizontal-flow immobilized biomass anaerobic reactor (HRT of 4 hours, temperature of 30±1°C), which was inoculated with granules from a UASB reactor treating poultry slaughterhouse wastewater. Since the observations were inconsistent and often contradictory, the effect of carbohydrates on protein degradation in anaerobic wastewater treatment systems is still largely unknown and needs more detailed investigation.

1.6 Scope of this thesis

The advantages of anaerobic treatment of protein-rich wastewaters were earlier discussed (section 1.1). This concept is extremely relevant for Vietnam where food production is of significant economic importance and the climate favours anaerobic treatment. So far research activities geared mainly towards anaerobic digestion of complex bio-wastes and particulate materials, and anaerobic degradation of (dissolved) proteins only has been investigated in few studies. The consequence of interaction between biopolymers such as carbohydrates and proteins is largely unknown; existing results of research are conflicting. Objectives of this thesis is to increase the knowledge on anaerobic conversion of proteins, focussing on the hydrolysis step for reactor design and operation to accomplish sufficient protein degradation. Understanding the degradation of proteins and in particular the hydrolysis step with respect to kinetics of the process and production of VFA and/or subsequent methane production under different environmental and process conditions will play a key role. The accomplished scientific knowledge will result in improved process design, enhancing the performance of anaerobic conversion of protein rich wastewater/waste.

Batch and CSTRs were used to study hydrolysis kinetics and affecting factors on hydrolysis of protein, and also to evaluate alternative reactor concepts. Chapter 2 presents results of batch experiments carried out under mesophilic conditions aimed to study the effect of pH and occurrence of methanogenesis on anaerobic hydrolysis and amino acid fermentation of gelatine, which was used as a model for dissolved proteins. In Chapter 3, the effect of starch (a model carbohydrate) and fermentation product (VFA) concentrations on protein hydrolysis and degradation is explored in batch experiments at methanogenic and non-methanogenic conditions. The results of Chapters 2 and 3 strongly suggest that hydrolysis of protein is

suppressed by the low pH and by presence of VFA, but not by the presence of methanogenesis or carbohydrates.

In Chapter 4, the possibility of enhanced protein hydrolysis at low pH after long-term exposure of biomass to pH 5 in a CSTR is studied and compared with reactor performance at neutral pH. Different SRTs were applied while maintaining a high protein concentration in the influent. The kinetic behaviour, accumulation of amino acids and recovery of products are considered in the design and operation of anaerobic systems treating protein containing wastewater. In the continuous reactors receiving high protein concentrations, the presence of amino acids during protein degradation at short SRT correlated with limited protein hydrolysis. Chapter 5 discusses the potential inhibitory effect of these protein hydrolysates viz. amino acids, on protein hydrolysis and compares different mathematic kinetic models.

From the knowledge that is gained, implications for the design and operation of anaerobic systems treating protein rich wastewaters and wastes to harvest CH_4 or VFA are discussed in Chapter 6. A case study in Vietnam with two different protein rich wastewaters (slaughterhouse and meat processing wastewater and whey containing wastewater) was taken as the example to illustrate the potential of anaerobic resource recovery. Finally, recommendations and suggestions are given for future research.

Chapter 2. Protein hydrolysis and fermentation under methanogenic and acidifying conditions

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Abstract

Many kinds of wastewaters contain appreciable quantities of protein. Anaerobic processes are suitable for the treatment of wastewater high in organics to achieve pollution control and recovery of energy as methane and hydrogen, or intermediates for production of biofuels and valuable biochemicals. A distinction between protein hydrolysis and amino acid fermentation, especially for dissolved proteins, is needed to target which step is truly rate-limiting and to effectively harvest bioproducts during anaerobic conversion of these wastewaters. This study explored mesophilic anaerobic hydrolysis and amino acid fermentation of gelatine, as a model for dissolved proteins, at pH 7 and at pH 5. The results showed that at pH 7 protein hydrolysis (first-order rate of 0.15 h^{-1}) was approximately 5 times faster than acidification of the hydrolysis products (first-order rate of 0.03 h^{-1}), implying that not hydrolysis but acidification was the rate limiting step in anaerobic dissolved protein degradation. This was confirmed by (temporary) accumulation of amino acids. Nineteen different amino acids were detected during the first 8 incubation hours of gelatine at neutral pH and the total chemical oxygen demand (COD) of these nineteen amino acids was up to approximately 40 % of the COD of the gelatine that was added. Furthermore, the presence or absence of methanogenic activity did not affect the rates of protein hydrolysis and acidification. Still, protein hydrolysis was suppressed at pH 5. Shifting the initial pH from neutral to acidic conditions (pH 5) inhibited protein degradation and changed the volatile fatty acids (VFA) product profile. Based on the findings in this study, a solid retention time for anaerobic degradation of protein rich wastewaters in continuous reactor systems can be defined to accomplish appropriate treatment requirements without over-estimation.

Keywords: Proteins, hydrolysis, amino acid fermentation, methanogenic conditions, non-methanogenic conditions.

2.1 Introduction

Anaerobic digestion is widely used for the treatment of high strength wastewaters and organic wastes since it can combine pollution control with the recovery of methane or hydrogen as a green source of energy. Besides, volatile fatty acids (VFAs), important intermediates in anaerobic processes, recently have gained a lot of attention because they are platform chemicals for the production of more valuable compounds such as bio-based plastics, medium chain length fatty acids and other organic acids for bio-electrochemical systems (Bengtsson et al., 2008, Chang et al., 2010, Kleerebezem et al., 2015, Tamis et al., 2015). Proteins are one of major compounds in wastewaters and wastes. Proteins account for 20-40% COD (chemical oxygen demand) in domestic wastewater, and up to 60-75% COD in sewage sludge and food wastewaters such as from the dairy, beverage, slaughterhouse and fish-processing industry (Yuan et al., 2006, Hassan and Nelson, 2012, Carvalho et al., 2013, Liu et al., 2015, Ma et al., 2016, Arantes et al., 2017, Handous et al., 2019). Microorganisms cannot take up proteins directly, but need extracellular proteases to cleave proteins in amino acids and small peptides, which can be subsequently taken up, metabolized to volatile fatty acids, ammonium and sulfide under acidogenic conditions and finally converted to methane under methanogenic conditions (Tang et al., 2005). Amino acids and peptides can also be used to synthesize cell proteinaceous matter, in particular when sufficient energy is present in the form of carbohydrates (Bach et al., 2005).

Anaerobic degradation of proteins is reported to be slower compared to degradation of other biopolymers (Breure and Van Andel, 1984, Breure et al., 1986a, Breure et al., 1986b, Yu and Fang, 2001, Arslan et al., 2016). For example, carbohydrates are considered to be favourable acidified than proteins in dairy wastewater (Yu and Fang, 2001). Similarly, Khiewwijit et al. (2015b) observed that proteins were the main residual compounds after anaerobic treatment of domestic wastewater. Also, protein containing wastewaters have been reported to result in low biogas yields, foaming and biomass wash-out and a deteriorated effluent quality (Perle et al., 1995, Hassan and Nelson, 2012, Tanimu et al., 2015). Information about protein fermentation is insufficient. Proteins such as gelatine and casein were observed to be hydrolysed only to a minor extent under acidic conditions, either because of a reduced protease activity at low pH (Breure and Van Andel, 1984, Breure et al., 1986a, Breure et al., 1986b, Yu and Fang, 2001) or because of a lack of methanogenic activity under these conditions (Miron et al., 2000). Sasaki et al. (2011) observed that thermophilic acidification of protein (gelatine, casein and bovine

serum albumin) was enhanced by the presence of hydrogen-scavenging methanogens. Besides, presence or accumulation of intermediates as acetic acid during anaerobic degradation of dissolved protein could reduce protein hydrolysis rate in an anaerobic, mesophilic saline environment (González et al., 2005) .

Most studies concluded that protein hydrolysis is the rate-liming step while subsequent amino acid fermentation was fast (Ramsay and Pullammanappallil, 2001, Flotats et al., 2006, Vavilin et al., 2008). However, this conclusion may be questionable because it was based on ammonium release rates, which does not allow for a distinction between protein hydrolysis and amino acid fermentation (Flotats et al., 2006, Vavilin et al., 2008). Free amino acids and peptide concentrations were rarely measured in studies focussing on anaerobic treatment of protein-rich wastewater. Although Breure and Van Andel (1984) and Miron et al. (2000) mentioned the presence of amino acids during protein degradation, they did not sufficiently quantify their concentrations to be able to compare hydrolysis and acidification rates. More knowledge is available from protein degradation in the rumen. Broderick et al. (1991) observed accumulation of peptides and amino acids within the first 2 hours after feeding ruminal bacteria with silages. Later, Cardozo et al. (2004) found in continuous fermenters receiving a daily diet of forage considerable concentrations of peptides and amino acids after 8 hours feeding. These findings indicate that amino acid fermentation or deamination could be the rate liming step during anaerobic protein degradation.

In the present study we explored the hydrolysis and degradation of gelatine as a model dissolved protein under methanogenic and non-methanogenic conditions at a neutral pH, and at a low pH of 5. For this purpose batch experiments were employed with an inoculum taken from a continuous fermenter that was fed with milk to represent a microbial population adapted to wastewater from the dairy industry. Protein degradation was followed in time, not only based on COD concentrations and gas production, but also the protein concentration and the amino acid and VFA concentration and composition.

2.2 Materials and Methods

2.2.1 Experimental set-up

2.2.1.1 Substrate

The model protein was gelatine, CAS no. 9000-70-8 (Merck, for microbiology, 1.04070.0500). The gelatine was completely dissolved in heated demi-water (40-50˚C). The pH of the gelatine solution was 5.0-5.5 and the concentration of gelatine below 2% to ensure a random coil configuration of gelatine and negligible electrostatic disturbance that might change the protein structure (Mao et al., 2006). It was shown by others that the pH in the experimental range of 5-7 did not influence gelatine structure and solubility (Mattison et al., 1995). The main characteristics of the gelatine are shown in Table 2.1.

Table 2.1. Main characteristics of the protein used in this experiment.

Characteristics	g TS	g VS	g COD	g TN
Protein (Gelatine)	0.953±0.004	0.952±0.004	1.150±0.013	0.139±0.001

Data are measured per gram gelatine and expressed in mean ± standard (n=10).

2.2.1.2 Inoculum and nutrient medium

The seed sludge for the batch tests was harvested after an operational period of 150 days from a continuous fermenter that was operated at a volumetric loading rate of 2 g COD L^{-1} d^{-1} and was fed with fresh milk. The fermenter had a working volume of 10 L (total volume 14 L), and was operated at 35°C. The pH in the reactor was 7.3. The total solids (TS) and volatile solids (VS) of the sludge were 19 g L^{-1} and 12 g L^{-1}, respectively. The nutrient medium for the batch tests was adapted from Angelidaki et al. (2009) except that NH_4Cl was not added because sufficient nitrogen was already present in the sludge inoculum (total nitrogen (TN) of 3.8 g L^{-1} and NH_4-N of 3.6 g L^{-1}). Each liter of the nutrient medium at pH 7 contained 2.18 g Na_2HPO_4; 1.06 g KH_2PO_4; 48 mg $CaCl_2.2H_2O$; 54 mg $MgSO_4.7H_2O$; 1.2 mg $FeCl_2.4H_2O$; 1.2 mg $CoCl_2.6H_2O$; 0.3 mg $MnCl_2.4H_2O$; 0.018 mg $CuCl_2.2H_2O$; 0.03 mg $ZnCl_2$; 0.03 mg HBO_3; 0.054 mg $(NH_4)_6Mo_7O_{24}.4H_2O$; 0.06 mg $Na_2SeO_3.5H_2O$; 0.03 mg $NiCl_2.6H_2O$; 0.6 mg EDTA (tripex II); 0.216 ml HCl 36%; 0.3 mg Resazurin. The medium at pH 5 was prepared to be identical to the medium at pH 7, except KH_2PO_4 (3.128 g.L^{-1}) and none of Na_2HPO_4.

2.2.1.3 Anaerobic batch experiments

The experiments were carried out in triplicate at 35°C in 2.6 L side-port-bottles (liquid volume of 0.62 L), which were continuously shaken at 60 rpm for 300 hours. Three different sets of test bottles were prepared: (i) Gelatine bottles at pH 7 with a gelatine concentration of 1.46 (± 0.015) g COD L^{-1} and an inoculum of 7.0 (± 0.05) g VS L^{-1} (non-adjusted pH of the culture); (ii) Gelatine-BES bottles similar to the protein pH 7 bottles but with addition of 2-Bromoethanesulfonate sodium (BES, 0.02M) to inhibit growth of methanogens; (iii) Gelatine-pH 5 bottles similar to the Gelatine-pH 7 bottles with addition of hydrochloric acid (HCl, 0.075 M) to obtain a pH of 5. Blanks were prepared with seed sludge and medium but without gelatine. Before they were closed all the bottles were well-mixed, sampled for initial concentrations and flushed with N_2 gas for 30 minutes.

An additional test was conducted with (i) Gelatine and (ii) Gelatine-BES (pH 7) amended with NaCl (± 0.075 M) to verify that chloride (Cl^-) at this concentration in Gelatine-pH 5 did not have a negative effect on gelatine hydrolysis and degradation.

2.2.2 Sampling and analyses

During the first 8 hours gas and liquid samples were taken from the bottles with an interval of 2 hours. Afterwards, six more samples were taken from all bottles at 17, 23, 29, 48, 96, and 240 hours after start of the incubation. Five additional samples were taken from Gelatine-pH 5 bottles after 120, 144, 168, 192, and 264 hours.

pH was measured by a pH meter (Hach, PHC 101, Seri No.162822568077, USA). The sludge samples were centrifuged (Eppendorf, Germany) at 10000 rpm for ten minutes and filtered with pre-washed 0.45 µm cellulose acetate membrane filters (Sartorius, Germany). The soluble fraction was analyzed for chemical oxygen demand (CODs), total nitrogen (TN) and ammonium (NH_4-N) using Hach Lange methods and test kits (LCK1014, LCK338, LCK303). Protein was determined using the Lowry method assay (Noble and Bailey, 2009) at 660 nm using gelatine as standard. Volatile fatty acids (VFAs) were quantified on a Trace gas chromatograph equipped with a Thermo TR-WAX column (30m x ID 0.32 mm x thickness of 0.25 µm) connected to a FID detector as described by Sudmalis et al. (2018). Amino acids were measured in the supernatant samples as described by Meussen et al. (2014) via high-

performance liquid chromatography (HPLC). Carbohydrate was determined by the phenol-sulfuric acid method (Dubois et al., 1956) at 490 nm using glucose as standard. Carbohydrate concentrations were only measured at 0, 17, 48 and 240 incubation-hours from all the bottles to verify the negligible effect of the presence of carbohydrate. It was confirmed that carbohydrate did not have an effect on protein hydrolysis and cell synthesis because the carbohydrate concentrations in all the bottles were identical at 0.04-0.05 g COD L^{-1} and did not change over the time.

Gas pressure in the head space, as a measure for biogas production, was determined by TSI Certifier FA Plus (USA, model 4088A, SN 40880735005). Gas composition (CH_4, CO_2, H_2 and N_2) was quantified by gas chromatography-8A (Shimadzu, Japan) equipped with a compact materials Unibeads C 60/80 mesh column (Φ3mm, length 2m) connected to a thermal conductivity detector (argon as carrier gas). Sludge inoculum and gelatine substrate solution of the batch tests were analysed for TS and VS using standard methods (APHA-AWWA-WEF, 2017).

2.2.3 Calculations

The rate of protein hydrolysis depends on the sludge concentration. However, in the batch tests a constant sludge concentration was applied. Therefore, to be able to compare hydrolysis rates first-order hydrolysis kinetics were assumed as proposed by Batstone et al. (2002) and the concentration of hydrolyzed protein in time was described by:

$$P_{hydrolyzed}(t) = P_{added} \cdot (1 - \exp(-k_h \cdot t)) \qquad \text{(eq. 2.1)}$$

With $P_{hydrolyzed}(t)$ the (cumulative) concentration of hydrolyzed protein (g COD L^{-1}) after time t hours, P_{added} the initial concentration of protein (g COD L^{-1}), and k_h the first-order hydrolysis rate constant (h^{-1}). The protein concentration was calculated as COD from the measured soluble protein concentration using a conversion factor of 1.115 g COD g^{-1} gelatine (Table 2.1).

Subsequent acidification of the hydrolysis products was also described by first-order kinetics according to:

$$P_{acidified}(t) = P_{end-acidified} \cdot (1 - \exp(-k_a \cdot t)) \quad \text{(eq. 2.2)}$$

Where $P_{acidified}(t)$ is the sum of the measured VFA concentration and methane production after time t hours, both expressed in g COD L^{-1}, $P_{end-acidified}$ is the sum of the measured VFA concentration and methane production at the end of the tests (in g COD L^{-1}) and k_a is the first-order acidification rate constant (h^{-1}).

2.3 Results and discussion

2.3.1 Effect of methanogenic conditions on gelatine degradation

Previous research indicated that methanogenesis stimulates anaerobic protein degradation (Miron et al., 2000, González et al., 2005, Sasaki et al., 2011). Figure 2.1 shows the hydrolyzed and acidified gelatine concentrations at pH 7 (left) and at pH 7 where methanogenesis was inhibited with 2-bromoethanesulfonate (BES), (right), which was confirmed by a lack of methane production. In both cases hydrolysis as well as acidification could be described by the first-order kinetics of equations (2.1) and (2.2), see in section 2.2.3. Interestingly, in contrast to the literature the occurrence of methanogenesis did not affect the hydrolysis and acidification rate. This observation was confirmed from amino acid measurements, as will be explained in section Amino acid production and fermentation.

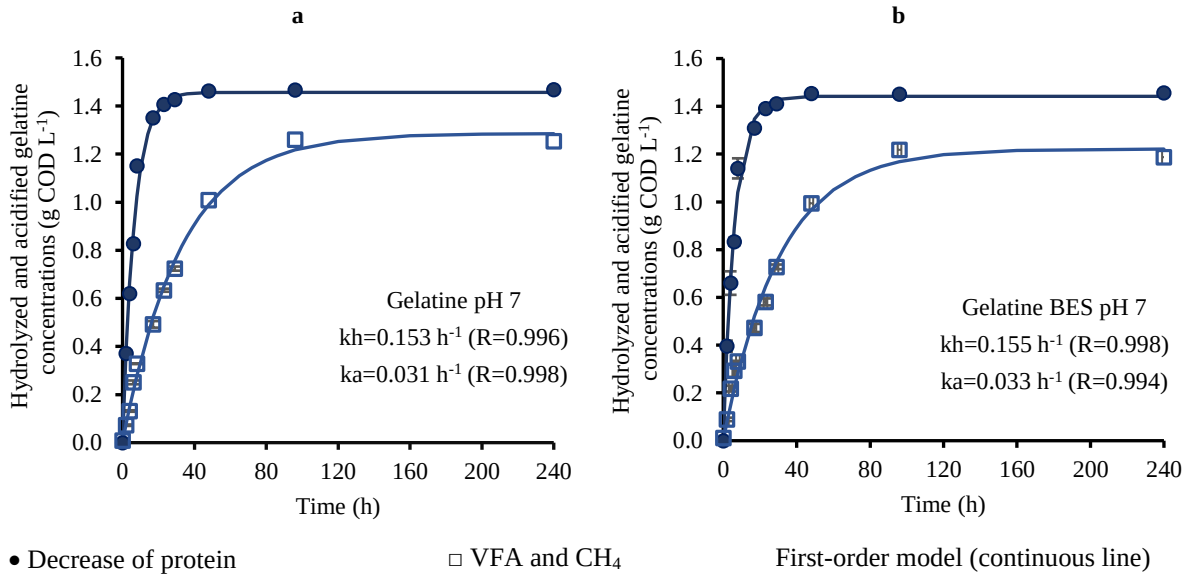

Figure 2.1. First-order model for hydrolyzed and acidified gelatine concentrations in Gelatine-pH7 (a) and Gelatine-BES-pH7 (b) in the batch experiment at 35°C. (Data plotted the mean and standard deviation)

More than 99% of the initial 1.40 g COD L^{-1} of (dissolved) gelatine was hydrolysed during the experiments, whereas 1.25 and 1.20 g COD L^{-1} of acidification products were measured under methanogenic and non-methanogenic conditions, respectively. This difference between the extent of hydrolysis and acidification can be explained by part of the COD being used for biomass production (see section 2.3.5 for more details).

The first-order rate constant for protein hydrolysis (k_h) (0.153 and 0.155 h^{-1}, under methanogenic and non-methanogenic conditions, respectively) were much higher than the first-order rate constants of acidification (k_a) (0.031 and 0.033 h^{-1}). This implies that not protein hydrolysis but acidification of the hydrolysis products was the rate limiting step for anaerobic dissolved protein degradation.

Sanders et al. (2002) showed that the dissolved protein (gelatine) hydrolysis rate was related to sludge concentration and to gelatine concentration. In their tests, comparable to ours, the gelatine hydrolysis rate was modelled using a zero-order kinetics model during the initial incubation hours. We observed an initial hydrolysis rate of 0.137 and of 0.140 g COD. L^{-1} h^{-1} during the first 8 hours of the batch tests, which is in accordance with the results of Sanders et al. (2002) (0.15 g COD L^{-1} h^{-1}) at a similar VS-sludge inoculum to COD-gelatine concentration ratio of 5 g volatile solids (VS) g^{-1} COD.

The first-order hydrolysis rate constants of 0.153-0.155 h^{-1} are higher than those reported by others (Breure and Van Andel, 1984, Tommaso et al., 2003). This may be explained by the calculation of the hydrolysis rate constant based on methane production (Mahmoud et al., 2004) and/or ammonium production (Flotats et al., 2006, Lee et al., 2015), which may have underestimated the hydrolysis rate. However, it is appreciated that many other factors such as the type of protein and biomass inoculum (Tommaso et al., 2003, Mahmoud et al., 2004, Vavilin et al., 2008), the biomass to protein ratio (Sanders et al., 2002) and temperature (Flotats et al., 2006) may also explain the differences.

2.3.2 Effect of low pH on gelatine hydrolysis

The pH variation in the Gelatine and Gelatine-BES at pH 7 during the experiment was negligible. The pH in Gelatine-pH 5 bottles increased to pH 5.5 during the first 48 hours, but then stabilized at this value.

Figure 2.2 shows the concentration of hydrolyzed gelatine in the bottles at pH 5 together with VFA production and pH. Nearly no methane was formed. After an initial hydrolysis rate of approximately 0.05 g COD L^{-1} h^{-1} gelatine degradation stagnated between 8 and 48 hours. After 48 h, gelatine hydrolysis took off again, albeit at a much lower rate of 0.006 g CODL^{-1} h^{-1}. These rates are 2-25 times lower than the initial hydrolysis rate of 0.137 and of 0.140 g COD L^{-1} h^{-1} observed in the experiments at pH 7, under methanogenic and non-methanogenic conditions, respectively. Breure and Van Andel (1984) found in a chemostat system at 30°C a gelatine hydrolysis rate at pH 5 that was twice as low as at pH 7.

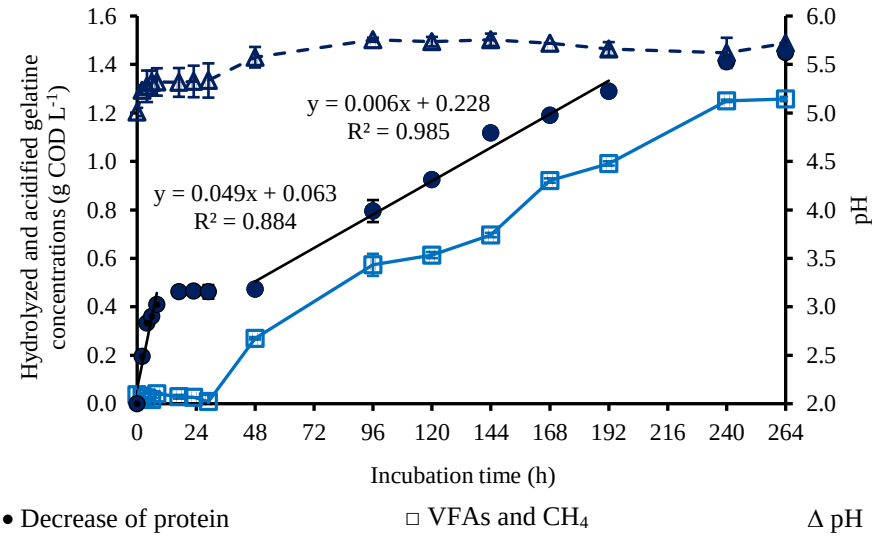

Figure 2.2. The depletion of gelatine concentration and hydrolysis rate at different periods, change of acidified gelatine concentrations and pH during incubation time at Gelatine-pH 5 in the batch experiment at 35°C. (Data plotted the mean and standard deviation)

The hydrolysis of gelatine at pH 5 during the first 6-8 hours was probably related to the presence of proteolytic enzymes in the inoculum which, to a certain extent, were still active. The inhibition of gelatine hydrolysis between 8 and 48 hours may be related to a negative effect of a low pH on the activity of the hydrolytic enzymes. A similar effect was observed by Lu et al. (2004) with a low protease activity under acidic conditions when starting up anaerobic digestion

of municipal solid waste. Breure and Van Andel (1984) reported an optimum pH of proteases of 7.5 whereas at a pH of 5 this was reduced by 50%. This negative effect may be due to an electrostatic repulsion among charged active sites of the proteolytic structure at pH 5. Similarly, at low pH attachment of the gelatine to cell bound proteases becomes more difficult as has been reported for ruminal organisms (Broderick et al., 1991, Vavilin et al., 2008). Finally, at low pH the fraction of undissociated VFA is higher, which has a negative effect on microbial growth (Mouriño et al., 2001, Dijkstra et al., 2012, Infantes et al., 2012) and herewith perhaps also on the excretion of proteolytic enzymes. To our knowledge, an effective hydrolysis of proteins at acidifying conditions has not been reported yet in literature, although adaptation to a low pH cannot be excluded (Breure et al., 1986a, Perle et al., 1995).

2.3.3 Amino acid production and fermentation

In the Gelatine (Figure 2.3) and Gelatine-BES bottles, nineteen different amino acids were detected during the first 8 hours of incubation, at concentrations up to 2 mM. Glycine, alanine, proline and glutamic acid were detected at the highest concentrations. The total COD of these 19 amino acids was as high as 0.60 g COD L^{-1} in the Gelatine bottles and 0.63 g COD L^{-1} in the Gelatine-BES bottles, which is equivalent to approximately 40 % of the COD of the gelatine that was added. From 8 hours onwards only very small amounts (0.01-0.1 mM) of amino acids were detected, and apparently these were readily fermented to VFAs. The temporary accumulation of amino acids at pH 7 under methanogenic and non-methanogenic conditions confirms that the initial hydrolysis rate of gelatine was much faster than the amino acid fermentation rate. Because amino acid accumulation was similar under methanogenic and non-methanogenic conditions this confirms that methanogenic conditions are not a prerequisite to obtain fast protein hydrolysis. So far, concentrations of different amino acids have not been measured or reported for anaerobic degradation of protein rich (waste)waters. However, in studies with ruminal microorganisms accumulation of free amino acids during degradation of food-containing proteins was observed by Broderick et al. (1991) and Cardozo et al. (2004). Clearly, our results imply that amino acid fermentation can be the rate limiting factor for protein degradation not only in the rumen (Bach et al., 2005) but also in anaerobic wastewater treatment reactors. Therefore, this should be taken into account when designing such reactors (also see section 2.3.6).

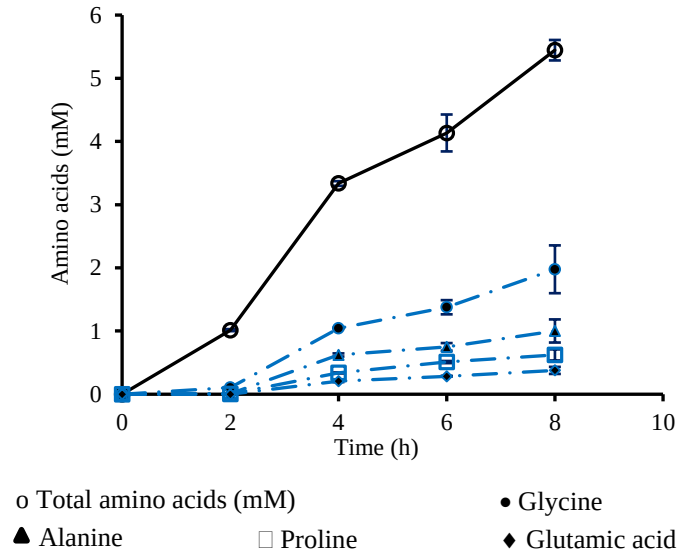

Figure 2.3. The concentration of total amino acids and of glycine, alanine, proline and glutamic acid during the first 8 hours of anaerobic degradation of gelatine at pH 7, at 35°C. (Data plotted the mean and standard deviation)

In the pH 5 bottles after 8 hours only 0.01-0.2 mM of amino acids were detected, equivalent to a total of 0.07 g COD L^{-1}. This agrees with the observation made earlier that hydrolysis of gelatine into amino acids at this low pH was very slow. Apparently, at pH 5 acidification was not the rate-limiting step.

Figure 2.4 shows the amino acids detected during 8 hours of incubation time under the different conditions along with the "theoretical" composition of gelatine (Gelatin Handbook, 2012). The amino acid composition at pH 7 was similar to the amino acid composition of gelatine, except for a slightly higher percentage of alanine by 8% and the absence of hydroproline. This indicates that gelatine hydrolysis was rather unselective. At pH 5, a lower contribution of glycine (by 7%) and a higher contribution of alanine (by 16%) were detected after 8 hours of incubation. Apparently at both pHs alanine was more slowly acidified to VFAs than the other amino acids.

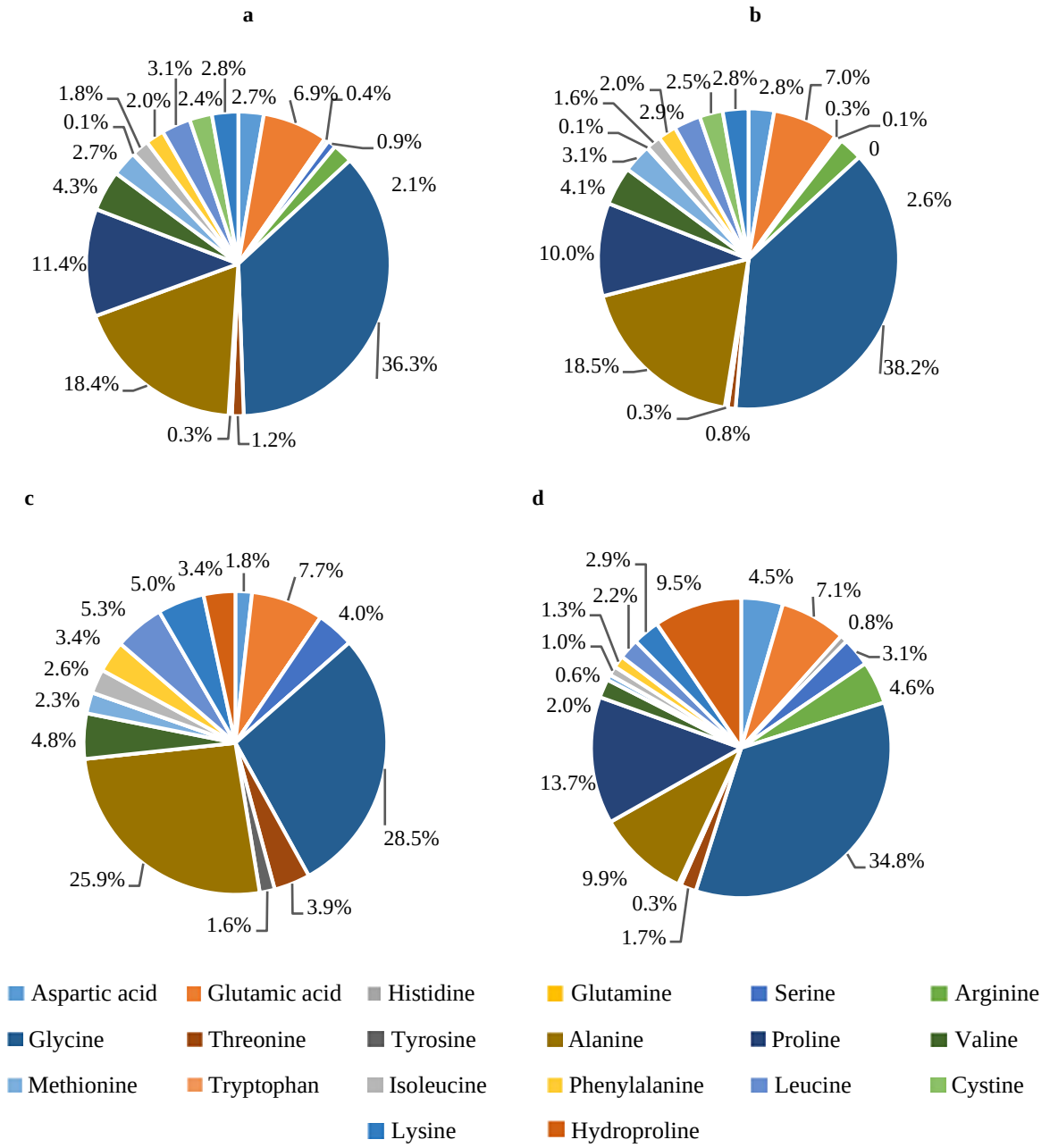

Figure 2.4. Percentage of amino acids (in total mM) from Gelatine-pH7 (a), Gelatine-BES-pH7 (b), and Gelatine-pH 5 (c) after 8 hours of incubation at 35°C; Data expressed as the mean value (n=3, standard deviation less than 5%). Theoretical amino acids composition of gelatine (referenced from Gelatin handbook, 2012) shown in Figure 2.4d.

2.3.4 VFA production

Figure 2.5 shows the VFA produced during gelatine degradation at pH 7 under non-methanogenic conditions (left) and at pH 5 (right). Because methanogenesis was absent, in both cases VFA accumulated, with acetate accounting for 45% of the total VFA.

At pH 7, VFA was rapidly produced during the first 50 hours of incubation. Acetate reached a concentration of 0.55 g COD L^{-1} and the total VFA concentration was 1.14 g COD L^{-1}, equivalent to 83% of the gelatine-COD that was added. At pH 5 almost no VFA was detected until after 29-48 hours of incubation. This slower production of VFA at pH 5 compared to pH 7 was already explained above as hydrolysis of gelatine into amino acids at pH 5 was lower than at pH 7. Another difference between pH 5 and pH 7 was that at pH 5 n-valerate was the second most abundant VFA that was produced while it was produced the least at pH 7, showing that the VFA spectrum is determined by the pH (Kleerebezem et al., 2015). The VFA profiles are partly in accordance with others (Erfle et al., 1982, Breure and Van Andel, 1984, Kleerebezem et al., 2015) who observed that shifting pH from 7.0 to 5.0 gradually decreased the production of acetate and butyrate and promoted the production of (n-)valerate from proteins. This can be explained by a lower energy expenditure to excrete larger VFAs compared to smaller molecules. As a result, at lower pH valerate production is more favourable.

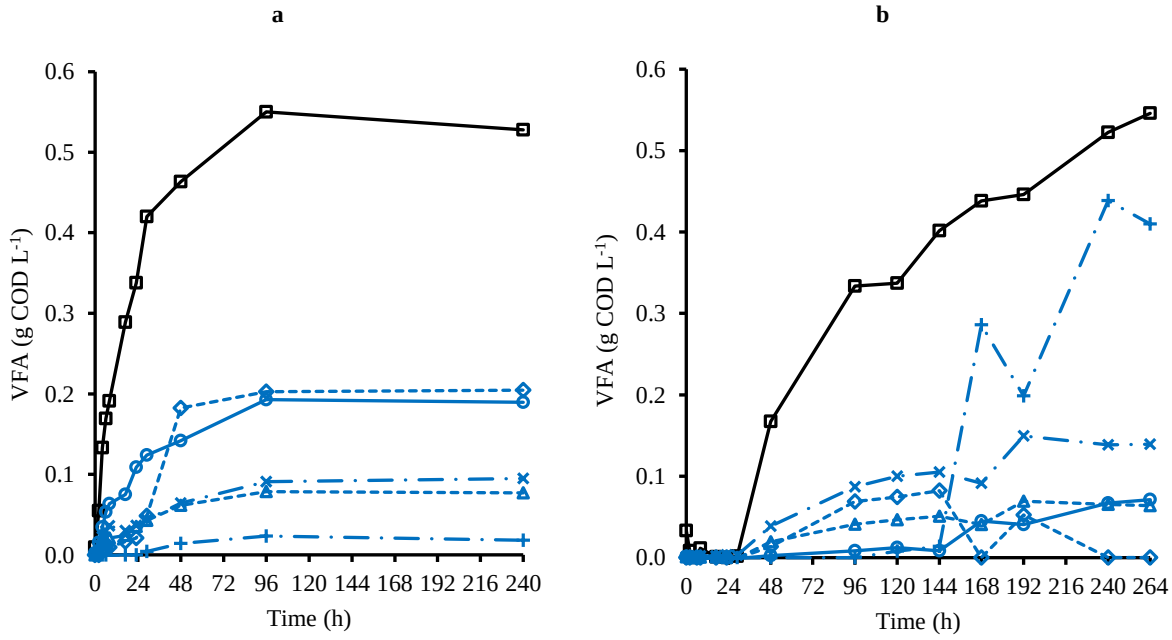

Figure 2.5. Concentration of VFAs from gelatine degradation at pH7 (a) versus at pH5 (b) in the batch experiment at 35°C. Data expressed as mean value (n=3, with standard deviation less than 5%). Acetate (□), propionate (○), i-butyrate (Δ), n-butyrate (◊), i-valerate (x), n-valerate (+).

2.3.5 COD mass balance

Figure 2.6 shows mass balances for the different batch experiments. Gelatine was completely (> 99%) converted at the end of the batch tests and the final products (methane and VFA) accounted for 83-86% of the COD, regardless of the pH and methanogenic activity. The missing 14-17% of the COD can be attributed to biomass production and is in a range of biomass yield of 0.12-0.36 g COD g^{-1} protein-COD reported by others (Breure and Van Andel, 1984, Ramsay and Pullammanappallil, 2001, Yu and Fang, 2001, Tang et al., 2005, Flotats et al., 2006).

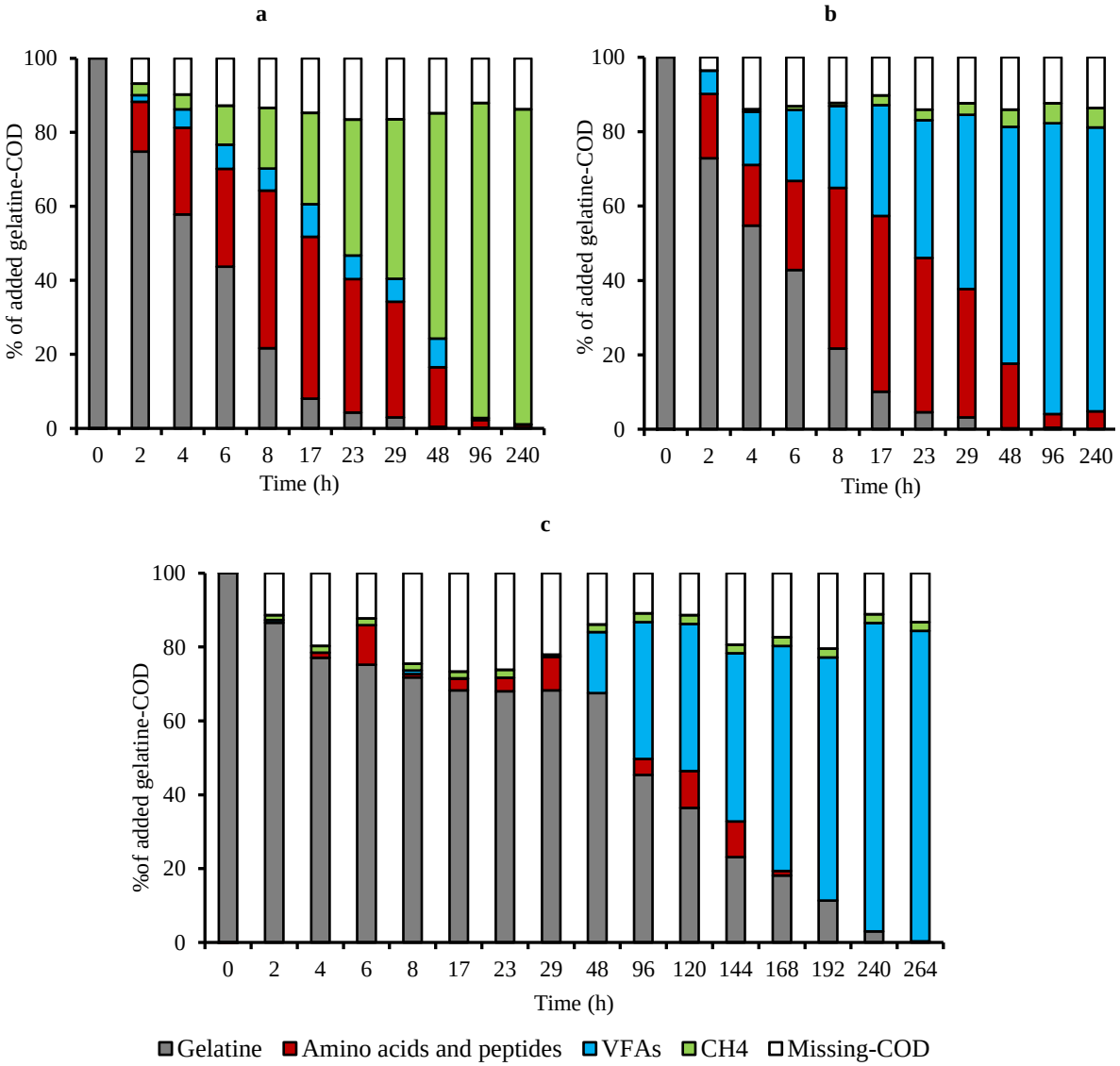

Figure 2.6. Anaerobic conversion of gelatine-COD to amino acids and peptides-, VFAs- and CH_4-COD in Gelatine-pH 7 (a), Gelatine-BES-pH 7 (b) and Gelatine-pH 5 (c) in the anaerobic batch experiment at 35°C. Data expressed as mean value (n=3, with standard deviation less than 5%)

2.3.6 Consequences for the design and operation of anaerobic reactors for protein-rich wastewaters

The results of this study clearly showed that not hydrolysis but subsequent acidification of the hydrolysis products is the rate limiting step in anaerobic conversion of dissolved proteins. Obviously this has consequences for the design of anaerobic treatment reactors. At pH 7 a hydrolysis rate constant of 0.15 h^{-1} and an acidification rate of 0.03 h^{-1} were found for gelatine. As an example, to avoid amino acid accumulation in a completely stirred tank reactor (CSTR) the volume of this CSTR should be 5 times bigger than the volume in case only protein hydrolysis would be taken into account. It is strongly recommended that the above difference between the rate of hydrolysis and acidification of the amino acids is also considered in models such as the anaerobic digestion model No.1 that are used for design purposes (Batstone et al., 2002).

Interestingly, the large difference between the hydrolysis and acidification rates offers the possibility to avoid the degradation of amino acid and design the reactor such that they can be recovered. For instance, valine, leucine and iso-leucine are important substrates for branched fatty acids formation such as iso-butyrate, iso-valerate and iso-caproate that can be harvested from fermenting protein-rich waste streams for potential branched chain elongation (Leeuw et al., 2019)

The production of VFA from waste streams has gained a lot of attention because they are considered important intermediates for the production of higher value products such as bioplastics. To this end reactors are operated at a short solids retention time to wash-out methanogens that otherwise would consume the VFAs. If the waste stream also contains an appreciable amount of (dissolved) proteins a low pH should be avoided at all times because it was shown that at pH 5 protein hydrolysis is approximately 20 times slower than at pH 7. Not only would this result in a lower VFA yield, but also in problems associated with the presence of proteins such as foaming, biomass wash-out and a deteriorated effluent quality (Perle et al., 1995, Hassan and Nelson, 2012, Tanimu et al., 2015). Adaptation or acclimation could potentially enhance protein hydrolysis at low pH (Perle et al., 1995, Gavala and Lyberatos, 2001), but this requires more research in this direction.

2.4 Conclusions

Batch experiments carried out with gelatine under mesophilic conditions (35°C) showed that the hydrolysis rate constant of gelatine was faster than the acidification rate, leading to accumulation of free amino acids. It is concluded that not hydrolysis but acidification can limit the fermentation rate of dissolved proteins. Methane formation did not stimulate the protein hydrolysis and acidification at neutral pH. Shifting the initial pH from neutral to acidic conditions (pH 5) inhibited protein degradation and changed the VFA product profile. The findings in this study can be used to define retention times needed for efficient anaerobic treatment of protein-rich wastewaters.

Chapter 3. Volatile fatty acids or methane from wastewater proteins – effect of presence of carbohydrates

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Abstract

This study aimed to assess the effect of carbohydrates on protein hydrolysis and potential implications for the design of anaerobic reactors for treatment of protein-rich wastewaters. Batch experiments were carried out with dissolved starch (Sta) and gelatine (Gel) at different ratios ranging from 0 to 5.5 under methanogenic conditions and up to 3.8 under non-methanogenic conditions, both at 35°C. The Sta/Gel did not have a direct effect on the gelatine hydrolysis rate constants under methanogenic (0.51±0.05 L g VSS^{-1} day^{-1}) and non-methanogenic conditions (0.48±0.05 L g VSS^{-1} day^{-1}). However, under non-methanogenic gelatine hydrolysis was inhibited by 64% when a spectrum of volatile fatty acids (VFA) was added at a VFA/Gel ratio of 5.9. This was not caused by the ionic strength exerted by VFA but by the VFA itself. These results imply that methanogenesis dictates the reactor design for methane production but hydrolysis does for VFA production from wastewater proteins.

Keywords: proteins, carbohydrates, volatile fatty acids, methanogenic, non-methanogenic.

3.1 Introduction

Anaerobic digestion is widely used for the treatment of wastewaters, converting organic pollutants into energy rich methane. Alternatively, short-chain volatile fatty acids (VFAs) can be produced from these pollutants to serve as platform chemicals, for instance for the production of more valuable compounds such as bioplastics (Kleerebezem et al., 2015, Tamis et al., 2015) or medium chain fatty acids (Leeuw et al., 2019). Carbohydrates and proteins are the dominant organic pollutants in many food related wastewaters and wastes, and together account for 60-90% of the chemical oxygen demand (COD) of dairy, beverage, slaughterhouse and food processing wastewaters. Of this COD 75-98% is biodegradable (Sayed et al., 1984, Behling et al., 1997, Palenzuela, 1999, Demirel et al., 2005, Hassan and Nelson, 2012). This implies that the proteins and carbohydrates have a huge contribution to energy or chemical recovery from such wastewaters. Moreover, fermentation of proteins may generate a rich mix of branched fatty acids, for instance iso-butyrate and iso-valerate. These are attractive substrates for chain elongation towards branched medium chain fatty acids (Leeuw et al., 2019). A lot of scientific as well as practical data about (separated) anaerobic degradation of proteins and carbohydrates is available. However, knowledge about the interaction between the biodegradation of these biopolymers is scarce and inconsistent, in particular regarding the effect of carbohydrates on protein degradation.

Breure et al. (1986a) operated a chemostat at pH 7 under non-methanogenic conditions. The chemostat was fed with 3.5 g L^{-1} of gelatine. Approximately 95% of the gelatine was hydrolysed and on carbon basis 89% of the gelatine could be recovered as VFA. When the feed was supplemented with 2 g L^{-1} of glucose the degree of gelatine hydrolysis was still high, but VFA recovery deteriorated. This was attributed to a retarded fermentation of the hydrolysis products of gelatine. In a similar experiment, but at a much higher glucose concentration of 10 g L^{-1}, Breure et al. (1986b) observed a reduction of gelatine hydrolysis from 96% to 77% and even to lower efficiencies at higher dilution rates. They suggested repression of the synthesis of extracellular proteases by glucose to be responsible for this phenomenon. However, the gelatine solution they used had been sterilised for 30 min at 110°C. This already could have resulted in (partial) hydrolysis of the gelatine (Karnjanapratum and Benjakul, 2015), and a misinterpretation of the results. Yu and Fang (2001) arrived at a similar conclusion when they observed in batch experiments at pH 5.5, also under non-methanogenic conditions, that protein

degradation did not start before the carbohydrates in the substrate (prepared from full-cream powder milk) were fully degraded. However, at such a low pH protein hydrolysis is inhibited (Duong et al., 2019), which may better explain their results than suppression by carbohydrates. In contrast to the above, Feng et al. (2009) found that rice carbohydrates improved protease activity in waste activated sludge approximately 10-fold. Also Elbeshbishy and Nakhla (2012), who added starch to bovine serum albumin (BSA) showed that the first-order hydrolysis rate constants of BSA increased by a factor of 1.5. It is noted however that in these last two studies particulate protein and carbohydrates were used and not only enzymatic reactions but also the particle surface available for hydrolysis may have been important (Sanders, 2001).

Under non-methanogenic conditions, VFA concentrations in the culture medium can be very high, in particular if significant amounts of carbohydrates are co-fermented. This raises the question if VFA can inhibit protein hydrolysis and/or subsequent amino acid fermentation. When Breure et al. (1986b) replaced glucose by a VFA mixture of 3 g L^{-1} the negative impact on gelatine hydrolysis was minimal. However, the effect of thermal sterilization on gelatine hydrolysis mentioned above was not accounted for. Flotats et al. (2006) concluded that concentrations up to 11.2 g VFA-COD L^{-1} did not affect gelatine hydrolysis at 55°C. Besides, the experiments of Flotats et al. (2006) were carried out under methanogenic conditions and VFA consumption by the methanogens may have alleviated a negative impact of VFA. Finally, also Veeken et al. (2000) could not find a relationship between VFA concentration (3-10 g L^{-1} at pH 7) and the hydrolysis of solid biowaste, but more specific details regarding protein hydrolysis and amino acid fermentation unfortunately were not reported. In contrast, González et al. (2005) reported that in a saline medium of 24 g NaCl L^{-1} and at pH 7 first-order hydrolysis rate constants of dissolved peptone were reduced by 2-4 times at acetate concentrations of 0.25 to 0.75 g L^{-1}. Also in the model of Angelidaki et al. (1999), VFA inhibition of hydrolysis was incorporated by a reduction coefficient of 0.33/(0.33+VFA). However, experimental data to support this and possible mechanisms were not mentioned.

In summary, literature information regarding the effect of carbohydrate on anaerobic protein degradation is scarce and inconsistent and therefore this effect needs to be further investigated. This is important to be able to design anaerobic reactor systems for the treatment of protein rich wastewaters, either to produce biogas or platform chemicals such as VFAs. For this purpose protein (gelatine) degradation was determined in the presence of carbohydrates (starch) and

VFA under methanogenic as well as non-methanogenic conditions at a pH of 6.5-7.5 and under mesophilic conditions (35°C). To be able to distinguish between the different steps in the anaerobic degradation pathway, gelatine and starch degradation were monitored based on concentrations of COD, protein, carbohydrate, amino acids, glucose, VFA and methane.

3.2 Materials and Method

3.2.1 Substrates

The model protein was gelatine (Gel), CAS no.9000-70-8 (Merck, for microbiology, 1.04070.0500) and the model carbohydrate was starch (Sta), CAS no. 9000-84-9 (Merck, GR for analysis ISO, 1.01252.0250). Gelatine and starch powder were dissolved in hot demineralised water (50°C) and after cooling to ambient temperature served as substrate stock solutions of 100 g COD L^{-1}. Main characteristics of the substrates are shown in Table 3.1.

Table 3.1. Main characteristics of the substrates used in this experiment. Data are measured per gram and expressed in average ± standard deviation (n=10).

Characteristics	TS, g	VS, g	COD, g	TN, g
Protein (Gel)	0.95±0.01	0.95±0.01	1.15±0.02	0.14±0.01
Carbohydrate (Sta)	0.98±0.01	0.98±0.01	1.12±0.02	-

VFAs were applied as a mixture consisting of 58% acetate, 27% propionate 9% butyrate and 6% valerate on COD basis, which is representative for the VFA profile that was obtained after starch fermentation under non-methanogenic conditions at pH 7 (section 3.3.2). This VFA mixture was prepared as a stock VFA solution of 100 g COD L^{-1}, with 54.3 g acetic acid (Ac, CAS no.64-19-7), 17.8 g propionic acid (Pro, CAS no.79-09-4), 4.9 g butyric acid (Bu, CAS no.107-92-6) and 2.9 g valeric acid (Val, CAS no.109-52-4) diluted in demi-water and neutralized with 5M NaOH to pH 7.0±0.2.

3.2.2 Inoculum and nutrient medium

The seed sludge was sampled from a full-scale anaerobic reactor that treated brewery wastewater. Sludge was sampled 108 and 360 days after the reactor had come into operation

for experimental set-up A and for experimental set-ups B and C, respectively (see section 2.3). The reactor was operated at a temperature of 30±3°C. The characteristics of the sludge samples taken on days 108 and 360 were very similar with total suspended solids (TSS) and volatile suspended solids (VSS) concentrations of 19.8±1.5 g L^{-1} and 15.0±0.5 g L^{-1}, respectively. Total COD of the sludge was 19.4±0.2 g L^{-1}, total nitrogen (TN) 0.35±0.08 g L^{-1} and the NH_4-N concentration was 0.12±0.01 g L^{-1}. Concentrations of dissolved residual proteins and carbohydrates in the seed sludge after degassing were 0.05±0.02 and 0.01±0.01 g L^{-1}, respectively. The pH of the sludge was 7.1±0.2.

The nutrient medium for the batch tests was adapted from Angelidaki et al. (2009) without the addition of NH_4Cl, since nitrogen was sufficiently present in the gelatine that was added to the tests.

3.2.3 Anaerobic batch experiments

Anaerobic batch experiments were carried out in triplicate at 35°C and at a pH between 6.5 and 7.5 in 2.6 L side-port-bottles, which were continuously shaken at 60 rpm for 240-456 hours. The initial gelatine concentration was 1.40±0.10 g COD L^{-1} in all bottles. Three series of experiments (A, B and C) were carried out (Table 3.2).

Table 3.2. Substrate composition and concentrations of the substrate mixtures of batch bottles in experimental set-up A, B and C. All data are expressed as average ± standard deviation (n=3), gelatine and starch concentrations in g COD L^{-1} and sludge concentrations in g VSS L^{-1}.

Experiment A: Gelatine and varying starch concentrations under methanogenic conditions.

Mixture	Sludge	Gelatine	Starch
Blank	8.4	0	0
Sta/Gel			
0	8.4	1.39±0.02	0
0.8	8.4	1.38±0.01	1.15±0.03
1.7	8.4	1.35±0.02	2.33±0.05
2.5	8.4	1.45±0.02	3.59±0.10
3.5	8.4	1.43±0.01	4.98±0.04
4.6	8.4	1.34±0.01	6.16±0.12
5.5	8.4	1.41±0.05	7.75±0.13

Experiment B: Gelatine and varying starch concentrations under non-methanogenic conditions.

Mixture	Sludge	Gelatine	Starch
Blank	2.8	0	0
only Sta	2.8	0	1.34±0.06
Sta/Gel			
0	2.8	1.37±0.01	0.01±0
1	5.2	1.40±0.04	1.35±0.05
1.8	8.0	1.47±0.07	2.67±0.07
2.7	11.0	1.47±0.06	4.02±0.10
3.8	13.6	1.40±0.03	5.36±0.23

Experiment C: Gelatine and varying VFA concentrations under non-methanogenic conditions.

Mixture	Sludge	Gelatine	VFA
Blank	6.5	0	0
VFA/Gel			
0	6.5	1.49±0.01	0.01±0
1.2	6.5	1.48±0.01	1.69±0.12
2.2	6.5	1.45±0.01	3.02±0.16
4.5	6.5	1.45±0.01	6.07±0.20
5.9	6.5	1.49±0.01	8.24±0.32

In experiment A the effect of starch on gelatine hydrolysis and degradation was studied under methanogenic conditions. The sludge and gelatine concentration in these tests were constant,

i.e. at 8.4 g VSS L^{-1} and 1.4±0.06 g COD L^{-1}, respectively. The starch concentration was varied to give a starch to gelatine COD ratio of 0 to 5.5.

In experiment B the interaction between starch and gelatine degradation was studied under non-methanogenic conditions. To inhibit methanogenesis all the bottles received 0.03M 2-bromoethanesulfonate (BES). Gelatine was added at a concentration of 1.4±0.08 g COD L^{-1} while the concentration of starch was varied to obtain a starch to gelatine COD ratio of 0 to 3.8. Unlike in experiment A, different sludge concentrations (2.8 to 13.6 g VSS L^{-1}) were applied to maintain a constant inoculum to substrate (I/S) ratio of 2.0±0.1 g VSS g^{-1} COD.

Experiment C was carried out to test if VFA, produced by carbohydrate fermentation, can inhibit protein degradation. Different VFA concentrations were added and the VFA to gelatine COD ratio varied between 0 and 5.9. The inoculum concentration was kept constant at 6.5 g VSS L^{-1}. Similar to set-up B, BES was added at 0.03 M to stop methanogenic activity.

All bottles were filled up to a working volume of 0.62 L. Blank bottles without substrate were prepared only containing seed sludge and nutrient medium, but otherwise they were treated similar to the test bottles. Prior to the experiment, the contents of the bottles were neutralized to pH 7 with 1M NaOH and sampled for the initial substrate and sludge concentrations. Thereafter, bottles were closed and flushed with N_2 gas for 20 minutes.

3.2.4 Sampling and analyses

During the first 8-10 h, gas and liquid samples were taken at an interval of 2-3 h. Afterwards, nine more samples were taken from all bottles after 17, 23, 29, 44-48, 72, 92-96, 116-120, 168, and 240 h. Two additional samples were taken from the bottles at a Sta/Gel ratio of 4.6 and 5.5 bottles in experimental set-up A after 336 and 456 h. Determination of pH, gas pressure and gas composition (CH_4, CO_2, H_2 and N_2) was performed as described by Duong et al. (2019). Total solids (TS) and volatile solids (VS) of gelatine and starch powder and TSS and VSS of sludge samples taken at the start and end of the tests were all measured according to standard methods (APHA-AWWA-WEF, 2017). The sludge samples were centrifuged (Eppendorf, Germany) at 10000 rpm for ten minutes and filtered with pre-washed 0.45µm cellulose acetate membrane filters (Sartorius, Germany). The supernatant was analysed for chemical oxygen

demand (COD), total nitrogen and ammonium (NH_4-N), as described by Duong et al. (2019). Protein was determined using the Lowry method assay (Noble and Bailey, 2009) at 660 nm using gelatine as standard. Amino acids were quantified in supernatant samples as described by Meussen et al. (2014) via high-performance liquid chromatography (HPLC) equipped with a Zorbax Eclipse AAA column (ID 4.6 x 150mm), Agilent. Carbohydrates (starch plus glucose) were determined by the phenol-sulfuric acid method (Dubois et al., 1956) at 490 nm using starch as standard. Glucose was measured by a D-glucose assay kit using Gopod reagent (McCleary et al., 2019). The starch concentration was subsequently calculated as the difference between these two measurements. Volatile fatty acids (VFAs) were quantified on a Trace gas chromatograph equipped with a Thermo TR-WAX column (30m x ID 0.32 mm x thickness of 0.25 µm) connected to a FID detector as described by Sudmalis et al. (2018).

3.2.5 Calculations

Gelatine hydrolysis (calculated from the measured decrease in soluble protein concentration using a conversion factor of 1.150 g COD g^{-1} gelatine) could best be described by first-order kinetics:

$$P_{Gel\text{-}hydrolyzed}(t) = P_{Gel\text{-}hydrolyzed\text{-}end}\cdot(1-\exp(-k_{h,Gel}\cdot X\cdot t)) \quad \text{(eq. 3.1)}$$

with $P_{Gel\text{-}hydrolyzed}(t)$ and $P_{Gel\text{-}hydrolyzed\text{-}end}$ the concentration of hydrolysed gelatine (g COD L^{-1}) at time t (day) and at the end of the experiments, respectively; $k_{h,Gel}$ the first-order gelatine hydrolysis rate constant normalized for the sludge concentration (L g^{-1} VSS day^{-1}), and X the volatile suspended solids concentration of the sludge (g VSS L^{-1}).

Similarly, acidification (increase of the sum of the concentration of VFA and methane, both expressed in g COD L^{-1}) and methanization (as g COD L^{-1}) were also best described by first-order kinetics:

$$P_{Acidified}(t) = P_{Acidified\text{-}end}\cdot(1-\exp(-k_a\cdot X\cdot t)) \quad \text{(eq. 3.2)}$$

$$P_{Methane}(t) = P_{Methane\text{-}end}\cdot(1-\exp(-k_m\cdot X\cdot t)) \quad \text{(eq. 3.3)}$$

with $P_{Acidified}(t)$ and $P_{Acidified\text{-}end}$ the produced sum of the concentration of VFA and methane at time t (day) and at the end of the experiments, respectively (g COD L^{-1}), $P_{Methane}(t)$ and $P_{Methane\text{-}end}$ the produced methane at time t (day) and at the end of the experiments (g COD L^{-1}) and k_a and k_m the first-order acidification rate and methanization rate constants normalized for the sludge concentration (L g^{-1} VSS day^{-1}).

The concentration of hydrolysed starch in time (using a conversion factor of 1.115 g COD g^{-1} starch) was more accurately described by zero-order kinetics, possibly because of the high affinity of the hydrolytic enzymes for starch and due to very fast starch hydrolysis, only a limited number of data-points were available in the lower range of starch concentrations:

$$P_{Sta\text{-}hydrolyzed}(t) = k_{h,Sta} \cdot X \cdot t \text{ for } t < t_k \text{ and } P_{Sta\text{-}hydrolyzed\text{-}end} \text{ for } t \geq t_k \quad \text{(eq. 3.4)}$$

with $P_{Sta\text{-}hydrolyzed}(t)$ and $P_{Sta\text{-}hydrolyzed\text{-}end}$ the concentration of hydrolysed starch (g COD L^{-1}) at time t (day) and at the end of the experiments, respectively and $k_{h,Sta}$ the zero-order starch hydrolysis rate constant normalized for the sludge concentration (g COD g^{-1} VSS day^{-1}).

First- and zero-order rate constants were estimated from the measurements using the least-squares method. The difference between measured COD in the supernatant of the bottles and the sum of the COD of the different compounds that were measured in this supernatant always was less than 2%. This implies the compound measurements were accurate and only those compounds that were measured (protein, carbohydrate, amino acids, glucose, volatile fatty acids and methane) were relevant.

3.3 Results and discussion

Rate constants were estimated for every single bottle and the regression coefficient always exceeded 0.98. Table 3.3 shows the average rate constants of triplicate bottles, together with their standard deviation (n=3). Figures A1-A4, B1-B3 and Figures C1-C2 of the Supplementary information (SI) show average measured concentrations and concentrations that were estimated according equations (3.1) to (3.4).

Table 3.3: First- and zero-order (for starch) rate constants based on COD of the gelatine, starch, VFA mixtures and methane production. All data are expressed as average ± standard deviation (n=3).

Experiment A Sta/Gel	$k_{h,Sta}$ g COD gVSS^{-1} day^{-1}	$k_{h,Gel}$ L g^{-1} VSS day^{-1}	k_a L g^{-1} VSS day^{-1}	k_m L g^{-1} VSS day^{-1}
0	-	0.58±0.02 (a)	0.17±0.01 (c)	0.08±0.01 (a)
0.8	0.84±0.04	0.55±0.01 (a)	0.23±0.01 (a)	0.06±0.01 (b)
1.7	0.78±0.03	0.57±0.02 (a)	0.21±0.01 (b)	0.06±0.01 (b)
2.5	0.87±0.04	0.47±0.01 (b)	0.20±0.01 (b)	0.05±0.01 (b)
3.5	1.05±0.01	0.48±0.01 (b)	0.18±0.01 (c)	0.05±0.01 (b)
4.6	1.19±0.01	0.45±0.01 (b)	0.17±0.01 (c)	0.04±0.01 (c)
5.5	1.37±0.01	0.43±0.01 (c)	0.16±0.01 (c)	0.04±0.01 (c)
Experiment B Sta/Gel	$k_{h,Sta}$ g COD gVSS^{-1} day^{-1}	$k_{h,Gel}$ L g^{-1} VSS day^{-1}	k_a L g^{-1} VSS day^{-1}	k_m L g^{-1} VSS day^{-1}
only Sta	0.95 ±0.05	-	0.25±0.02 (a)	-
0	-	0.54±0.03 (a)	0.15±0.03 (b)	-
1	0.87 ±0.05	0.44±0.04 (b)	0.10±0.01 (c)	-
1.8	1.18 ±0.08	0.47±0.05 (ab)	0.09±0.01 (c)	-
2.7	1.12 ±0.07	0.49±0.06 (ab)	0.09±0.01 (c)	-
3.8	1.10 ±0.10	0.47±0.05 (ab)	0.04±0.01 (d)	-
Experiment C VFA/Gel	$k_{h,Sta}$ g COD gVSS^{-1} day^{-1}	$k_{h,Gel}$ L g^{-1} VSS day^{-1}	k_a L g^{-1} VSS day^{-1}	k_m L g^{-1} VSS day^{-1}
0		0.45±0.01 (a)	0.12±0.01 (a)	-
1.2	-	0.38±0.05 (a)	0.11±0.01 (a)	-
2.2	-	0.25±0.03 (b)	0.07±0.01 (b)	-
4.5	-	0.25±0.02 (b)	0.06±0.01 (b)	-
5.9	-	0.16±0.01 (c)	0.04±0.01 (c)	-

Note: Data expressed the mean ± std; letters in parentheses indicate significant differences between values ($p<0.05$) with a>b>c>d. Values with the same letters are not significantly different. Values with ab are neither significantly different with those with a nor b.

As was already mentioned, the pH in the different bottles varied between 6.5 and 7.5. Anova regression statistics did not show a correlation between the pH and the rate constants (p-value > 0.1), which also agrees with others that pH in the range of 6.5 to 7.5 does not affect hydrolysis of dissolved proteins (Yu and Fang, 2003, Liu et al., 2012, Liu et al., 2015).

3.3.1 Effect of Sta/Gel ratio on process rates

Estimated zero-order rate constants for starch hydrolysis ($k_{h,Sta}$) in experiment A under methanogenic conditions and experiment B under non-methanogenic varied between 0.78 and 1.37 COD g^{-1} VSS day^{-1} (Table 3.3). Starch hydrolysis was very fast, and it took less than 6-12 hours before the starch was completely hydrolysed (Figures A1 and B1 in SI). As a consequence, only a few data points were available to estimate $k_{h,Sta}$, which makes it rather inaccurate. A consistent effect of the Sta/Gel ratio, or of the difference in conditions (methanogenic versus non-methanogenic) on starch hydrolysis could therefore not be discriminated from the data. Starch hydrolysis generally was much faster than gelatine hydrolysis. Partly this can be explained by a higher affinity of the biomass towards starch, but obviously this also is a property of the seed sludge that was sampled from an anaerobic treatment reactor for brewery wastewater. Typically, the carbohydrate and protein content of brewery wastewater vary between 45-50% and 20-25% on COD basis, respectively (Forssell et al., 2008, Westendorf et al., 2014) and therefore a higher starch compared to protein degrading capacity of the sludge can be expected. Please remark that also the acidification rate constant when only starch was added in experiment B (0.25 L g^{-1} VSS day^{-1}) was faster than acidification when only gelatine was added in the same experiment (Sta/Gel=0.15 L g^{-1} VSS day^{-1}), which probably can be explained by the same reason.

Irrespective of the conditions and the Sta/Gel ratio, gelatine hydrolysis always was much faster than acidification and under methanogenic conditions in experiment A acidification was much faster than methane production. Gelatine hydrolysis also was much faster than acidification in those test bottles that only received gelatine (Sta/Gel = 0). This implies that amino acid fermentation is a much slower process than hydrolysis of dissolved proteins, which also has been reported by Duong et al. (2019). The large differences in rates of gelatine hydrolysis, subsequent amino acid fermentation and methanogenesis ($k_{h,Gel} \approx 3.4\text{-}3.5 \times k_a \approx 7.3 \times k_m$) obviously have strong implications for the design of anaerobic reactors treating protein rich wastewaters with the aim to either produce methane or to produce one of the intermediate products amino acids or VFAs. This will be further discussed in section 3.3.4.

Under methanogenic conditions, gelatine hydrolysis rate constants $k_{h\text{-}Gel}$ were significantly different among Sta/Gel ratios and at the highest Sta/Gel ratio $k_{h,Gel}$ was 25% lower than the

rate in the absence of starch. Under non-methanogenic conditions no significant effect of the Sta/Gel ratio on $k_{h\text{-}Gel}$ could be observed (p value > 0.1) except at Sta/Gel ratio of 1 where $k_{h,Gel}$ was 18% lower than the rate in the absence of starch. Therefore it was unclear if starch itself could affect gelatine hydrolysis or its acidified products. This will be futher discussed in section 3.3. The average protein hydrolysis rates under methanogenic (0.51 ± 0.05 L g VSS^{-1} day^{-1}) and non-methanogenic conditions (0.48 ± 0.05 L g VSS^{-1} day^{-1}) were in the same order as reported by others, i.e. 0.53 L g VS^{-1} day^{-1} under methanogenic as well as under non-methanogenic conditions (Duong et al., 2019) and 0.59 L g VSS^{-1} day^{-1} for dissolved (meat) peptone hydrolysis at mesophilic conditions (Gonzalez et al. 2005).

Under both conditions the acidification rate (sum of amino acid and glucose acidification) decreased with the Sta/Gel ratio. That this effect was much stronger under non-methanogenic conditions can be explained by a higher VFA accumulation under these conditions, which will be further discussed in sections 3.3.2 and 3.3.3.

Finally, in experiment A methane production was slower at higher Sta/Gel ratio's. Also this can be explained by VFA production, more in particular by the production of propionic acid (Angelidaki et al., 1999, Siegert and Banks, 2005, Wang et al., 2009, Ma et al., 2011). For example, at a Sta/Gel ratio of 5.5 a maximum propionate concentration of 1.73 g COD L^{-1} was measured (Table S2 in SI), which exceeds propionate concentrations of 0.7-1.3 g COD L^{-1} that are reported to inhibit methanogenesis (Wang et al., 2009, Ma et al., 2011). The other acid concentrations, i.e acetate and butyrate, were well below inhibitory concentrations.

3.3.2 Mass-balances

Figures 1 shows COD mass-balances under methanogenic (experiment A at t=0, t=240 and also at t=456 h at Sta/Gel of 4.6 or higher) and non-methanogenic conditions (experiment B at t=0 and t=240 h), respectively. The corresponding data can be found in Table S1 of the SI.

By the end of the tests under methanogenic conditions, irrespective of the Sta/Gel ratio, more than 99% of the gelatine and starch were hydrolyzed and glucose and amino acids were absent (Figure 3.1). VFA was only present at a maximum of 0.1 % of the COD that was added at the start of the tests. Methane recoveries ranged between 82 and 89%.

Also under non-methanogenic conditions a complete hydrolysis of starch was achieved in all test bottles. In the absence of starch also gelatine hydrolysis was complete. However, although the hydrolysis rate constant did not decrease at higher Sta/Gel ratio's (Table 3.3), gelatine hydrolysis was incomplete in the presence of starch and gelatine contributed 3-4% to the COD mass balance at the end of the tests. This corresponds with an increase of the remaining gelatine concentration from 0 g COD L^{-1} at Sta/Gel = 0 to 0.25 g COD L^{-1} at Sta/Gel = 3.8 and a decrease of the gelatine hydrolysis efficiency of 100% at Sta/Gel = 0 to 82% at Sta/Gel = 3.8. This suggests a small fraction of the gelatine became unavailable for hydrolysis, which is possibly due to changes in the structure of the gelatine, or because of a lower protease production and/or activity at the end of the tests. This will be futher discussed in section 3.3.3. Also under non-methanogenic conditions no glucose or amino acids were detected at the end of the tests. The recovery of VFA was 89% in the absence of starch but significantly lower (82-84%) at the higher Gel/Sta ratio's, which is explained by the lower gelatine conversion in the presence of starch.

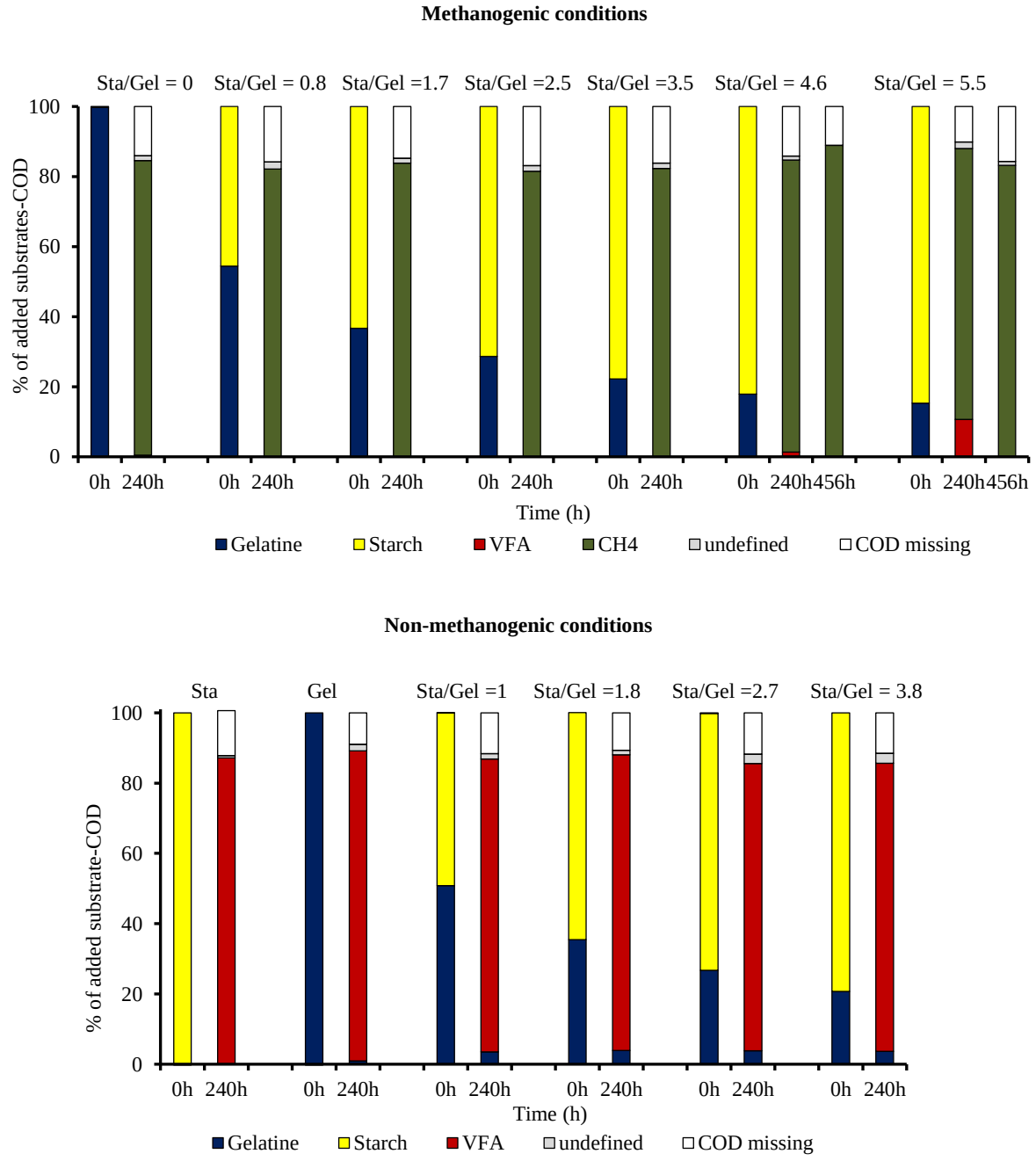

Figure 3.1. COD mass balances in experiment A (above) and experiment B (below) at different Sta/Gel ratios under methanogenic and non-methanogenic conditions, respectively. The data present average values of triplicate bottles. Standard deviations can be found in Table S1 of SI.

VFA product spectra of the tests in experiment B under non-methanogenic conditions are given in Figure 3.2. Acetate always was the most abundant VFA (49-58%), followed by propionate (21-26%). These results are similar to spectra observed by others and can be explained from the metabolic pathways and stoichiometry of gelatine and starch degradation (Breure et al., 1986b, Arslan et al., 2016, Regueira et al., 2020a). When starch was the only substrate valerate

accounted for 6% of the VFA that was produced but with gelatine alone this fraction (total of i-valerate and n-valerate) was almost 25%. This can be explained by valerate being the main product of deamination of proline, valine, isoleucine and leucine that are among the most abundant amino acids in gelatine (22-25% w/w)(Gelatin Handbook, 2012).

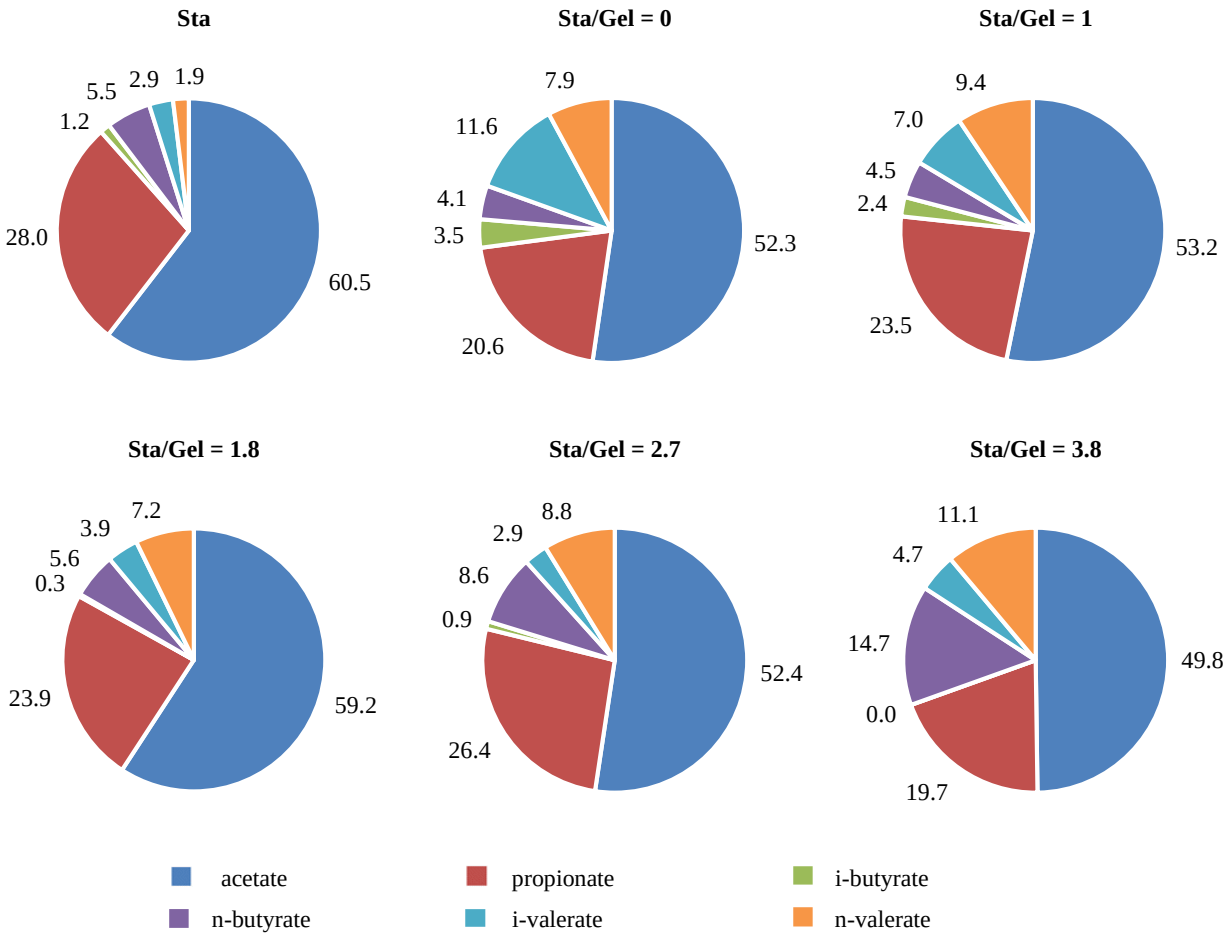

Figure 3.2. VFA product spectra in the tests of experiment B at different Sta/Gel ratio's under non-methanogenic conditions.

Finally, if the missing fraction of COD at the end of the tests can be attributed to biomass growth, under non-methanogenic conditions in experiment B this was 11.3 ± 1.4 %. Such a yield for acidifying and acetogenic biomass is in accordance with values reported by others (Breure and Van Andel, 1984, Breure et al., 1986b, Ramsay and Pullammanappallil, 2001, Yu and Fang, 2001, Tang et al., 2005). Under methanogenic conditions this yield was 14.4 ± 2.2 %, i.e. about 3.2% higher, caused by additional growth of methanogenic biomass, which also is in agreement with others (Breure and Van Andel, 1984, Stams, 1994, van Lier et al., 2020).

3.3.3 VFA inhibition of protein hydrolysis

Several researchers claim that VFAs do not inhibit protein hydrolysis (Breure et al., 1986b, Flotats et al., 2006). Also, the gelatine hydrolysis rates in experiments A and B (Table 3.3) did not indicate such inhibition, although under non-methanogenic conditions in experiment B at higher Sta/Gel ratios higher residual gelatine concentrations were measured. Still, a negative impact of VFA cannot be excluded, simply because in this experiment the major fraction of the gelatine was already hydrolysed by the time that significant VFA concentrations were produced. For example, by the time that already more than 75% of the gelatine was hydrolysed the VFA concentration was still below 3.0 g COD L^{-1} (Figure B2-B3, SI). In practice, in continuously operated reactors aiming to produce VFA, much higher VFA concentrations can be expected. For this reason experiment C was performed at different VFA/Gel ratio's (Table 3.2) to further investigate a potential negative impact of VFA on protein hydrolysis. Results in Table 3.3 (Experiment C) shows the effect of the VFA/Gel ratio on the first-order gelatine hydrolysis and acidification rate constants.

Clearly, VFA has a strong negative effect: at a VFA/Gel ratio of 5.9 (initial VFA concentration of 8.2 g COD L^{-1}) both the gelatine hydrolysis rates and the acidification rates were reduced to 64% of their values in the absence of VFA. The COD mass-balance (Table S1, SI) shows this resulted in an increase of the residual gelatine concentrations from 0 g COD L^{-1} when no VFA was added to 0.24 g COD L^{-1} at the highest VFA/Gel ratio of 5.9, corresponding to hydrolysis efficiencies of 100 and 84%, respectively. Please note that no conclusions can be drawn from the decreasing acidification rate constant at higher VFA/Gel ratios as this rate constant reflects the production of VFA (equation 3.2) and is the result of coupled protein hydrolysis and amino acid acidification. The highest concentrations of free amino acids was of about 2-4 mM after 8h-incubation, which is similar to concentrations measured in the absence of VFA (Figure C3, SI). A negative effect of amino acids on hydrolysis can therefore be excluded.

At pH 7 more than 99% of VFA is present in dissociated form and thus contributes to ionic strength. It is known that higher ionic strengths can have an effect on the structure of proteins, including enzymes. The structure of gelatine however is rather stable, even at high ionic strengths (Gelatin Handbook, 2012). It is therefore unlikely that the reduced gelatine hydrolysis at high VFA concentrations is caused by an ionic strength related effect of VFAs on the structure of gelatine. Figure 3.3 shows the gelatine hydrolysis rate constants as a function of the

(calculated) ionic strength in the test bottles of Experiments A, B and C and in previous batch tests where a higher ionic strength was induced by adding NaCl (Duong et al., 2019). In the latter tests, an ionic strength up to 105 mM did not have a negative effect on gelatine hydrolysis while the negative effect of VFA observed in Experiment C already occurred starting at a much lower ionic strengths of 40-50 mM. VFA contributed to approximately 50% of this ionic strength. This strongly indicates that gelatine hydrolysis is specifically inhibited by VFA and not by the ionic strength exerted by this VFA. To the best of our knowledge, a mechanistic explanation of this inhibitory effect is not available. We speculate that the VFA (i) directly affect the structure of gelatine or the structure or activity of existing proteases, (ii) cause suppression of protease production, and/or (iii) give reduced growth of protease producing biomass as was reported by González et al. (2005) at 0.25-0.75 g acetate L^{-1} at pH 7. If and to what extent these mechanisms reduce protein hydrolysis needs to be further investigated.

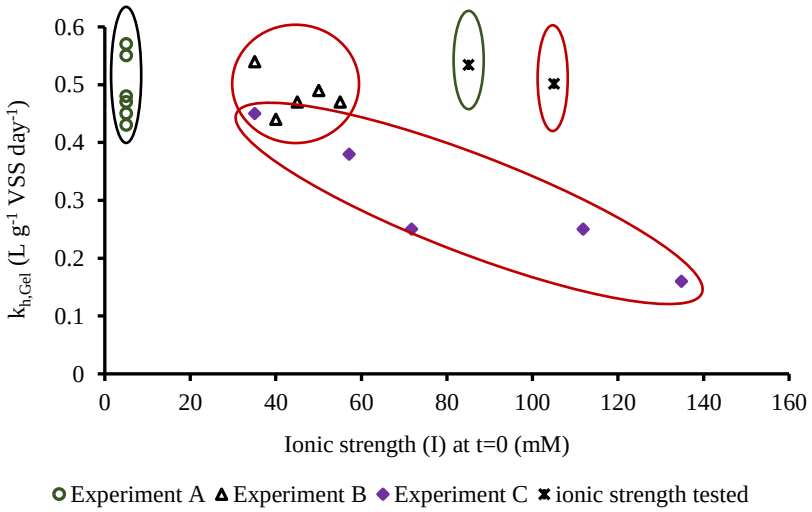

Figure 3.3. Gelatine hydrolysis rate constants (the average values, $k_{h,\ Gel}$) as a function of ionic strength concentrations in experiment A, B, C and in the ionic strength tests excluding VFA (*). The green circles express the data under methanogenic conditions and the red ones under non-methanogenic conditions.

3.3.4 Consequence for design of anaerobic reactor systems for treatment of protein rich wastewaters

The results in Experiments A and B showed that carbohydrates as such do not directly affect the rate at which proteins are hydrolysed. However, high VFA concentrations cause inhibition of protein hydrolysis as was demonstrated in experiment C (Table 3.3). For well-designed, continuously operating anaerobic reactors with methane as the desired end product this will not present a problem. Methanogenesis is much slower than hydrolysis of dissolved proteins such

as gelatine (experiment A) and dictates reactor design. At a solids retention time (SRT) high enough to avoid their wash-out, methanogens will keep the VFA concentration at a sufficiently low level to prevent inhibition of protein hydrolysis.

If VFA is the desired end product, i.e. under non-methanogenic conditions, high VFA concentrations can be expected to cause a strong reduction of protein hydrolysis rate to an extent that it becomes the rate limiting process. For example, at a product concentration of 8 g VFA-COD L^{-1} (Experiment C) the protein hydrolysis rate was reduced by a factor of approximately 3 with serious consequences for reactor construction and operational costs. This can only be overcome by active recovery of the VFA from the fermentation broth, for instance by extraction or electrodialysis processes (Aktij et al., 2020) or by applying a very long SRT. The mechanism by which VFA inhibit hydrolysis of dissolved proteins remain unclear. Moreover it cannot be excluded that during long-term operation of a continuous reactor on protein-rich wastewater the microbial population or the enzymatic machinery of the existing population will adapt to accommodate higher protein hydrolysis rates such as have been found for casein hydrolysis (Perle et al., 1995).

The results of experiments A and B also showed that the acidification rate is significantly lower than the rate of protein hydrolysis. This suggests it is possible to design a protein hydrolysis reactor followed by (active) amino acid recovery. However, this requires a complex lay-out in which a (small) fraction of the protein rich wastewater is fed to a second reactor for the production of the protein hydrolysing enzymes. These enzymes should be efficiently separated from the fermentation broth or effluent and subsequently be added to the hydrolysis reactor in sufficient amounts. This would be an interesting option but clearly needs to be investigated in more detail and is probably merely economically feasible at relatively high protein fractions in the wastewater.

3.4 Conclusions

Batch experiments carried out with a model dissolved protein (gelatine, Gel) and a model carbohydrate (starch, Sta) at 35°C under methanogenic and non-methanogenic conditions showed that the protein hydrolysis rate was not directly affected by starch. However, protein

hydrolysis was strongly inhibited by a mixture of different VFAs, which reduced the rate by 64±2%. For anaerobic reactors that aim to produce methane from protein rich wastewaters this does not present a problem as the VFA concentration can be maintained at a sufficiently low level. However, for VFA producing reactors this has implications as protein hydrolysis could be rate limiting.

Supplementary Information of Chapter 3

Table S1: Concentrations of products and residual substrates of batch bottles in experimental set-up A, B and C (t=240 h, except for Sta/Gel = 4.6 and 5.5 in Experiment A t = 456h). All data are expressed as average ± standard deviation (n=3), in g COD L^{-1}.

Experiment A	Sta/Gel						
	0	0.8	1.7	2.5	3.5	4.6	5.5
Gelatine	0	0	0	0	0	0.01±0.01	0.01±0.01
Starch	0	0	0	0	0	0	0
VFA	0	0	0	0	0	0	0
CH_4	1.17±0.09	2.08±0.03	3.09±0.11	4.11±0.13	5.28±0.20	6.66±0.15	7.61±0.24

Experiment B		Sta/Gel				
	Sta	0	1	1.8	2.7	3.8
Gelatine	0	0.01±0.01	0.10±0.02	0.16±0.01	0.21±0.02	0.25±0.02
Starch	0	0	0	0	0	0
VFA	1.17±0.02	1.21±0.01	2.29±0.04	3.48±0.06	4.49±0.04	5.54±0.04

Experiment C	VFA/Gel				
	0	1.2	2.2	4.5	5.9
Gelatine	0	0.13±0.01	0.10±0.01	0.23±0.01	0.24±0.01
VFA*	1.28±0.01	1.13±0.02	1.06±0.09	0.93±0.04	0.93±0.02

*: concentrations of VFA produced from the added gelatine

Table S2: Maximum (experiment A) and final (experiment B) VFA concentrations (g COD L^{-1}) at different Sta/Gel ratios.

Experiment A: gelatine with varying starch concentrations at methanogenic conditions

Mixtures	Acetate	Propionate	Butyrate	Valerate	Total VFA
Sta/Gel = 0	0.09	0.17	0.05	0.05	0.32
Sta/Gel = 0.8	0.16	0.45	0.08	0.03	0.23
Sta/Gel = 1.7	0.19	0.65	0.08	0.03	0.41
Sta/Gel = 2.5	0.21	0.99	0.10	0.02	1.09
Sta/Gel = 3.5	0.19	1.31	0.10	0.05	1.58
Sta/Gel = 4.6	0.65	1.37	0.06	0.10	1.87
Sta/Gel = 5.5	0.73	1.73	0.47	0.18	2.65

Experiment B: gelatine with varying starch concentrations at non-methanogenic conditions

Mixtures	Acetate	Propionate	Butyrate	Valerate	Total VFA
Sta/Gel = 0	0.69	0.26	0.09	0.18	1.22
Sta/Gel = 1	1.24	0.57	0.16	0.37	2.34
Sta/Gel = 1.8	2.12	0.86	0.17	0.38	3.53
Sta/Gel = 2.7	2.39	1.19	0.43	0.52	4.53
Sta/Gel = 3.8	2.79	0.92	0.93	0.94	5.58

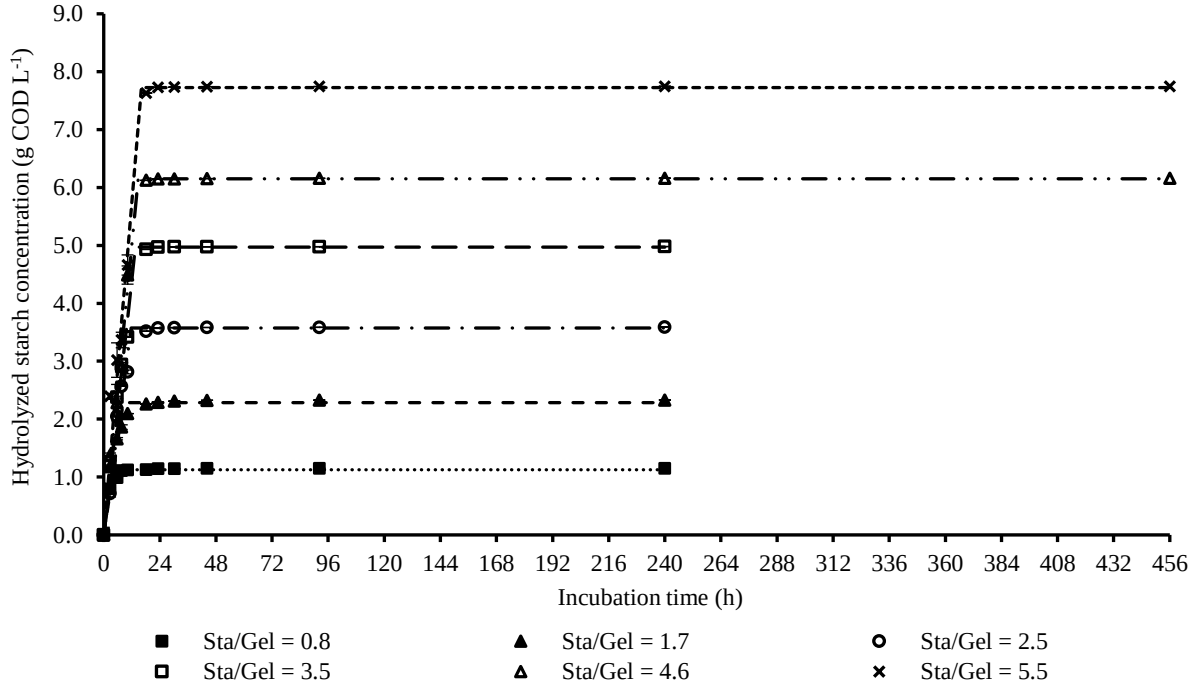

Figure A1. Zero-order kinetics of starch hydrolysis at different Sta/Gel ratios under methanogenic conditions in experiment A. The data points represent average values from triplicate bottles. The standard deviation always was less than 10%.

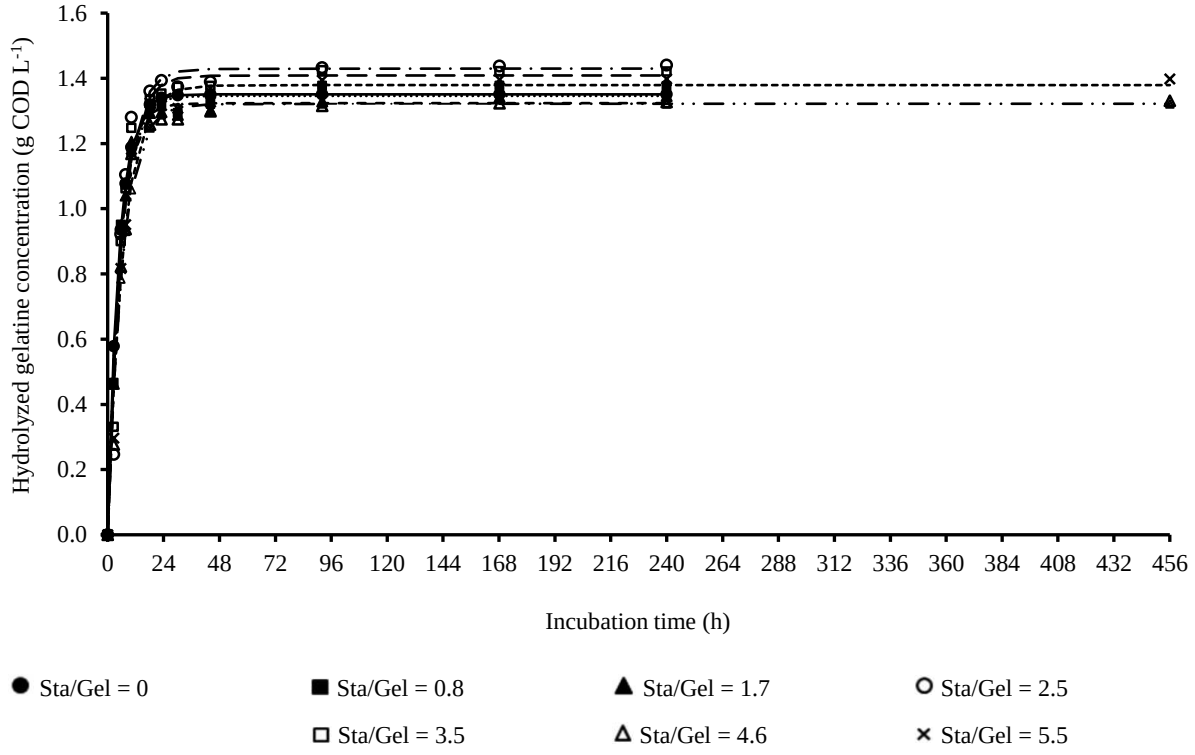

Figure A2. First-order kinetics of gelatine hydrolysis at different Sta/Gel ratios under methanogenic conditions in experiment A. The data points represent average values from triplicate bottles. The standard deviation always was less than 10%.

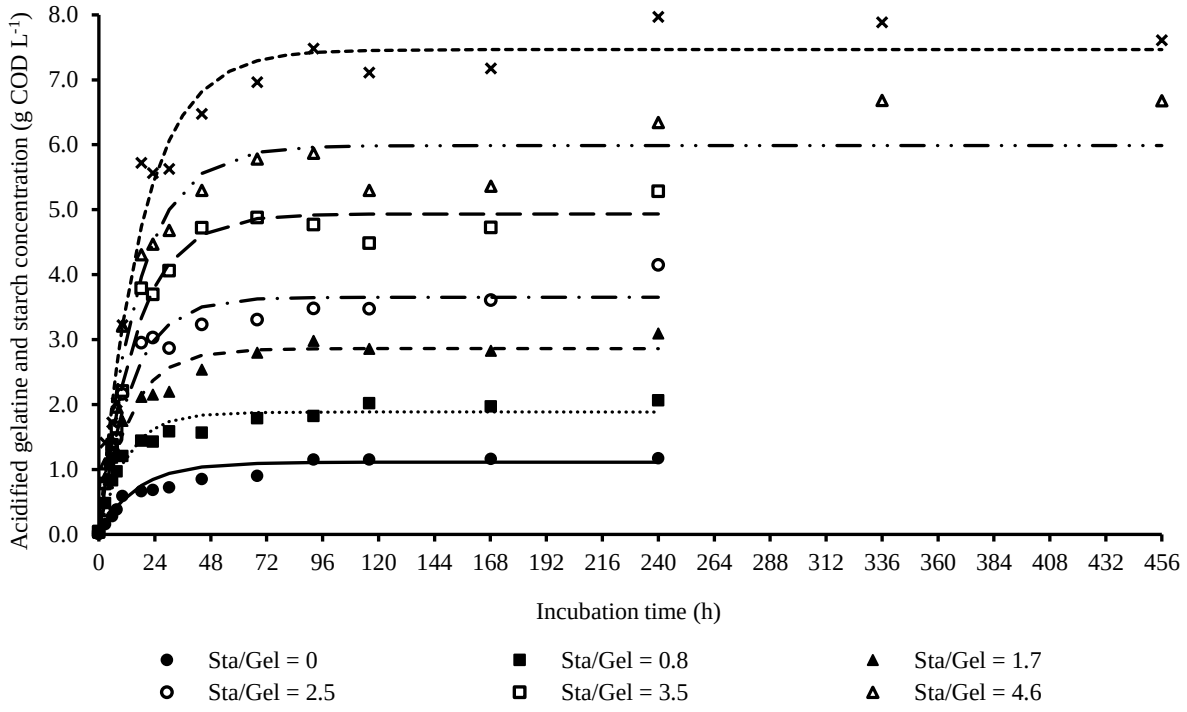

Figure A3. First-order kinetics of VFA plus methane production at different Sta/Gel ratios under methanogenic conditions in experiment A. The data points represent average values from triplicate bottles. The standard deviation always was less than 10%.

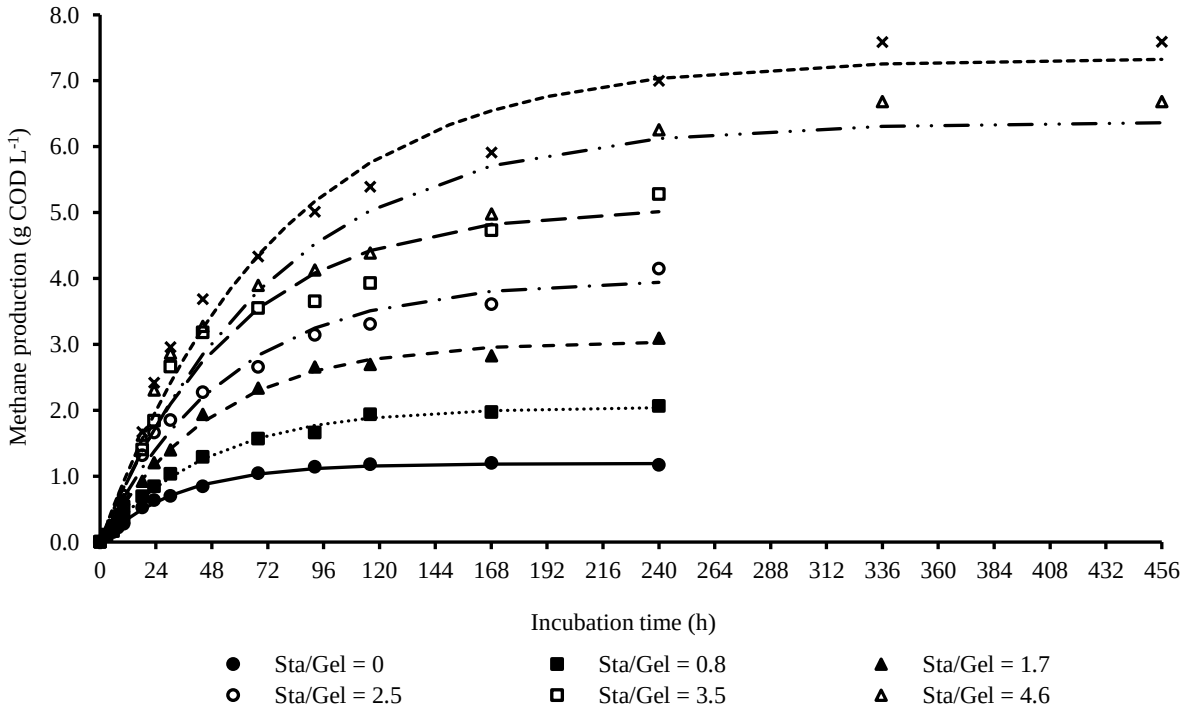

Figure A4. First-order **k**inetics of methane production at different Sta/Gel ratios under methanogenic conditions in experiment A. The data points represent average values from triplicate bottles. The standard deviation always was less than 10%.

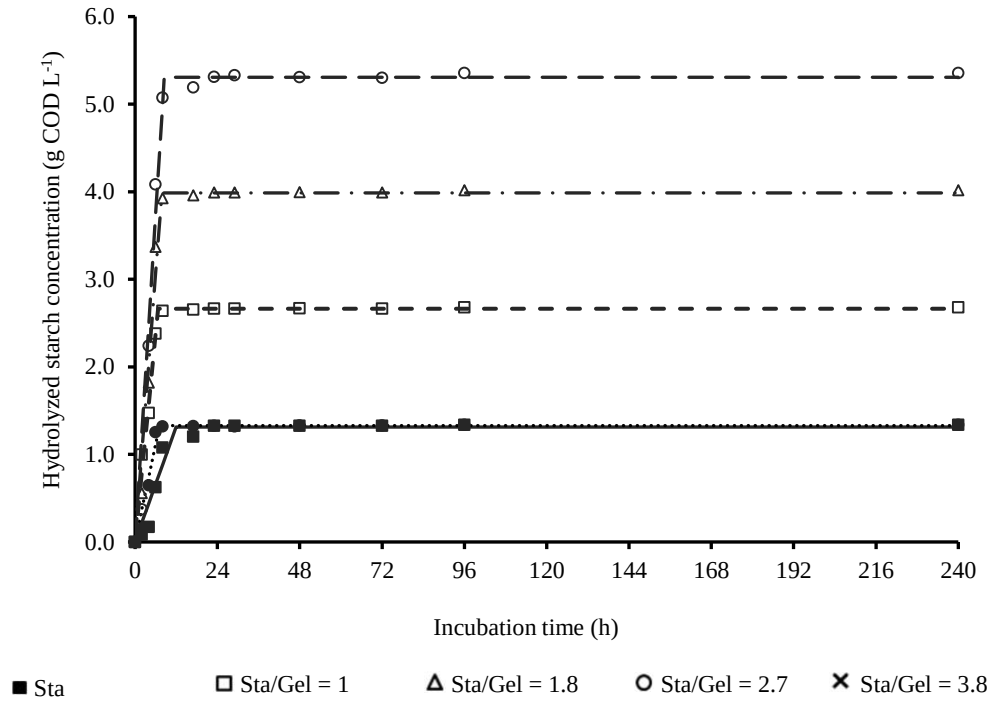

Figure B1. Zero-order kinetics of starch hydrolysis at different Sta/Gel ratios under non-methanogenic conditions in experiment B. The data points represent average values from triplicate bottles. The standard deviation always was less than 10%.

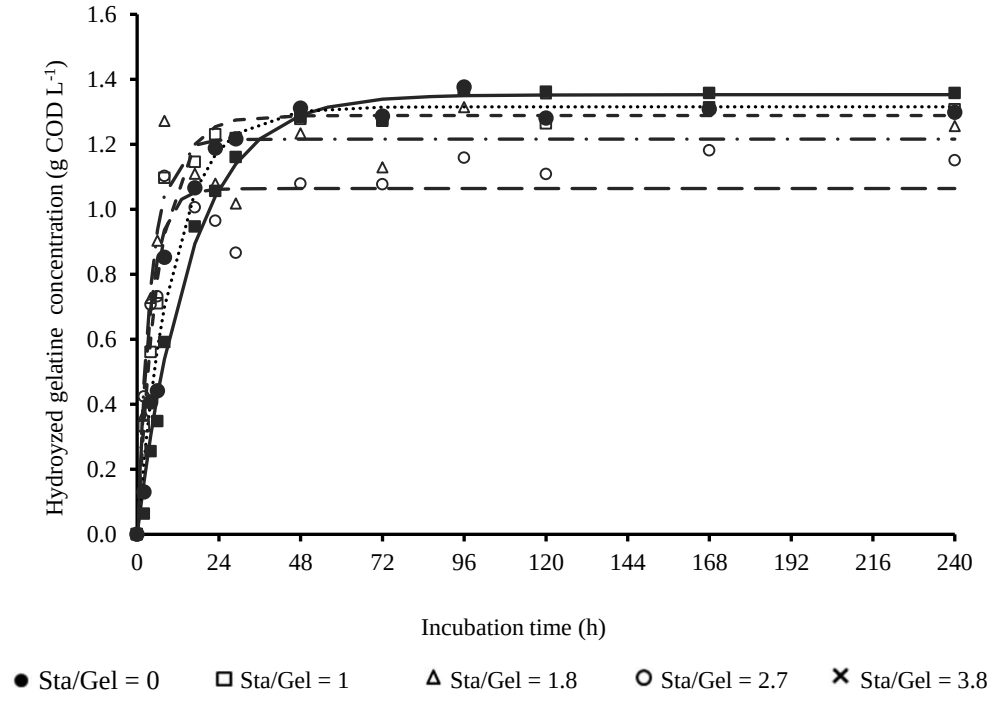

Figure B2. First-order kinetics of gelatine hydrolysis at different Sta/Gel ratios under non-methanogenic conditions in experiment B. The data points represent average values from triplicate bottles. The standard deviation always was less than 10%.

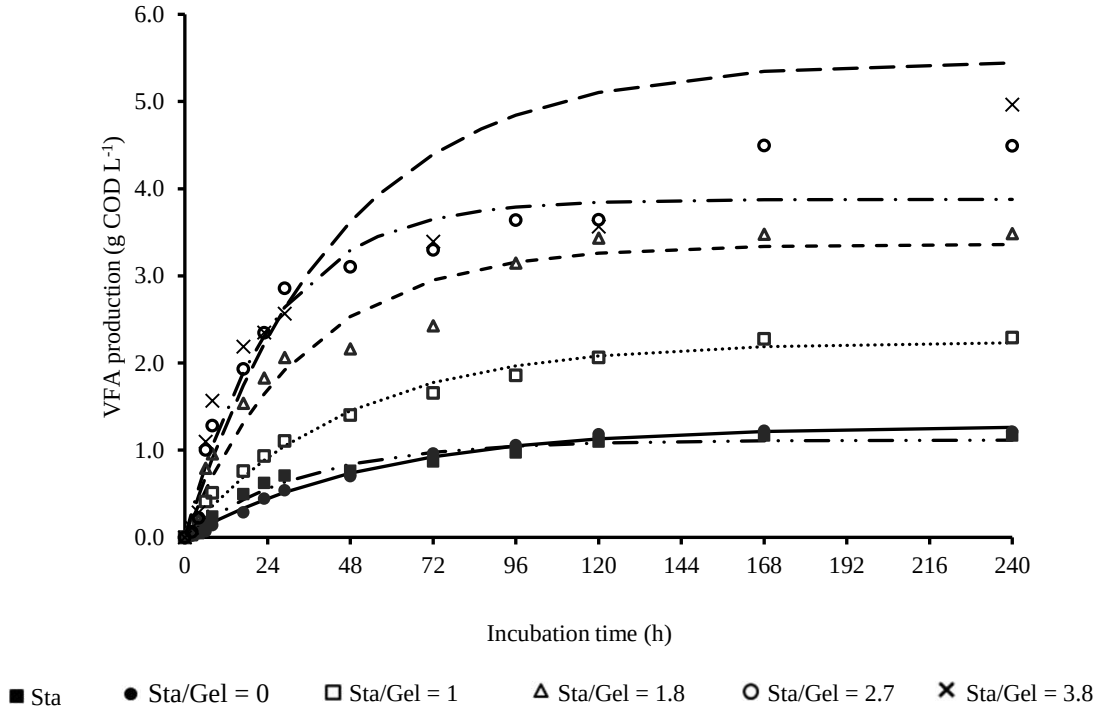

Figure B3. First-order kinetics of VFA production at different Sta/Gel ratios under non-methanogenic conditions in experiment B. The data points represent average values from triplicate bottles. The standard deviation always was less than 10%.

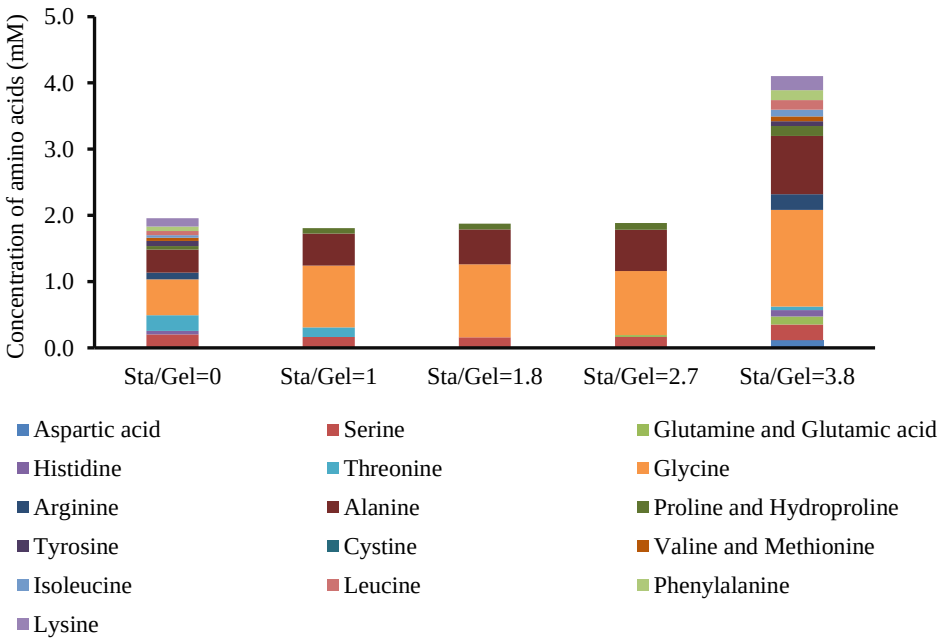

Figure B4. Concentration of amino acids at the different Sta/Gel ratios and at 8h incubation, non-methanogenic conditions in experiment B at 35°C. The data points represent average values from triplicate bottles. The standard deviation always was less than 5%.

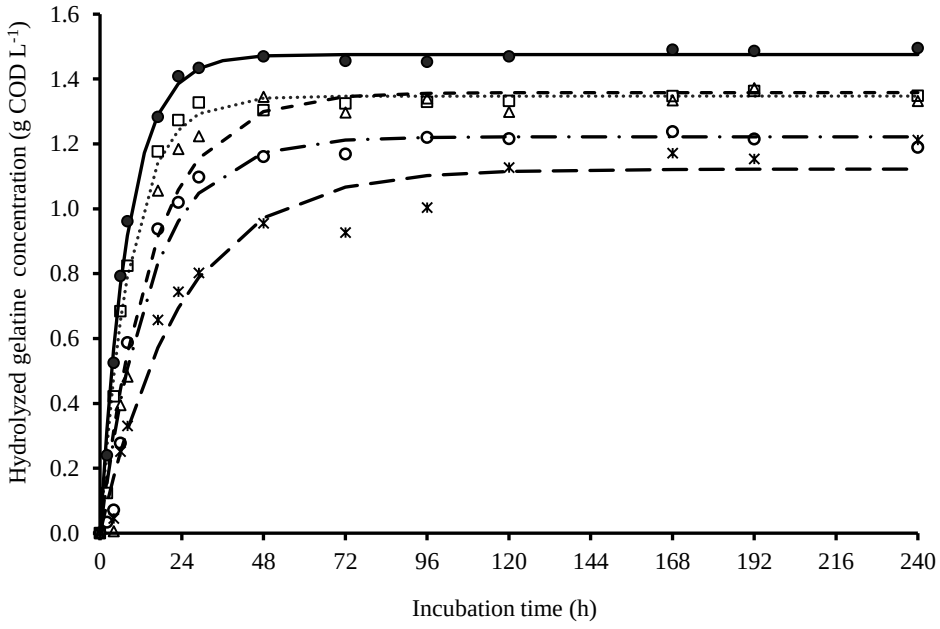

Figure C1. First-order kinetics of gelatine hydrolysis at different VFA/Gel ratios under non-methanogenic conditions in experiment C. The data points represent average values from triplicate bottles. The standard deviation always was less than 10%.

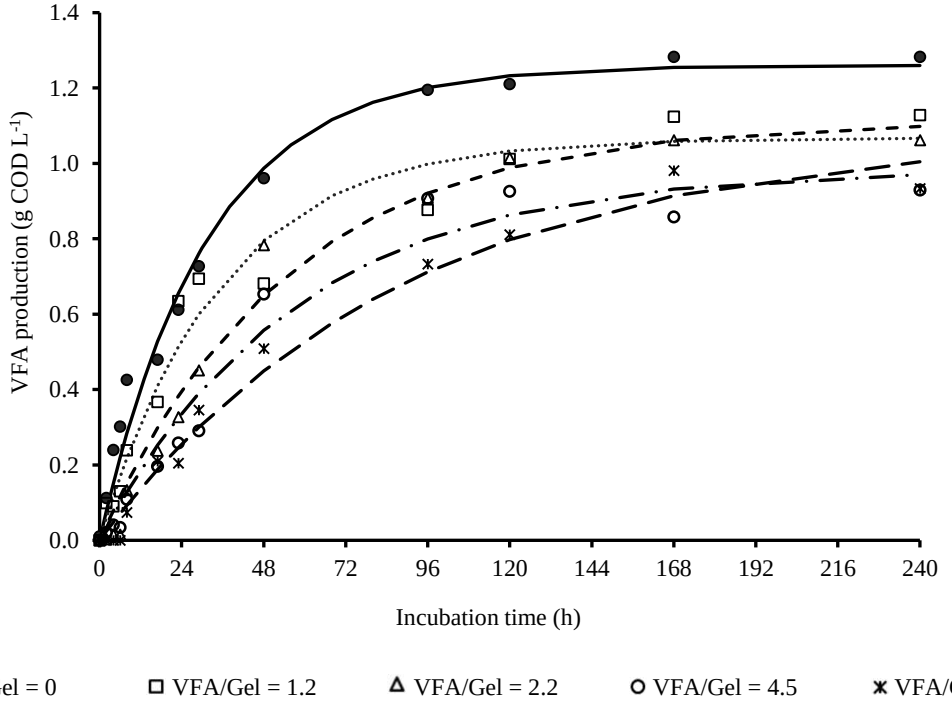

Figure C2. First-order kinetics of VFA production at different VFA/Gel ratios under non-methanogenic conditions in experiment C. The data points represent average values from triplicate bottles. The standard deviation always was less than 10%.

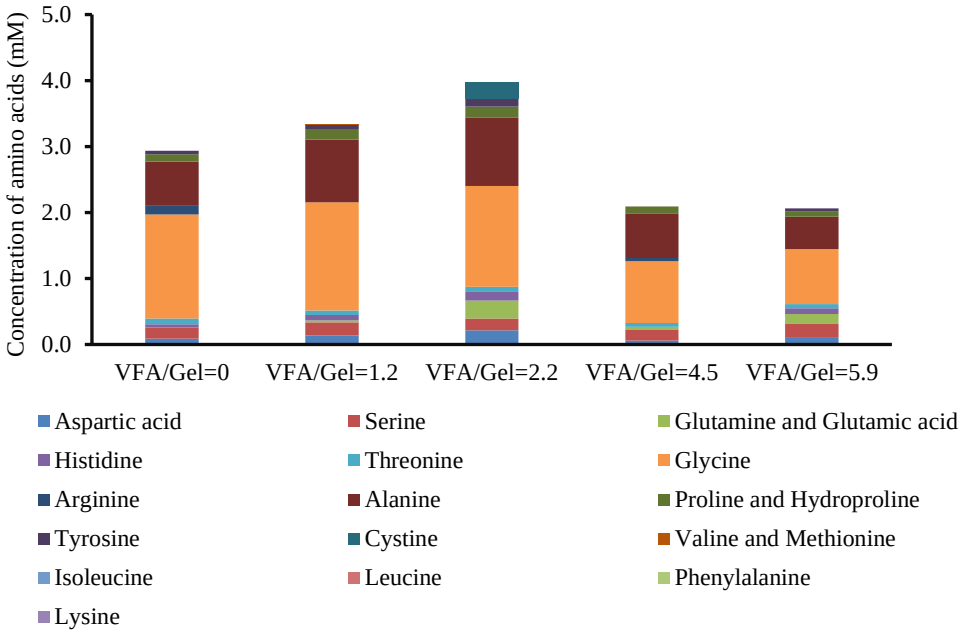

Figure C3. Concentration of amino acids at the different VFA/Gel ratios in experiment C and at 8h incubation, non-methanogenic conditions at 35°C. The data column represent average values from triplicate bottles. The standard deviation always was less than 5%.

Chapter 4. Effect of solid retention time on protein hydrolysis and acidogenesis at pH 5 and pH 7

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Abstract

Anaerobic fermentation can be used to recover volatile fatty acids (VFA) from high-strength wastewaters and organic wastes. However, many waste(waters) contain considerable concentrations of proteins and knowledge about anaerobic conversion of protein into VFAs is limited. In this study the effect of the solids retention time (SRT) and pH on dissolved protein conversion into VFAs was investigated in completely stirred tank reactors (CSTRs) operated at 35°C. Even after a long-term exposure of the biomass to pH 5, the hydrolysis rate constant for protein (0.05 L g^{-1}VSS day^{-1}) was still much lower than at pH 7 (0.62 L g^{-1}VSS day^{-1}). The highest volumetric VFA productivity of 2.3 g COD L^{-1} day^{-1} was obtained at pH 7 and at an SRT of 10 days. For complete removal of protein a longer SRT is required.

Keywords: protein hydrolysis, acidogenesis, retention times, low pH, long-term exposure.

4.1 Introduction

Protein is a major organic constituent of wastewaters and wastes, accounting for 20-75% of the chemical oxygen demand (COD) of e.g. meat and fish-processing, slaughterhouse, cheese whey and beverage wastewaters (Palenzuela, 1999, Masse and Masse, 2000, Carvalho et al., 2013). These protein-rich waste streams are attractive substrates for anaerobic treatment to generate energy-rich methane while simultaneously achieving the objective of pollution control (Zeeman et al., 2008, De Schouwer et al., 2019, van Lier et al., 2020). Alternatively, volatile fatty acids (VFA) can be produced as valuable intermediates of anaerobic degradation processes because they provide chemical building blocks for compounds in the bio-based economy such as bioplastics, biopolymers in textiles and cleaning agents (Kleerebezem et al., 2015, Tamis et al., 2015, Arslan et al., 2016, Regueira et al., 2020b). Anaerobic conversion of complex biowastes (containing mixtures of proteins, carbohydrates, fats and other compounds) has been extensively studied (Fra-Vázquez et al., 2020, van Lier et al., 2020). However, the necessary information on how to optimize the first steps in the conversion of proteins, i.e. hydrolysis and fermentation, is lacking.

Protein hydrolysis is inhibited at low pH (Duong et al., 2019, Fra-Vázquez et al., 2020), but the underlying mechanism is poorly understood. Several studies suggested a low pH may negatively affect the activity of hydrolytic microorganisms and/or of the proteases they produce (Breure and Van Andel, 1984). The strategy of overloading anaerobic reactors with biodegradable COD to enforce a low pH and subsequent inhibition of methanogenesis is often used to obtain VFA from complex organic wastes. However, little is known about the VFA production efficiency from protein using this strategy (Arslan et al., 2016). The COD content in protein containing food-processing wastewaters can be as high as 30-45 g L^{-1} (Yu and Fang, 2001, Bengtsson et al., 2008, Khatami et al., 2021) and under non-methanogenic conditions this could result in inhibition of protein degradation by high concentrations of end products VFAs (González et al., 2005). Yu and Fang (2001) found that the acidification degree of milk reduced from 50% to 30% when the COD increased from 4 to 30 g COD L^{-1}. Perle et al. (1995) observed that acclimation of the inoculum sludge could improve solubilization (i.e. hydrolysis) of casein at neutral pH in batch tests. However, it is unknown if hydrolysis can also be improved by long-term exposure of biomass to lower pH values.

Hydrolysis is generally considered to be the rate-limiting step during anaerobic degradation of particulate organics, which explains why hydrolysis rate constants reported in literature usually are based on the formation rate of end products such as methane and ammonium. However, in a previous study we showed that at pH 7 and under methanogenic conditions amino acid fermentation was significantly slower than hydrolysis of dissolved protein (Duong et al., 2019). The hydrolysis rate constant and operational conditions, mainly the solid retention time (SRT) and pH, will affect protein conversion efficiency. Information on the effect of the SRT on protein hydrolysis, acidification efficiency and VFA product spectrum in continuous anaerobic systems is limited. A number of studies suggested that a short SRT, which is economically more favorable, will result in limited conversion (Bengtsson et al., 2008, Bevilacqua et al., 2020a). This would explain the low VFA product yields, ranging from 0.2 to 0.5 g VFA-COD per g gelatine- or casein-COD observed in reactors operated at SRTs of 5-36 h (Breure and Van Andel, 1984, Yu and Fang, 2003, Bevilacqua et al., 2020a, Bevilacqua et al., 2020b). More information about the effect of SRT and pH is needed to be able to optimize the conversion efficiency for protein-rich waste(waters).

In this study, we explored the effect of pH and SRT on protein degradation with the objective to produce VFAs, so under non-methanogenic conditions. For this purpose two continuous stirred-tank reactors (CSTR) were inoculated with sludge from an anaerobic reactor treating brewery wastewater. The reactors were operated at pH 5 and 7, and at SRTs of 12-30 days and 6-12 days, respectively. Gelatine, a model (dissolved) protein, was fed to the reactors at a concentration of approximately 29 g COD L^{-1}. Protein hydrolysis kinetics were determined from CSTR measurements and in batch experiments with biomass that was sampled from these CSTRs. Protein degradation was assessed from protein, amino acid and VFA concentrations.

4.2 Materials and Methods

4.2.1 Inoculum and substrate characteristics

The seed sludge for the two CSTRs was obtained from a full-scale anaerobic reactor treating brewery wastewater and which was operated at a temperature of 30 ± 3°C. The sludge had the following characteristics: total suspended solids (TSS) 18.6 ± 0.5 g L^{-1}, volatile suspended

solids (VSS) 10.3 ± 0.1 g L^{-1}, total COD (COD_{tot}) 19.3 ± 0.3 g L^{-1}. Total nitrogen (TN) and ammonium (NH_4^+-N) were 0.32 ± 0.03 and 0.12 ± 0.05 g L^{-1}, respectively. The pH of the sludge was 7.3 ± 0.1.

Gelatine was used as a (soluble) model protein (CAS no.9000-70-8, Sigma-Aldrich), and applied as feedstock solution of 25 ± 1.0 g gelatine, equivalent to 28.6 ± 1.2 g COD dissolved in 50°C-heated demi-water and supplemented with micro nutrients as described in Angelidaki et al. (2009). The feedstocks for each CSTR were kept in a water bath of 40 ± 2°C to avoid gelation during feeding into the CSTR.

4.2.2 Continuous experiments

The continuous experiments were performed in two double-walled plastic CSTRs, each with a working liquid volume of 20 L and a headspace of 7 L. The temperature was kept constant at 35 ± 1°C by a water mantle and water bath (AS One, Japan). The pH of two CSTR were controlled at pH 5.0±0.1 and pH 7.0 ± 0.1 by HCl (1N) or NaOH (1N) addition. The reactors were inoculated at an initial biomass concentration of 8.6 g VSS L^{-1}. In both reactors 2-bromoethanesulfonate (BES, 20mM) was added at day 0 to inhibit methanogenic activity and additional doses of BES (10mM) were applied to CSTR pH 7 on days 275, 360 and 516.

The CSTRs were operated at different SRTs according to the schedule in Table 4.1. The reactors were assumed to be in 'steady state' when during at least three consecutive SRTs the effluent concentrations of protein and VFA gave less than 20% variation. The influent and effluent flow rates were set at 5.9 mL min^{-1} in a 5-min cycle (1 minute on and 4 min off) to set an SRT of 12 days at the start of the experiments. During 600 operational days the SRT of the CSTR operated at pH 7 was decreased from 12 days to 10-8-6 days and back to 8-10 days. The CSTR at pH 5 was operated for 480 days and the SRT was subsequently increased from 12 to 20 and 30 days.

Table 4.1. Operation strategies for CSTR at pH 5 and 7 and 35°C.

pH 5	Period	0-150	151-290*	291-480			
	SRT (day)	12	20	30			
pH 7	Period	0-60	61-102	103-156	157-206	207-516*	517-600
	SRT (day)	12	10	8	6	8	10

Note: * temperature dropped (day 203-213 in CSTR at pH 5 and day 260-270 in CSTR at pH 7) due to water bath broke down during these periods

The CSTRs were sampled from the influent (±10 mL) and effluent valves (±50 mL). pH and concentrations of total chemical oxygen demand (COD_{tot}) and protein in the influent were determined two times per week and total nitrogen (TN) once per month. pH, total suspended solids (TSS), volatile suspended solids (VSS), COD_{tot}, COD of the supernatant (COD_{sol}), protein, and VFA concentrations in the effluent were assessed 2-3 times a week. Analyses of concentrations of amino acid, total peptides, TN and ammonium (NH_4^+-N) were carried out on selected samples during steady state periods. Gas production was measured daily via liquid-displacement columns connected to the CSTRs. Samples to determine the gas composition (CH_4, CO_2, H_2 and N_2) were taken from the gas sampling valve of each reactor and analysed once a week.

4.2.3 Batch experiments

Several batch experiments were set-up to determine the kinetics of protein hydrolysis by the biomass in the CSTRs, sampled during steady states at different SRTs. The batch experiments were carried out in triplicate at 35 ± 1°C in 0.23 L serum bottles (working liquid volume of 0.15 L), continuously shaken at 60 revolutions per minute (rpm) for 240 h. The batch medium at pH 7 was adapted from Angelidaki et al. (2009). NH_4Cl was not added because sufficient nitrogen was already present in the gelatine. The medium at pH 5 was identical to that at pH 7, except for Na_2HPO_4 which was replaced with 3.13 g KH_2PO_4 L^{-1}.

The biomass was collected from the effluent, and was allowed to settle in a beaker for 2-3 days to obtain a concentrated sludge (VSS above 17 g L^{-1}). The concentrated biomass was added to batch bottles to achieve a working concentration of 2.8 ± 0.2 g VSS L^{-1}. Dissolved gelatine was added at a concentration of 1.4 ± 0.05 g COD L^{-1}. Before the bottles were closed with rubber stoppers and aluminium caps, the contents were carefully mixed, sampled for initial concentrations and flushed for a short period of time with N_2 gas until methane no longer was detected in the head space.

Blanks were prepared containing only biomass inoculum and medium, thus without gelatine addition. BES (20mM) was added into pH 7 batch bottles to inhibit methane formation. Also,

a batch test was conducted with gelatine at pH 5 and pH 7 without inoculum to verify that no chemical hydrolysis of gelatine occurred at 35 ± 1°C.

In the batch experiments, gas and liquid samples were taken at an interval of 2-3 h during the first 8-10 h. Subsequently 8 more samples were taken towards the end of the experiment. The samples were analysed as described in (Duong et al., 2019). A lack of methane production in all bottles showed that methanogenesis was effectively inhibited (data not shown).

4.2.4 Analyses

Gas composition (CH_4, CO_2, H_2 and N_2) was quantified by injecting the gas sample in a Shimadzu 8A (Shimadzu, Japan) GC equipped with a compact materials Unibeads C 60/80 mesh column (Φ3 mm, length 2 m) connected to a thermal conductivity detector (argon as carrier gas). pH was measured by a pH meter (Hach, PHC 101, Seri No.162822568077, USA). The determination of total solids (TS) and volatile solids (VS) of gelatine was done according to standard methods (APHA-AWWA-WEF, 2017). Digestate (effluent) and sludge samples were analysed for TSS, VSS, and COD_{tot} using the standard methods. The digestate samples were centrifuged (Eppendorf, Germany) at 10000 rpm for ten minutes and filtered with pre-washed 0.45 µm cellulose acetate membrane filters (Sartorius, Germany). The soluble fraction was analyzed for CODs, TN and NH_4-N using Hach Lange methods and test kits (LCK1014, LCK338, LCK303). Protein was determined using the Lowry method assay (Noble and Bailey, 2009) at 660 nm using gelatine as standard. Total peptides were analysed in the supernatant samples as described by Cuchiaro and Laurens (2019). VFAs were quantified on a Trace gas chromatograph equipped with a Thermo TR-WAX column (30 m x ID 0.32 mm x thickness of 0.25 µm) connected to a FID detector as described by Sudmalis et al. (2018). Amino acids were measured in the supernatant samples as described by Meussen et al. (2014) via high-performance liquid chromatography (HPLC).

4.2.5 Calculations

Hydrolysis, acidification, VFA yield and biomass yield in the CSTRs were calculated as follows:

Degree of hydrolysis: $H = \frac{P_i - P}{P_i} \cdot 100\ (\%)$ (eq. 4.1)

Degree of acidification: $A = \frac{COD_{VFA}}{P_i} \cdot 100\ (\%)$ (eq. 4.2)

VFA yield: $Y_{VFA} = \frac{COD_{VFA}}{P_i - P}\ \left(g\ COD_{VFA}\ g^{-1} COD_{hydrolyzed\ protein}\right)$ (eq. 4.3)

Biomass yield:

$Y_{sludge} = \frac{COD_{tot,eff} - COD_{sol,eff}}{P_i - P}\ \left(g\ COD_{sludge}\ g^{-1} COD_{hydrolyzed\ protein}\right)$ (eq. 4.4)

With P_i the influent protein concentration and P the effluent protein concentration (g COD L^{-1}), using a conversion factor of 1.150 g COD g^{-1}gelatine; COD_{VFA} the total COD concentration of volatile fatty acids (VFA, g COD L^{-1}); $COD_{tot,eff}$ the total COD of the effluent and $COD_{sol,eff}$ the COD of the supernatant of the effluent (g COD L^{-1}).

A first-order model was used to estimate (with a least-squares method) hydrolysis rate constants from the protein concentrations:

$$P = \frac{P_i}{1 + k_h \cdot X \cdot (SRT - SRT_{min})}$$ (eq. 4.5)

With k_h the hydrolysis rate constant, normalised for the sludge concentration (L g^{-1}VSS day^{-1}); SRT is the solid retention time in the CSTR (day); SRT_{min} (day) the minimum SRT below which hydrolysis no longer takes place due to wash-out of hydrolysing microorganisms; X the VSS concentration of sludge in the CSTR (g VSS L^{-1}).

The COD mass balance was evaluated from the influent COD, effluent COD_{tot} and COD_{sol}, and COD of fermented products in the effluent including the liquid and off-gas to guarantee that the analytical measurements covered all the important compounds. In none of the CSTRs a significant amount of gas was produced (0.1-0.3 L day^{-1}) and the COD content of the gas (hydrogen and methane) always was less than 0.1% of the influent COD. Thus, non-methanogenic conditions in both continuous reactors were assured.

First-order protein hydrolysis rate constants were estimated from the results of the batch experiments with the following equation:

$$P_{\text{hydrolyzed prot}}(t) = P_{\text{added prot}} \cdot (1 - \exp(-k_{h-batch} \cdot X \cdot t)) \quad \text{(eq. 4.6)}$$

With $P_{\text{hydrolyzed prot}}(t)$ the (cumulative) concentration of hydrolyzed protein (g COD L^{-1}) after time t (day), $P_{\text{added prot}}$ the concentration of protein (g COD L^{-1}) in the batch experiments; $k_{h-batch}$ the first-order hydrolysis rate constant of protein (L g^{-1}VSS day^{-1}); X the VSS concentration of the seed sludge in batch tests (2.8 g VSS L^{-1}). The COD mass balance in the batch experiments was evaluated according to a similar procedure as applied for the CSTRs.

4.3 Results and Discussion

The COD mass balances in the experiments indicated that all important intermediates and products in protein degradation pathways were identified. The COD mass balances of the CSTRs operated at pH 5 and pH 7 can be found in the Supplementary information (Figure S1, SI). The gap in the COD mass balances always was less than 10%. Reactor performance was affected by an unforeseen temperature drop from 35°C to 25°C for 10 days (Table 4.1), but recovered from this without having to take operational measures.

4.3.1 Protein hydrolysis and acidification at pH 5 and pH 7

The CSTRs (Figure 4.1 and Table 4.2 for pH 5; Figure 4.2 and Table 4.3 for pH 7) showed distinct differences with respect to the degree of hydrolysis and acidification in response to SRT and pH. In general, as expected, a longer SRT gave higher sludge concentrations, hydrolysis and acidification efficiencies, and VFA yields (for pH 7). It should be noted however that the VFA yields for the CSTR operated at pH 5 of 0.68-0.74 g COD_{VFA} $g^{-1}COD_{hydrolyzed\ protein}$ statistically were not different. The VFA yield at pH 7 and at SRT 12 days of 0.72 g COD_{VFA} $g^{-1}COD_{hydrolyzed\ protein}$ did not follow the trend, and was lower than expected. Perhaps this is because CSTR operation started at this particular SRT (Figure 4.2), and full acclimation of the inoculum was not yet achieved after 60 days. The average biomass yield of the reactors ranged between 0.08 and 0.13 g COD_{sludge} $g^{-1}COD_{hydrolyzed\ protein}$, which is in accordance with values reported by others (Breure and Van Andel, 1984, Tang et al., 2005, Bevilacqua et al., 2020b).

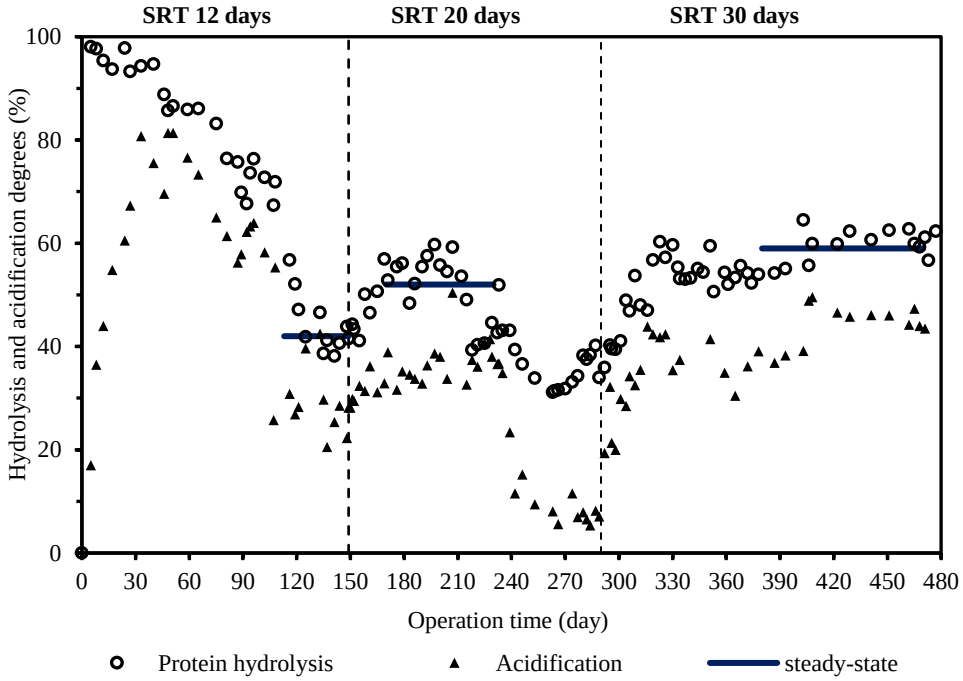

Figure 4.1. Protein conversion at CSTR pH 5 and 35°C.

Table 4.2. Organic loading rate (OLR), sludge concentration (X), hydrolysis degree of gelatine (H), VFA yield (Y_{VFA}), and biomass yield (Y_{sludge}) at different SRTs for operation of a CSTR at pH 5.

pH 5	SRT 12 days	SRT 20 days	SRT 30 days
OLR (g COD L^{-1} day^{-1})	2.31 ± 0.10	1.42 ± 0.05	0.95 ± 0.10
X (g VSS L^{-1})	0.9 ± 0.2 (a)	1.3 ± 0.1 (b)	1.5 ± 0.2 (b)
H (%)	42 ± 5 (a)	52 ± 5 (b)	59 ± 9 (c)
Y_{VFA} (g COD_{VFA} $g^{-1}COD_{hydrolyzed\ protein}$)	0.68 ± 0.08 (a)	0.73 ± 0.06 (a)	0.74 ± 0.05 (a)
Y_{sludge} (g COD_{sludge} $g^{-1}COD_{hydrolyzed\ protein}$)	0.11 ± 0.02 (a)	0.12 ± 0.02 (a)	0.12 ± 0.02 (a)

Note: Data expressed the mean ± std at the steady state at different SRT. Letters in parentheses indicate significant differences between values ($p<0.05$) with a<b<c. Values with the same letters are not significantly different.

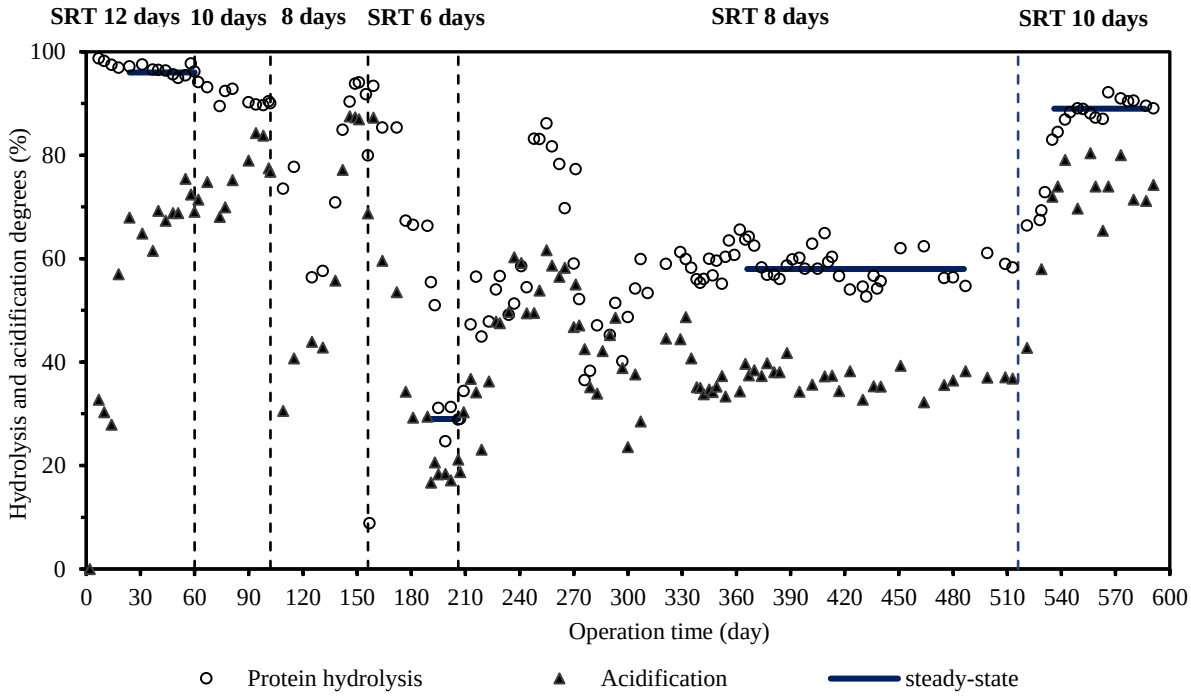

Figure 4.2. Protein conversion at CSTR pH 7 and 35°C. Data at SRT 10 days (day 60-102) and at SRT 8 days (day 102-155) varied more than 20%, therefore the latter periods (day 366-486 for SRT 8 days and day 536-583 for SRT 10 days) were used for calculation.

Table 4.3. Organic loading rate (OLR), sludge concentration (X), hydrolysis degree of gelatine (H), VFA yield (Y_{VFA}), and biomass yield (Y_{sludge}) at different SRTs for operation of a CSTR at pH 7.

pH 7	SRT 6 days	SRT 8 days	SRT 10 days	SRT 12 days
OLR (g COD L^{-1} day^{-1})	4.58 ± 0.29	3.58 ± 0.10	2.94 ± 0.09	2.31 ± 0.04
X (g VSS L^{-1})	0.5 ± 0.1 (a)	1.0 ± 0.2 (b)	1.3 ± 0.3 (b)	2.3 ± 0.3 (c)
H (%)	35 ± 2 (a)	55 ± 5 (b)	92 ± 2 (c)	96 ± 1 (d)
Y_{VFA} (g COD_{VFA} $g^{-1}COD_{hydrolyzed\ protein}$)	0.51 ± 0.04 (a)	0.62 ± 0.04 (b)	0.84 ± 0.05 (d)	0.72 ± 0.04 (c)
Y_{sludge} (g COD_{sludge} $g^{-1}COD_{hydrolyzed\ protein}$)	0.08 ± 0.01 (a)	0.08 ± 0.01 (a)	0.08 ± 0.01 (a)	0.12 ± 0.02 (b)

Note: Data expressed the mean ± std at the 'steady state' at different SRT. Letters in parentheses indicate significant differences between values ($p<0.05$) with a<b<c<d. Values with the same letters are not significantly different.

For both CSTRs from the protein measurements a first-order hydrolysis rate constant k_h and a minimum SRT_{min} were estimated according to equation (4.5) (Figure 4.3). In spite of the long-term exposure of the biomass to pH 5 the estimated hydrolysis rate constant at pH 5 (0.05 L g^{-1}VSS day^{-1}) was more than 12 times lower than at pH 7 (0.62 L g VSS^{-1} day^{-1}). The estimated SRT_{min} to avoid wash-out of hydrolytic biomass at pH 7 was 4.4 days. A reliable estimation for

the minimum SRT at pH 5 unfortunately is not available, but is expected to be somewhere between 4.4 and 12 days.

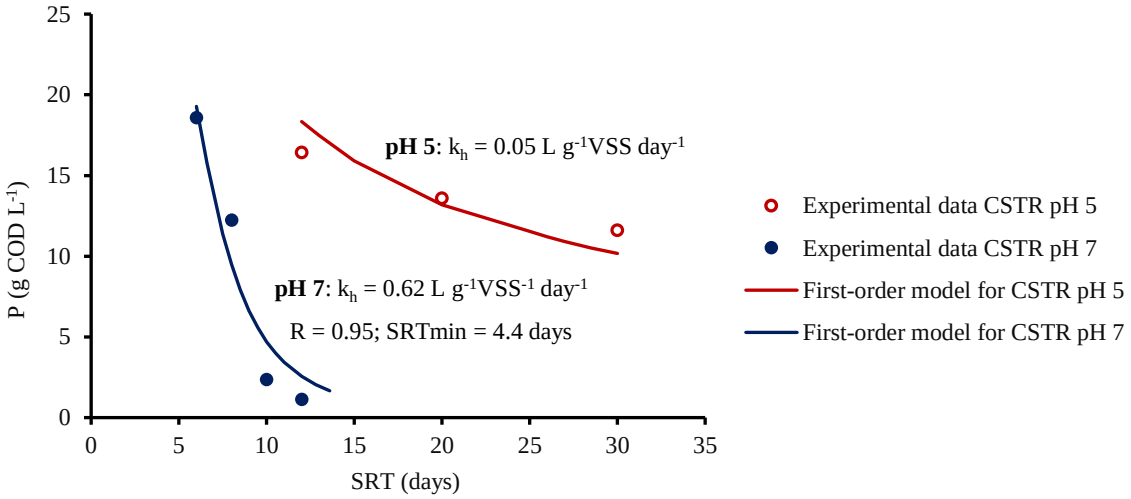

Figure 4.3. Effluent protein concentrations (the mean) at different retention times and first-order model for protein hydrolysis in the CSTRs at pH 5 and pH 7.

This hydrolysis rate constant for protein at pH 7 (0.62 L g VSS^{-1} day^{-1}) is higher than hydrolysis rate constants that were previously found in batch experiment fed with gelatine (0.54 L g VSS^{-1} day^{-1}) (chapter 3). However, these batch experiment were carried out with biomass that was previously fed with substrate not only containing protein but also carbohydrates. Therefore per gram VSS it can be expected to have a lower abundancy of protein degrading microorganisms and a thus a lower specific hydrolysis rate constant. Obviously, the hydrolysis rate for the dissolved gelatine at pH 7 is higher than those found by others for particulate proteins (0.33 L g VSS^{-1} day^{-1}) (Elbeshbishy and Nakhla, 2012). For lower pH the literature provides limited information on first-order hydrolysis rate constants for (dissolved) proteins. The average hydrolysis rates for gelatine in this study as well as results from other studies can be found in the SI (Table S1). In general our hydrolysis rate of 0.35-1.08 g $COD_{hydrolyzed\ gelatine}$ $g^{-1}VSS$ day^{-1} at pH 5, were higher than for instance a rate of 0.38 g $COD_{hydrolyzed\ gelatine}$ $g^{-1}VSS$ day^{-1} obtained from upflow reactors operated at pH 5 by Yu and Fang (2003).

4.3.2 Concentration of amino acids and rate-limiting step for protein degradation at pH 5 and pH 7

Figure 4.4 shows the amino acid composition in the effluent during steady state conditions.

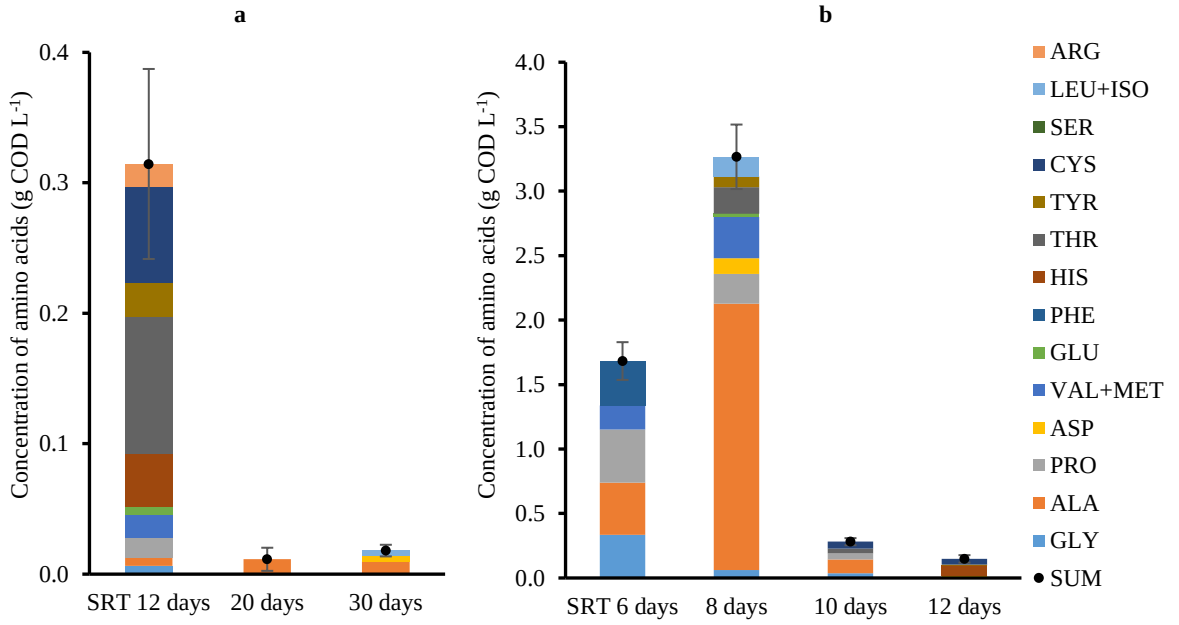

Figure 4.4. Concentrations of amino acids at pH 5 (a) and pH 7 (b) at different SRT. Data expressed the mean and standard deviation (only for the sum) during steady state of different SRT phases. (GLY: Glycine; ALA: Alanine, PRO: Proline and Hydroproline, ASP: Aspartic acid; VAL+MET: Valine and Methionine, GLU: Glutamine and Glutamic acid, PHE: Phenylalanine, HIS: Histidine, THR: Threonine, TYR: Tyrosine, CYS: Cystine, SER: Serine, LEU+ISO: Leucine and Isoleucine, ARG: Arginine)

At pH 5 and an SRT of 12 days the total amino acid concentration was 0.31 g COD L^{-1} (Figure 4.4a), which is equivalent to 2% of the concentration of hydrolysed protein. At SRTs of 20 and 30 days amino acid concentrations were even lower. At pH 7 significantly higher amino acid concentrations were measured, in particular at the shorter SRTs of 6 days (1.68 g COD L^{-1}) and 8 days (3.27 g COD L^{-1}). Apparently at the shorter SRTs hydrolysis of gelatine was faster than conversion of the intermediate amino acids into VFAs, a phenomenon that was also observed by Duong et al. (2019). At longer SRTs hydrolysis rather than amino acid conversion became the rate limiting step, resulting in much lower amino acid concentrations. We do not have an explanation for the higher effluent concentration of amino acids that was observed at SRT 8 days compared to SRT 6 days.

At pH 7, the concentration of the different amino acids in the effluent at SRT 6 or 8 days (Figure 4.4b) was proportional to their presence in the gelatine, i.e glycine and proline, valine and methionine (the amino acid composition of gelatine is the same as in Duong et al (2019) and can be found in the SI (Figure S2). This suggests non-specific degradation of these individual

amino acids during anaerobic degradation of gelatine. Only alanine at an SRT of 8 days was present at higher concentrations than expected. We also observed accumulation of alanine during previous batch tests (Duong et al., 2019). We do not have a mechanistic explanation for this.

4.3.3 VFA production and spectrum

VFA concentrations were measured to assess the VFA yield (Figure 4.5).

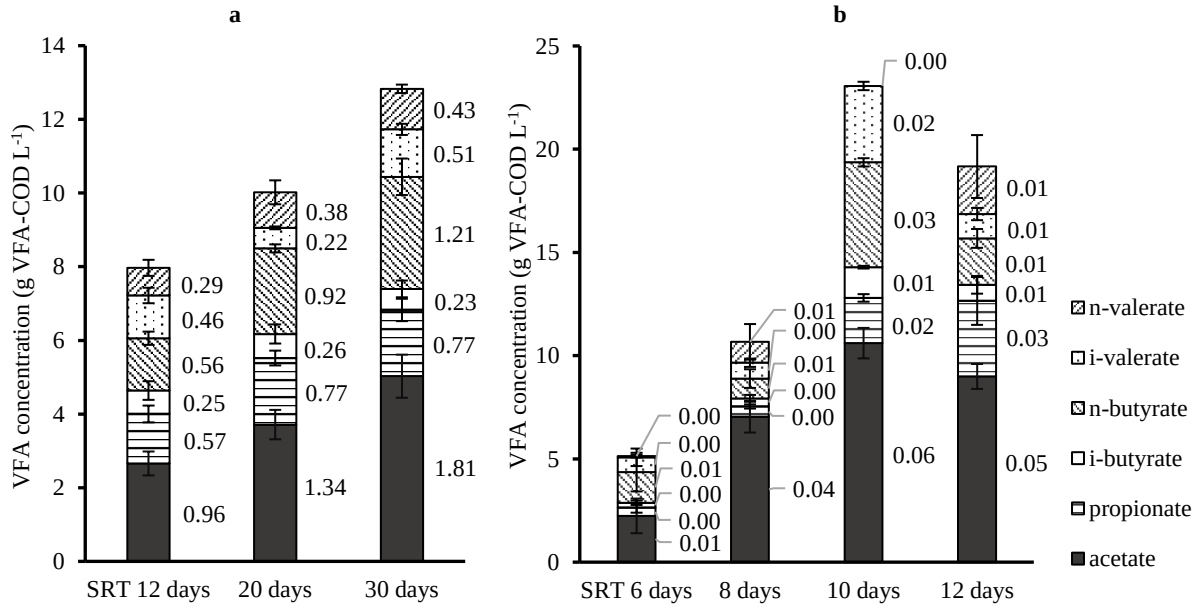

Figure 4.5. VFA concentration and compositions at pH 5 (a) and pH 7 (b) at different SRT. Data expressed the mean and standard deviation during steady state periods of different SRT phases. Number presents the average of undissociated VFA concentration of acetic, propionic, i-butyric, n-butyric, i-valeric and n-valeric at different SRT at pH 5 and pH 7, respectively to the chart columns.

At pH 5 the VFA concentration increased from 8 to 13 g COD_{VFA} L^{-1} when the SRT was increased from 12 days to 30 days (Figure 4.5a). The VFA spectra were nearly similar, irrespective of the SRT with approximately 33-39% acetate, 14-17% propionate, 26-30% butyrate and 15-24% valerate. This relatively stable VFA spectrum suggests that VFA production pathways did not shift as a response to a changing SRT.

Please note that at pH 5 the concentration of undissociated VFA was as high as 3.1-5.0 g COD L^{-1} (Figure 4.5a), which exceeds the inhibitory thresholds of undissociated acids to hydrolysing/fermenting bacteria reported by others, i.e. 0.8 g COD L^{-1} for acetic acid (González

et al., 2005), and 0.6 g COD L^{-1} for propionic acid and butyric acid (Xiao et al., 2016). These undissociated acids can pass the cell membrane and dissociate in the cell. As a result bacteria have to spend significant amounts of energy to regulate the pH inside the cell (Pratt et al., 2012). This reduces the growth rate and associated hydrolytic enzyme production and most likely explains the poor performance of the CSTR operated at pH 5 compared to the CSTR operated at pH 7.

With the exception of SRT 12 days also at pH 7 the VFA concentrations (5 and 23 g COD_{VFA} L^{-1}) increased with the SRT (Figure 4.5b). At this pH the concentration of undissociated VFA is much lower than at pH 5, resulting in less inhibition and higher VFA concentrations compared to pH 5. However, dissociated VFA may still limit protein hydrolysis rates to a certain extent, which will be further discussed in section 3.4.

Unlike at pH 5 the VFA spectra at pH 7 were significantly affected by the SRT. The proportion of propionate at SRTs of 6 and 8 days (8 and 5%) and n-butyrate at SRT of 8 days (9%) were lower compared to SRTs 10 and 12 days (9-19% for propionate and 12-22% for n-butyrate). This can be explained by incomplete degradation of their "parent" amino acids, i.e. methionine at SRTs of 6 or 8 days and alanine at SRT of 8 days (Figure 4.4b).

Comparing the VFA spectra of pH 5 and pH 7 at SRT of 12 days shows lower acetate (33%) and higher butyrate (26%) and valerate (24%) proportions at pH 5 than at pH 7 (acetate of 47%, butyrate of 16% and valerate of 18%). Probably this can be explained by the lower amount of energy that the microorganisms have to spend on excretion of valerate and butyrate compared to acetate (Rodríguez et al., 2006).

4.3.4 Batch tests at lower product/substrate concentrations give higher hydrolysis rate

To investigate if hydrolysis is inhibited by the relatively high product concentrations in the CSTRs, batch tests with CSTR biomass and a low gelatine concentration (1.4 g COD L^{-1}) were carried out. For this purpose sludge was sampled from the reactors during steady state conditions (Table 4.4). The first-order model of equation (4.6) could describe hydrolysis of dissolved proteins in all the batch experiments ($R>0.95$). The protein hydrolysis rate constant of the sludge used to inoculate the CSTRs was also determined and was 0.39±0.01 L g^{-1}VSS

day^{-1}. This value is lower than the hydrolysis rate constant in the CSTR at pH 7 (0.62 L g^{-1}VSS day^{-1}), which can be explained by the property of the inoculum that was previously fed with the brewery wastewater containing both protein and carbohydrates, as discussed in section 4.3.1.

Table 4.4. First order hydrolysis constants for protein hydrolysis in batch experiments inoculated with biomass taken from CSTRs operated at pH 5 and pH 7 at different SRTs.

pH 5	SRT 12 days (day 139)	SRT 20 days (day 190)	SRT 30 days (day 393)	SRT 30 days (day 480)
k_h (L g^{-1}VSS day^{-1})	0.14	0.15	0.34	0.32
pH 7	SRT 6 days (day 193)	SRT 8 days (day 431)	SRT 10 days (day 600)	SRT 12 days (day 50)
k_h (L g^{-1}VSS day^{-1})	n.a	0.98	0.86	0.77

Note: Data of k_h were expressed the mean of the triplicates (with standard deviation less than 10%); n.a: not available.

The hydrolysis rate constants at pH 5 (0.14-0.34 L g^{-1}VSS day^{-1}, Table 4.4) were 3-6 times higher than the rate constant estimated from the CSTR data of 0.05 L g^{-1}VSS day^{-1}. Most likely this can be explained by the lower VFA concentrations (total VFA below 1.6 g COD L^{-1}) in the batch medium. The similar batch hydrolysis rate constants at day 393 and day 480, both with sludge sampled at an SRT of 30 days, suggest that a longer exposure time did not result in acclimation of the biomass to pH 5. We cannot explain why the batch kinetic constants at SRTs 20 and 12 days (0.14-0.15 L g^{-1}VSS day^{-1}) were considerably lower than the values at SRT 30 days (0.32-0.34 L g^{-1}VSS day^{-1}).

At pH 7, batch hydrolysis rate constants, ranging between 0.77 and 0.98 L g^{-1}VSS day^{-1} (except at SRT 6 days) were also higher than the rate constant determined from the CSTR data of 0.62 L g^{-1}VSS day^{-1}, although the difference was not as high as for pH 5. This indicates that high VFA concentrations (> 1.6 g COD L^{-1}, chapter 3), even at pH 7 can inhibit protein hydrolysis, also suggested by González et al. (2005) and Angelidaki et al. (1999).

4.3.5 Consequences for the design and operation of reactors treating high-strength protein wastewaters

The results clearly showed that at pH 5 anaerobic protein hydrolysis is supressed and it was not possible to improve hydrolysis by long-term exposure of the biomass to this pH. As a consequence, a very long SRT would be needed to achieve an acceptable protein removal and VFA productivity. In our research, an SRT of 12 days would give a maximum productivity of 0.7 g COD_{VFA} L^{-1} day^{-1} with a very limited protein removal of 42%. Higher values reported in other studies can be explained by the higher sludge concentrations compared to those in our research (Yu and Fang, 2003) and the use of (partly) already hydrolyzed proteins (Breure and Van Andel, 1984, Bevilacqua et al., 2020b) (Table S1, SI). Higher sludge concentrations, smaller reactor volumes and higher volumetric VFA productivities are possible by applying sludge retention, i.e. with membrane bioreactors, biofilm systems or, preferably, with granular sludge systems. For example, Yu and Fang (2003) used an upflow sludge bed reactor with a sludge concentration of 10.8 g VSS L^{-1} and achieved a VFA productivity of 4.2 g COD_{VFA} L^{-1} day^{-1} from gelatine at pH 5. Biomass granulation at pH 5 was demonstrated with glucose as substrate (Tamis et al., 2015) but the question remains if this is also possible for protein-rich wastewater. In addition, future research perhaps should also focus on finding appropriate inocula that are able to grow on protein at low pH.

The low hydrolysis rate constants at pH 5 probably were caused by the presence of high concentrations of undissociated VFA. This can be avoided if the VFA is actively removed from the reactor, for example by electrodialysis processes such as proposed by Aktij et al. (2020). At the non-inhibitory VFA conditions, the hydrolysis rate of protein at pH 5 is expected to increase by 3-6 times to levels comparable to the values found in the batch experiments (Table 4.4). This would reduce the reactor volume and make this process more attractive. The implementation of such a separation system however would significantly increase the production costs.

The results of this study clearly showed that protein hydrolysis is much more efficient at pH 7 than at pH 5. Therefore, maintaining a neutral pH can be one of the effective solutions for harvesting VFA from acid-stressed protein containing waste streams, i.e food processing waste, kitchen waste, slaughterhouse wastewater, cheese waste, etc. The maximum volumetric VFA productivity at pH 7 of 2.3 g COD_{VFA} L^{-1} day^{-1}, was achieved at an SRT of 10 days. An SRT shorter than 10 days would result in too low a hydrolysis degree and is insufficient to give high

VFA productivity. At longer SRTs more protein is converted but the volumetric VFA productivity decreases. It should be noted that at SRT 10 days with the highest VFA productivity still approximately 8% of the proteins is discharged with the effluent. This implies that the optimum SRT will largely depend on the purpose of the reactor treating protein-rich wastewaters: a high removal of protein or a high VFA volumetric productivity. Furthermore, at pH 7, it will be more difficult to prevent methanogenesis, in particular at SRT of 10 days or longer. More research is needed to determine how methanogenic activity can be effectively limited under these conditions.

4.4 Conclusions

The effect of the solid retention time and pH on (dissolved) protein hydrolysis and amino acid fermentation was investigated. At pH 5 hydrolysis (0.05 L g^{-1}VSS day^{-1}) was more than 12 times slower than at pH 7 (0.62 L g^{-1}VSS day^{-1}), probably because of the inhibitory effect of undissociated VFA. At pH 7, the SRT (6-12 days) had a significant effect on protein hydrolysis, VFA yield and spectrum. The optimum volumetric VFA productivity was 2.3 g COD_{VFA} L^{-1} day^{-1} at SRT 10 days. Complete removal of protein requires longer SRTs.

Supplementary Information of Chapter 4

A. COD mass balance

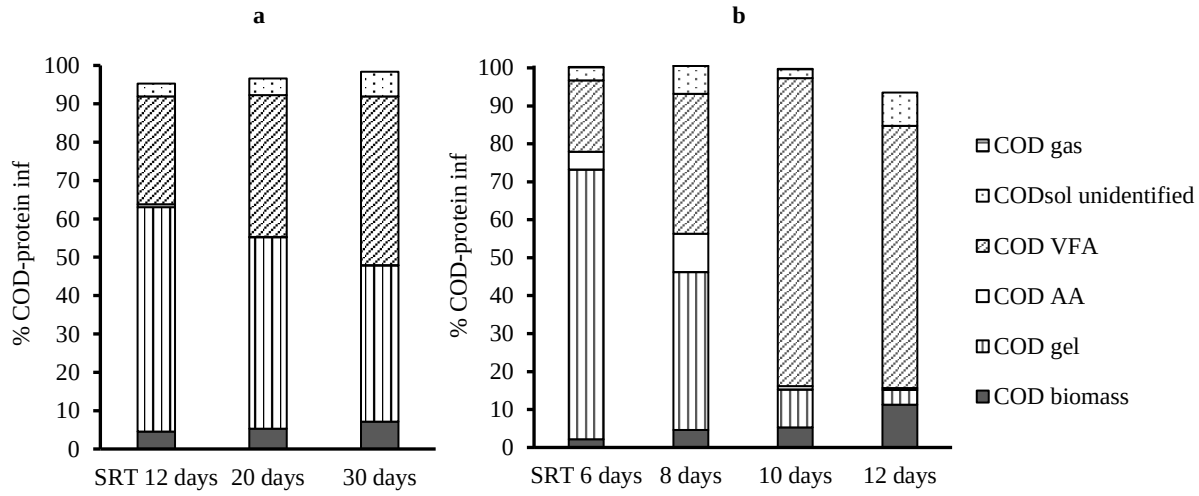

Figure S1. COD mass balance at pH 5 (a) and pH 7 (b) and different SRT with gelatine as substrate. The products are given as percentage of the influent COD. Data expressed the mean with standard deviation less than 5% during steady state periods of different SRT phases. The "unidentified" COD mass was the gap between the measured COD of supernatant and the sum of the identified products including unhydrolyzed protein, amino acids, volatile fatty acids, hydrogen and methane. The unidentified COD compounds (less than 8%) could be dipeptides, which was confirmed by the difference of concentrations of total peptides (data not shown) with sum of concentrations of amino acids via HPLC analysis.

B. Amino acid composition of the gelatine, defined in Gelatin Handbook (2012).

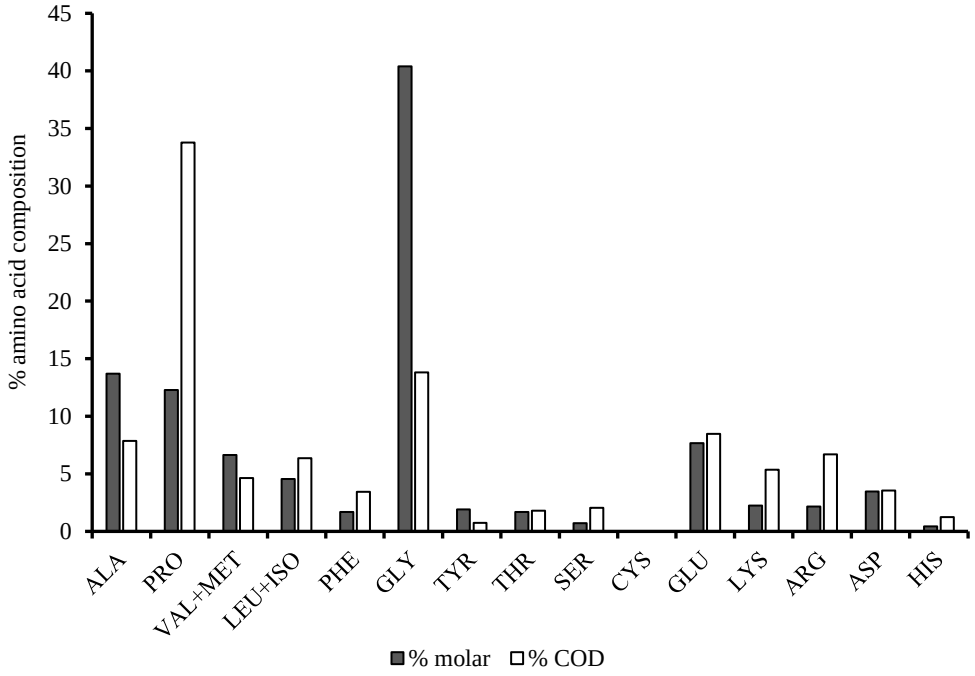

Figure S2. Amino acid composition (% molar and COD) of the gelatine. (ALA: Alanine, PRO: Proline and Hydroproline, VAL+MET: Valine and Methionine, LEU+ISO: Leucine and Isoleucine, PHE: Phenylalanine, GLY: Glycine, TYR: Tyrosine, THR: Threonine, SER: Serine, CYS: Cystine, GLU: Glutamine and Glutamic acid, LYS: Lysine, ARG: Arginine, ASP: Aspartic acid, HIS: Histidine)

C. Protein hydrolysis and acidification at pH 5-7 in this study and in previous studies

Table S1. Overview of process parameters, hydrolysis and acidification of protein and at pH 5-7 in this study and in previous studies.

Process parameters: pH, types of substrate (Substrate), influent protein concentration (P_i, g COD L^{-1}), system of experiments (System), temperature (T, °C) and solid retention time (SRT, days); **Hydrolysis**: degree of hydrolysis (H, %), average rates (rate, g $COD_{hydrolyzed\text{-}protein}$ g^{-1}VSS day^{-1}) and first-order rate constants (k_h, L g^{-1}VSS day^{-1}); **Acidification**: VFA yield (Y_{VFA}, g COD_{VFA} g^{-1} $COD_{hydrolyzed\text{-}protein}$) and volumetric VFA productivity (q_{VFA}, g COD_{VFA} g^{-1}L day^{-1}).

Process parameters						**Hydrolysis**			**Acidification**		**References**
pH	Substrate	Pi	System	T	SRT	H	rate	k_h	Y_{VFA}	q_{VFA}	
5	Gelatine	4.0	Upflow reactor	37	0.5[a]	85	0.38		0.55	4.2	(1)
5.3	Gelatine	8.6	Chemostat	30	0.4	-	-		0.32	6.7	(2)
5	Hydrolysed casein	8	CSTR	25	1				0.30	2.4	(3)
	Hydrolysed gelatine	8			1.5				0.20	1.1	
5	Gelatine	28.6	CSTR	35	12	42	1.08		0.68	0.6	(4)
					20	52	0.57	0.05	0.73	0.5	
					30	59	0.35		0.74	0.4	
7	Gelatine	4.0	Upflow reactor	37	0.5[a]	96	0.40		0.62	5.2	(1)
7	Gelatine	8.6	Chemostat	30	0.3	-	-		0.73	15.7	(2)
					0.4				0.52	15.0	
7	Hydrolysed casein	8	CSTR	25	1				0.50	4.0	(3)
	Hydrolysed gelatine	8			1.5				0.40	2.1	
7	Gelatine	28.6	CSTR	35	6	35	3.21		0.51	0.8	(4)
					8	55	1.97	0.62	0.62	1.3	
					10	92	2.08		0.84	2.3	
					12	96	0.96		0.72	1.6	
7	Gelatine	6.3	batch tests	55				0.65[b]			(5)
7	Gelatine	1.4	batch tests	35				0.54			(6)
7	BSA	5	batch tests	35				0.33[c]			(7)

Note:

[a] : hydraulic retention time

[b] : unknown sludge concentration

[c]: at methanogenic conditions

- : hydrolysis data not used because gelatine thermal treatment before feeding in the chemostat

(1): (Yu and Fang, 2003)

(2): (Breure and Van Andel, 1984)

(3): (Bevilacqua et al., 2020a)

(4): this chapter

(5): (Flotats et al., 2006)

(6): (chapter 3)

(7): (Elbeshbishy and Nakhla, 2012), k_h of 0.65 day^{-1} and VSS of sludge of 1.96 g L^{-1}.

Chapter 5. Anaerobic protein hydrolysis kinetics under non-methanogenic conditions

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Abstract

This study aimed to investigate the potential inhibitory effect of amino acids on protein hydrolysis by testing different models for the kinetics of hydrolysis: first-order kinetics, first-order kinetics including amino acid inhibition and Contois kinetics. The modelling results and statistical tests showed that the non-competitive amino acid inhibition model provided the best fit between model and experimental data for gelatine hydrolysis at different solid retention times (6-12 days) in a completely stirred tank reactor (CSTR) at pH 7 and 35°C under non-methanogenic conditions. Although this suggests amino acids indeed inhibit protein hydrolysis experimental evidence still is required and the mechanism associated with this inhibition should be further investigated.

Keywords: proteins, amino acids, modelling, hydrolysis kinetics, inhibition.

5.1 Introduction

Proteins are hydrolysed to peptides and amino acids. Under anaerobic conditions the latter are subsequently converted into volatile fatty acids (VFA) and methane (McInerney, 1988). Assessment of the kinetics and degradation performance of protein rich waste(water)s are generally based on the intermediate and end products, i.e. ammonium and methane (Vavilin et al., 2008), based on the assumption that hydrolysis always is the rate limiting step (Flotats et al., 2006, Vavilin et al., 2008). However, recently it was shown in batch experiments (chapter 2) as well as in CSTRs operated at short solids retention times (SRTs) (chapter 4) that also amino acid conversion to VFAs can be the rate limiting step. This may have serious consequences because this results in relatively high amino acid concentrations that are suspected to inhibit protein hydrolysis (Glenn, 1976, Palenzuela, 1999, Miron et al., 2000, Bar-Even et al., 2011). To test this hypothesis of amino acid inhibition on protein hydrolysis three different kinetic models for (dissolved) protein hydrolysis, including an inhibition model, were investigated for the accuracy at which they describe the steady state experimental results of a CSTR with gelatine as the feed substrate. This CSTR was operated at 35°C, at pH 7, under non-methanogenic conditions and an SRTs between 6 and 12 days.

5.2 Materials and methods

5.2.1 Model description

The models describe the hydrolysis process of proteins to yield amino acids in a CSTR operated at steady state. The mass balance for proteins is:

$$\frac{1}{SRT} . (P_i - P) - r_h = 0 \qquad \text{(eq. 5.1)}$$

With P_i and P the influent and effluent protein concentration of the CSTR, respectively (g COD L^{-1}) using a conversion factor of 1.150 g COD g^{-1} gelatine and r_h the hydrolysis rate, (g COD $L^{-1}day^{-1}$).

Three different models to describe the rate of hydrolysis r_h (Table 5.1) were tested. Model A assumes that protein hydrolysis follows first-order kinetics with respect to the protein

concentration and includes a linear dependency on the biomass concentration (eq. 5.2). Model B assumes that protein hydrolysis suffers from non-competitive amino acid inhibition (eq. 5.3). Other inhibition forms (uncompetitive and competitive) were evaluated as well (data not shown), but could not describe the experimental results and therefore were discarded for further evaluation. The non-competitive model B is commonly used for hydrolysis of biopolymers and (Angelidaki et al., 1999, Batstone et al., 2002). Model C assumes that protein hydrolysis follows Contois kinetics, i.e. the hydrolysis rate is controlled by the ratio between protein and biomass (eq. 5.4).

Table 5.1. Models featuring different hydrolysis rate descriptions.

Model	Hydrolysis rate	Mathematical expression	
Model A	No inhibition	$r_h = k_h \cdot P \cdot X$	(eq. 5.2)
Model B	Non-competitive AA inhibition	$r_h = k_h \cdot P \cdot X \cdot \frac{1}{1 + \frac{AA}{K_I}}$	(eq. 5.3)
Model C	Contois kinetics	$r_h = k_h \cdot \frac{P/X}{K_S + P/X} \cdot X$	(eq. 5.4)

With k_h the hydrolysis rate constant (L g^{-1}VSS day^{-1} for model A and B, and g $COD_{protein}$ g^{-1}VSS day^{-1} for model C), X the biomass concentration (g VSS L^{-1}), AA the amino acid concentration (g COD L^{-1}), K_I the amino acid inhibition constant (g COD L^{-1}) and K_S the Contois half-saturation constant (g $COD_{protein}$ g^{-1}VSS).

The kinetic models were used to calculate the protein concentration in the reactor effluent (P) and the degree of hydrolysis H:

$$H = \frac{P_i - P}{P_i} \times 100\ (\%) \qquad \text{(eq. 5.5)}$$

5.2.2 Experimental input data for the models

Table 5.2 shows the set of experimental data regarding hydrolysis and acidification of gelatine, obtained during steady state operation of non-methanogenic CSTRs at SRTs of 6-12 days, pH 7 and 35°C (chapter 4). These data were used to test the hydrolysis models of Table 5.1. The COD mass balances at each steady state were closed for at least 90%, indicating the high quality of the experimental results.

Table 5.2. Concentrations of protein in the influent and in the effluent, and concentrations of amino acids, VFA and biomass in the effluent at steady states of CSTR (chapter 4).

SRT (days)	Pi (g COD L^{-1})	P (g COD L^{-1})	AA (g COD L^{-1})	H (%)	X (g VSS L^{-1})
6	28.61±0.58	18.63±2.65	1.68±0.15	35±2	0.5±0.1
8	27.20±2.12	12.13±0.90	3.27±0.25	55±5	1.0±0.2
10	29.53±0.55	2.11±0.40	0.28±0.03	92±2	1.3±0.3
12	28.35±0.48	1.03±0.25	0.15±0.03	96±1	2.3±0.3

Note: No methane and very little hydrogen was produced, the COD of gas always less than 0.1% COD influent. Non-methanogenic conditions was assured at all the steady-states.

5.2.3 Parameter estimation

The model parameters were estimated by minimisation of the root-squared mean deviation (RMSD) between the experimental data and the data obtained by calculation of the degree of hydrolysis H at the different SRTs (eq. 5.6). The kinetic models were implemented in MATLAB (R2016a) and RMSD minimisation was performed using the command *lsqnonlin* (trust-region-reflective algorithm).

$$\mathrm{RMSD} = \frac{1}{n} \cdot \sqrt{\sum_{i=1}^{n} \left(\hat{H}_i(\theta) - H_i\right)^2} \qquad \text{(eq. 5.6)}$$

with n is the number of steady state experiments at different dilution rate values (n=4), $\hat{H}$ is the calculated hydrolysis degree, H is the experimental hydrolysis degree value, θ is the vector of parameters being estimated. The subscript i refers to each steady state for the different SRTs.

5.2.4 Model comparison (F-test)

To compare and decide which kinetic model gives the best fit of the experimental data a F-test was performed as described in Turner et al. (2015) (eq. 5.7):

$$F = \frac{(RSS_1 - RSS_2)/(df_1 - df_2)}{(RSS_2/df_2)} \qquad \text{(eq. 5.7)}$$

With F is the F-statistic, RSS_1 and RSS_2 are the sum of squared residuals of model 1 and 2 and df_1 and df_2 are the respective degrees of freedom (number of data points (n) – number of parameters (p)) for model 1 and 2, respectively. The RSS value can be calculated as $RSS = RMSE^2 \cdot n$.

The F-statistic and the degrees of freedom were used to determine the p-value (probability value) with the built-in fcdf function in MATLAB (eq. 5.8). If the p-value is lower than the defined significance level (α), 5% in this case, the null hypothesis (i.e. model 2 does not provide a better fit to the experimental data than model 1) can be rejected:

$$P = 1 - \text{fcdf}(F, df1 - df2, df2) \qquad \text{(eq. 5.8)}$$

The F-test has as requisite that the models being compared are nested (i.e. model 1 is a simplified version of model 2). Model B can be easily transformed into model A by selecting an infinite value of K_I (i.e. no inhibition is considered) but model C is only partially nested as it can only be transformed into model A if the K_S value is assumed to be negligible with respect to the protein to biomass concentration ratio. Therefore, the Akaike information criterion (AIC) was used (eq. 5.9) to compare the three models (Turner et al., 2015). The model featuring the lowest AIC value can be considered as the option that allows the best description of experimental observations while avoiding overparameterization.

$$AIC = n \cdot \ln\left(\frac{RSS}{n}\right) + 2 \cdot (p + 1) \qquad \text{(eq. 5.9)}$$

With n the number of data points.

5.3 Results and Discussion

5.3.1 Results model

Model calibration results (Figure 5.1 and Table 5.3) show that all of the proposed models can qualitatively describe the observed trend in the experimental data regarding the hydrolysis degree, i.e. an increase of the hydrolysis degree H with the SRT. However, model B (non-

competitive amino acid inhibition) gave the best model fit, as indicated by the lowest RMSD value (Table 5.3).

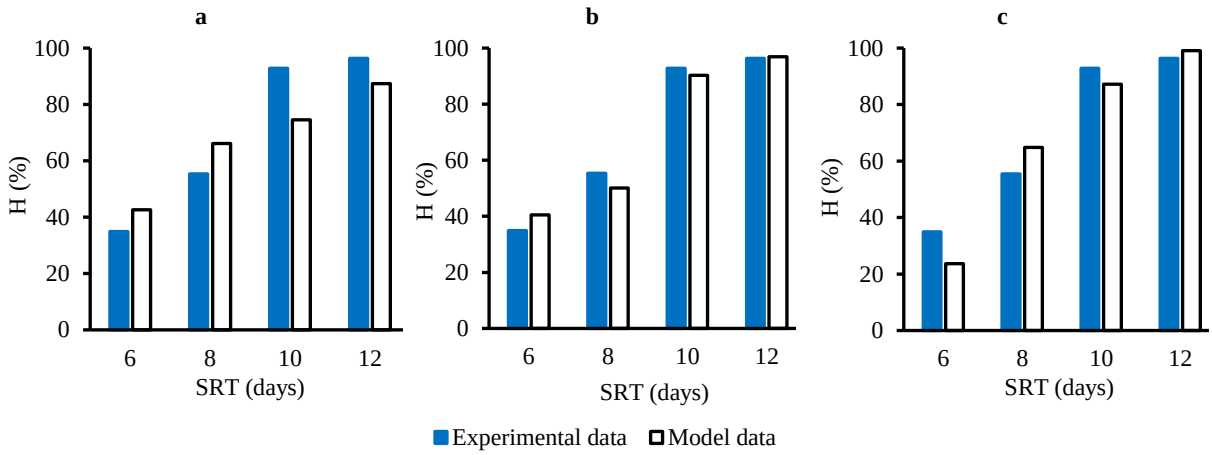

Figure 5.1. Model results with estimated parameters (grey bars) and experimental results (blue bars) concerning hydrolysis degree at different SRT values. Each subfigure considers a different hydrolysis description according to the models of Table 5.1: Model A (a), Model B (b) and Model C (c).

Table 5.3. Model calibration results for each of the models of Table 5.1 and RMSD values obtained in the calibration. *The value was constrained by a lower bound in the estimation procedure of 0.1 g $COD_{protein}$ g^{-1}VSS.

Model calibration results	**k_h**	**Ki (inhibition) or Ks (Contois)**	**RMSD**
Model A: No inhibition	0.25 (L g^{-1}VSS day^{-1})		0.0615
Model B: Non-competitive inhibition	1.75 (L g^{-1}VSS day^{-1})	0.25 (g $COD_{amino\ acids}$ L^{-1})	0.0191
Model C: Contois	2.24 (g $COD_{protein}$ g^{-1}VSS day^{-1})	0.1* (g $COD_{protein}$ g^{-1}VSS)	0.0410

Importantly, unlike model A (no inhibition) and C (Contois), model B was able to correctly predict the sudden increase in hydrolysis degree from the SRT 6-8 days to SRT 10-12 days. This suggests that protein hydrolysis was indeed inhibited by the higher amino acid concentrations at the shorter SRTs. It should be noted that the hydrolysis rate constant k_h in Table 5.3 of 0.25 L g^{-1} VSS day^{-1} is lower than the 0.62 L g^{-1} VSS day^{-1} that was estimated in chapter 4 from the same experimental data. This can be explained because for estimation of the latter value a slightly different first-order model was used that included a minimum SRT below which hydrolytic biomass completely washed-out of the reactor.

5.3.2 Model comparison

According to the RMSE results, models B and C provide a better fit than model A, but at the cost of an additional parameter. To verify that model B does not provide a better description of the experimental data because of overparameterization, an F-test was performed between models A and B and between models A and C (Table 5.4). In the first comparison, the p-value was lower than our chosen significance value (α=0.05), indicating that statistically model B provided a better fit. However, model C statistically did not provide a better fit.

Table 5.4. F-test results (eq. 5.5-5.6) for comparing model A and B and model A and C.

Comparison	RSS_1	RSS_2	df_1	df_2	F	P
Model A vs model B	0.0606	0.0058	3	2	18.7118	0.0495
Model A vs model C	0.0606	0.0269	3	2	2.5045	0.2543

As mentioned in section 2.4, models A and C cannot truly be considered nested models, therefore not complying the F-test requirements. For that reason, the AIC was also calculated (Table 5.5), leading to the same conclusions that model B provided the lowest AIC, i.e. gave the best model fit.

Table 5.5. AIC value of the three models proposed.

Models	AIC value
Model A	-12.7610
Model B	-20.1112
Model C	-14.0087

5.3.3 Discussion

Kinetic model B with amino acid inhibition of protein hydrolysis gave the best model fit of the experimental data. However, the question remains if amino acid inhibition really took place as we have no solid experimental evidence for this. Free amino acids have been identified as an end-product inhibitor of protease formation and activity (Glenn, 1976, Sandhya et al., 2006) in the case of ruminal bacteria (e.g. Sales-Duval et al. (2002)). Specifically for wastewater treatment hardly any information is available about this phenomenon.

During hydrolysis of milk proteins, soy proteins, rice and wheat proteins, the IC50 (the concentration to achieve 50% inhibition of protease activity) of hydrolysates ranged from 0.03-2.1 g L^{-1} (equivalent to approximately 0.04-3.1 g COD L^{-1}) depending on type of amino acid, proteases and protease concentration (Deng, 2018, Deng et al., 2018). It can therefore not be excluded that the relatively high amino acid concentrations at the shorter SRTs of 6-8 days of 1.7–3.3 g COD L^{-1} (Table 5.2) were indeed inhibitory for gelatine hydrolysis.

Gelatine mainly consists of glycine, proline and alanine. These are hydrophobic amino acids and also accounted for 68-73% of the total concentration of unconsumed amino acids at the shorter SRTs (chapter 4). Perhaps protein hydrolysis is specifically inhibited by these hydrophobic amino acids, as was suggested by others (Glenn, 1976, Palenzuela, 1999, Miron et al., 2000, Bar-Even et al., 2011). Glenn (1976) described an example involving the repression of exo-protease synthesis by the presence/ of proline and isoleucine in several hydrolytic bacterial genera. Additionally, hydrophobic amino acids from fish skin hydrolysates (gelatine) were demonstrated as inhibitors of several proteases (Bar-Even et al., 2011).

5.4 Conclusions

A non-competitive amino acid inhibition model for protein hydrolysis gave the best fit for experimental data obtained from a CSTR that converted gelatine into VFAs under non-methanogenic conditions. This suggests that at short SRTs (<8 days), where amino acid conversion into VFAs is slower than gelatine hydrolysis, the relatively high amino acid concentrations can inhibit gelatine hydrolysis. However, it is appreciated more experimental evidence for this phenomenon is required as well as more research to elucidate the inhibition mechanisms.

Chapter 6. General Discussion and Outlook

6.1 Introduction

Anaerobic treatment processes are widely used for the treatment of organic wastewater and wastes, because they allow pollution control with simultaneous energy recovery. Hydrolysis of biopolymers is generally considered the rate limiting step in anaerobic treatment. Many waste streams, in particular those generated by the food industry, contain appreciable amounts of protein. This may cause severe problems such as foaming, biomass washout and a poor effluent quality (chapter 1). Therefore, this research focussed on the anaerobic degradation of protein with an emphasis on the hydrolysis step in relation to the presence of other organic compounds (e.g. carbohydrates), environmental factors (e.g. pH), process conditions (e.g. solids retention time, SRT), microbiological activity (e.g. methanogenic activity) and intermediate and end products (e.g. amino acids and volatile fatty acids, VFA). Knowledge about this gives direction on how to solve problems associated with (insufficient) protein degradation and gives more insight in how to design and operate anaerobic treatment processes for protein rich wastewaters. In the following sections, the results of this study and implications for the design and operation of anaerobic systems treating protein rich wastewaters and wastes are discussed as well as the potential for anaerobic resource recovery with Vietnamese slaughterhouse, meat processing and whey containing wastewater as an example. Finally, recommendations for future research are given.

6.2 Most important findings

Figure 6.1 summarizes the main findings in this thesis. Protein degradation is significantly affected by pH (chapter 2 and 4). Although most hydrolytic bacteria have an optimum pH between 5 and 7 (chapter 1), the hydrolysis rate constant for protein at pH 5 (0.05 L g^{-1} VSS day^{-1}) was much lower than at pH 7 (0.62 L $gVSS^{-1}$ day^{-1}). The difference is explained by the inhibitory effect of the large fraction of undissociated VFA at pH 5. Even long-term exposure (480 days) of the microbial population to pH 5 in a continuous stirred tank reactor (CSTR) reactor did not result in an improved hydrolysis of dissolved protein (chapter 4).

At pH 5 and between an SRT of 12 and 30 days, hydrolysis always was the rate limiting step of protein degradation (chapter 4). At pH 7 and at SRTs ≤ 8 days, the system shifted from being limited by hydrolysis to being limited by the conversion of amino acids to VFA. This resulted

in considerable concentrations of amino acids in the effluent from a CSTR, i.e. up to 3.3 g COD L^{-1}, when the CSTR was fed with 28.6 g COD L^{-1} of protein (chapter 4). An inhibitory effect of amino acids on protein hydrolysis has been suggested (Glenn, 1976, Sandhya et al., 2006) but unfortunately could not be verified experimentally as part of this thesis. Although mathematical modelling indeed indicated such inhibition may take place (chapter 5), it is appreciated that more experimental evidence is required to prove it.

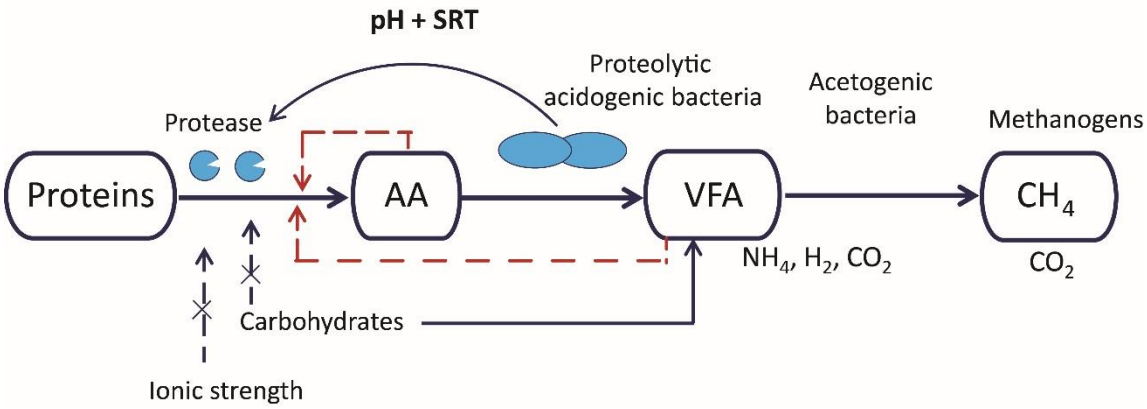

Figure 6.1. Effects of different environmental and microbial factors on dissolved protein hydrolysis. Note: Solid lines indicate the degradation scheme; dashed red lines indicate inhibitory effects (arrows point to the inhibited process); dashed black lines with (×) indicate that no inhibitory effect was observed. (AA: amino acids)

The pH also determined the VFA product spectrum. Gelatine degradation at pH 5 in the absence of methanogenesis led to a more pronounced production of valerate and/or butyrate compared to acetate (chapters 2 and 4). This can be explained by a lower energy expenditure to excrete larger VFA molecules compared to smaller molecules, as these carry less charge per mole of C-atoms (Rodríguez et al., 2006). The VFA spectra at pH 5 were similar between SRT 12-30 days, with approximately 33-39% acetate, 14-17% propionate, 26-30% butyrate and 15-24% valerate on COD basis. In contrast, at pH 7, the SRT did have an effect on the VFA spectrum, which can be partially explained by incomplete degradation of alanine at an SRT of 8 days. (chapter 4).

High VFA concentrations can be expected in reactors aiming to produce VFA. The effect of (dissociated) VFA on protein hydrolysis was investigated at pH 7 (chapter 3), and we observed strong (64% in rate constant) inhibition of protein hydrolysis by VFA. This was not caused by the ionic strength exerted by VFA but most likely by the VFA itself, as was also found by (Palenzuela, 1999). The inhibitory mechanism of VFA is not fully understood. It is

hypothesized that VFA (i) directly affect the structure of protein or the structure/activity of existing proteases, (ii) reduce the hydrolytic acidogenic bacteria growth rate and as a consequence the protease production, as was reported by González et al. (2005).

In contrast to what has been suggested in literature active methanogenesis did not stimulate the hydrolysis and acidification rate of protein as such (chapter 2). However, the presence of methanogens obviously will keep the VFA concentration at a sufficiently low level to prevent inhibition of protein hydrolysis (chapter 3).

6.3 Recommendations for anaerobic treatment of protein rich wastewater

Hydrolysis of dissolved protein could be very well described by a simple first-order model with a linear dependency on the biomass concentration (chapters 2 and 3). In chapter 4 it was shown that to describe protein hydrolysis in a CSTR a minimum SRT can be incorporated in this model to account for wash-out of the hydrolytic biomass.

Irrespective of the SRT, at pH 5 hydrolysis always was the rate limiting step of protein conversion into VFA, and thus will dictate reactor design (chapter 4). However, protein hydrolysis was extremely slow and therefore a huge reactor volume would be required, which may not be economically viable unless a cheap and excellent biomass retention system can be applied. This will be addressed later. In batch experiments at pH 7 (chapter 2) it was found that protein hydrolysis is much faster than amino acid fermentation and the CSTR experiments of chapter 4 showed this indeed was the case at SRTs ≤ 8 days. As a result, relatively high concentrations of amino acids (1.7 – 3.3 g COD L^{-1}) can prevail. Methane production always is the slowest process (chapters 2 and 3). These differences in individual process rates offer the possibility to harvest VFA and even amino acids from protein rich waste(waters) as will be further explained below.

6.3.1 Methane production

Methane production only is possible if the pH is sufficiently high. At pH 5 for example this is not the case. If, at higher pH levels, pH 7 for instance, methane is the desired product, the

reactor design should be based on methanogenesis being the slowest process. At 35°C a typical minimum SRT to accomplish this would be 20 to 30 days (van Lier et al., 2020). However, this is only valid if the waste(water) is primarily comprised of dissolved proteins. In practice this usually is not the case and an appreciable fraction of the proteins will be present in particulate form. Because hydrolysis of particulate protein is much slower than of dissolved protein hydrolysis not methanogenesis, but hydrolysis will dictate reactor design. To accomplish a sufficiently high degree of hydrolysis, and to avoid high VFA concentrations that inhibit methanogenesis an SRT of at least 40-70 days is required (Sayed et al., 1988, Palenzuela, 1999, Tawfik et al., 2008, Graaff et al., 2010).

Systems such as anaerobic sludge bed or anaerobic membrane reactors can be applied to retain the biomass in the reactor and in this manner minimize the reactor volume. However, several studies claimed that foaming related to proteins and/or amino acids can occur in anaerobic reactors, causing poor settling biomass, blockage of three-phase separators and consequently less biogas production (Boe et al., 2012, Kougias et al., 2013, Tanimu et al., 2015). Although based on our experiments this cannot be verified, it is suspected that if a sufficiently long SRT is applied complete protein and subsequent amino acids conversion can be accomplished and herewith such issues can be prevented. It cannot be excluded however that a small fraction of protein is less amenable to degradation, even at long SRTs, and still can cause the problems mentioned above. This should be further investigated with realistic protein rich wastewaters.

6.3.2 VFA production

Figure 6.2 shows volumetric VFA productivities and protein removal efficiencies that were found at different SRTs for the CSTRs operating at pH 5 and pH 7 (data from chapter 4).

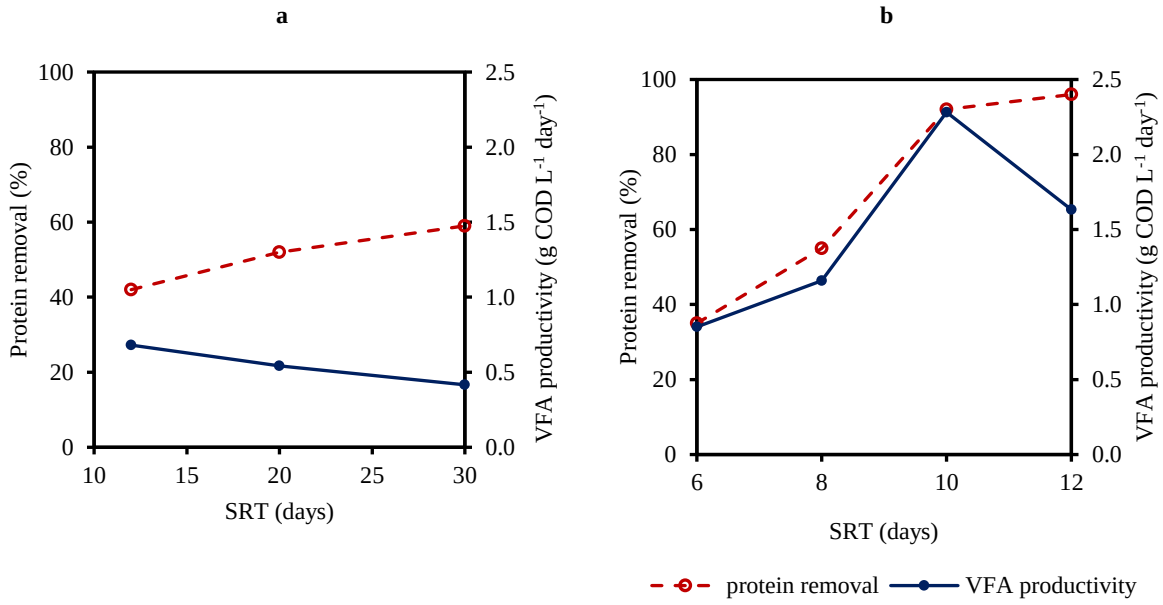

Figure 6.2. Protein removal efficiency and VFA productivity achieved with the CSTRs operating at pH 5 (a) and pH 7 (b).

The CSTR that was operated at pH 7 gave the highest volumetric VFA productivity of 2.3 g COD_{VFA} L^{-1} day^{-1} at an SRT of 10 days (Figure 6.2, b). Shorter SRTs gave insufficient protein hydrolysis to allow a high VFA production, at longer SRTs the reactor volume is used less efficiently. Please remark that at an SRT of 10 days only 92% of protein was removed. A longer SRT may be required to obtain an effluent low enough in protein such that it can be safely discharged. For example, to obtain a protein removal efficiency of more than 95% an SRT of at least 12 days would be required, albeit at the cost of a 35% lower VFA productivity.

Unfortunately, at an SRT of 10-12 days methanogenesis cannot be fully prevented unless, like in the CSTR experiments, a chemical inhibitor such as BES is dosed. However, chemical inhibitors or other methods such as heat shocks to prevent methanogenesis not always are effective and/or economically feasible. Also, operation at lower pH levels can be used to avoid methanogenesis, as was demonstrated in this thesis for pH 5. Figure 6.2 (a) shows this has serious implications for protein removal efficiency and VFA productivity. The highest VFA productivity of 0.7 COD_{VFA} L^{-1} day^{-1} obtained at an SRT of 12 days was 70% lower than the highest productivity of 2.3 g COD_{VFA} L^{-1} day^{-1} obtained at pH 7 (SRT 10 days). Also, the maximum protein removal efficiency only was 60%, achieved at an SRT of 30 days. Clearly this poor performance at pH 5 compared to pH 7 does not seem very attractive for a full-scale application. Possibly an optimum pH somewhere between pH 5 and 7 can be found that avoids

methanogenesis and at the same time still allows for an attractive VFA productivity. Alternatively, different biomass inocula may be found in low pH/protein-rich environments that are less sensitive to VFA inhibition and are able to perform better than our inoculum from a brewery wastewater treatment reactor that even after long-term exposure to pH 5 gave a poor performance.

The results of chapter 4 showed that the reduced performance at pH 5 was largely caused by the inhibitory effect of the (undissociated) VFA on protein hydrolysis. In theory this inhibition can be overcome by active recovery of the VFA, for example by electrodialysis (Aktij et al., 2020, Ramos-Suarez et al., 2021) or liquid-liquid extraction (Sprakel and Schuur, 2019). Separation of VFA from complex fermentation broths however still is very challenging and state-of-art technologies are not yet available. More technological developments in this direction are required to make them selective towards specific VFAs and to reduce their energy consumption and operational costs.

In a CSTR only very low biomass concentrations can be maintained, typically 1-3 g VSS L^{-1} when fed with 30 g COD L^{-1} of protein (chapter 4). A biomass retention system is therefore needed to increase the volumetric VFA productivities mentioned above. A few studies have shown successful granulation under acidifying conditions, albeit with glucose as substrate (Zoetmeyer et al., 1982, Tamis et al., 2015, Atasoy et al., 2019). For example, using a glucose concentration of 25 g COD L^{-1}, Tamis et al. (2015) were able to grow granular sludge at a pH of 4.0 to 5.5 with a VFA productivity of 150 to 300 g COD_{VFA} L^{-1} day^{-1}. To obtain similar productivities with protein as the substrate a biomass retention factor is needed of at least 60 at pH 7 and 200 at pH 5, corresponding to reactor biomass concentrations of approximately 80 and 180 g VSS L^{-1}, respectively. This would require an excellent sludge retention system and/or formation of granules. The question remains if this is possible with protein rich wastewaters. Yu and Fang (Yu and Fang, 2001, Yu and Fang, 2003) used granules, cultivated from synthetic dairy wastewater, to inoculate a laboratory up-flow reactor to degrade gelatin at pH 5.5. They found a somewhat lower similar specific hydrolysis rate (0.38 g COD g^{-1} VSS day^{-1}) as in our CSTR operated at pH 5 (0.4 –1.1 g COD g^{-1} VSS day^{-1}). However, more importantly, it is unclear if also on the long-term it would be possible to maintain robust and strong granules.

6.3.3 Amino acid production

At pH 7 and short SRTs (6-8 days) acidification in the CSRTs of chapter 4 was significantly slower than protein hydrolysis and this may offer the possibility to recover amino acids. This would require a more complex reactor lay-out in which a (small) fraction of the protein rich wastewater is fed to a bioreactor for production of protein hydrolysing enzymes. These enzymes should be separated from the fermentation broth and recycled to a hydrolysis reactor from which the amino acids are actively recovered. Although this would be an interesting concept it first needs to be explored in more detail on laboratory-scale. Considering its complexity and the need for active amino acid extraction the question remains if it would be economically attractive.

6.4 The potential of anaerobic resource recovery from protein rich wastewater

A case study in Vietnam with two different protein rich wastewaters was taken as the example to illustrate the potential of anaerobic resource recovery: (1) slaughterhouse and meat processing wastewater and (2) whey containing wastewaters. Based on findings in two previous studies by NUCE (project number 01C-09/02-2013-2, NUCE (2015) and MT-2019-33, FEE (2019)) the production volumes of these wastewaters in Vietnam were estimated (Table 6.1). The reference case is that all this wastewater is treated aerobically by the activated sludge process. In the new situation 50% of the wastewater is treated anaerobically such that either CH_4 or VFA is produced. For this purpose the parameters in Table 6.2 were used. Table 6.3 summarizes the main results, i.e. the 50% savings on energy production, sludge production and costs associated with the 50% reduction in activated sludge treatment plus the benefits associated with CH_4 or VFA production.

Table 6.1. Estimated volume of wastewater generated and COD load of slaughterhouse and meat processing, and whey containing wastewaters per year in Vietnam.

Kinds of wastewater (ww)	unit	Slaughterhouse	Meat processing	Whey containing
Estimated volume generated[a]	$Mm^3\ year^{-1}$	24	0.48	0.60
COD load of wastewater[b]	ton COD $year^{-1}$	240 000	52 800	30 000

Note: [a]: amount of food production adapted from FAO (2020) and EC (2020); amount of wastewater generation adapted from NUCE studies (NUCE, 2015 and FEE, 2019); [b]: adapted COD concentrations from Table 1.1 (chapter 1).

Table 6.2 Parameter values used in the calculations.

For aerobic treatment:	unit	Value used	Reference
O_2 consumption	g O_2 g $COD_{removed}^{-1}$	0.51	(Metcalf et al., 2004, Khiewwijit et al., 2015a)
Energy required for aeration	kg O_2 kWh^{-1}	1.5	
Sludge production	g VSS g $COD_{removed}^{-1}$	0.4	
CO_2 production	g CO_2 g $COD_{removed}^{-1}$	0.7	

For anaerobic treatment:			
Average VFA conversion efficiency	g COD_{VFA} g COD_{ww}^{-1}	0.7	(this study)
Recovery VFA yield		0.75	(Ramos-Suarez et al., 2021)
Sludge production from fermentation	g VSS g $COD_{removed}^{-1}$	0.08	(this study)
CO_2 production from fermentation	g CO_2 g $COD_{removed}^{-1}$	0.16	(Flotats et al., 2006)

CH_4 conversion efficiency	g CH_4 g $COD_{VFA\ removed}^{-1}$	0.23	(Gavala et al., 2003, Khiewwijit et al., 2015a)
Energy of methane	kWh kg CH_4^{-1}	13.9	
Sludge production from methanogenesis	g VSS g $COD_{VFA\ removed}^{-1}$	0.058	
CO_2 production from methanogenesis	g CO_2 g $COD_{VFA\ removed}^{-1}$	0.64	

note: stoichiometric equation with acetic acid (as VFA)

Cost	unit	Value used	Reference
Electricity cost	€ kWh^{-1}	0.094	(van Lier et al., 2020)
Electricity conversion efficiency of a CHP		0.4	
Sludge treatment and disposal cost	€ ton^{-1}	575	
Average VFA (acetic acid) market value	€ ton^{-1}	500	(Tecnon OrbiChem, 2019)

Table 6.3 Estimated potential for anaerobic resource recovery as CH_4 or VFA from Vietnamese slaughterhouse plus meat processing and whey containing wastewater, including 50% savings on aerobic activated sludge treatment.

Slaughterhouse and meat processing wastewater

Parameter		unit	Savings on aerobic treatment	Anaerobic treatment	
				CH_4	VFA
Energy/VFA					
	Energy cons./prod.	10^6 kWh year^{-1}	50	328	0
	VFA production	ton VFA-COD year^{-1}	0	0	76 860
	Savings/benefits	M€ year^{-1}	4.7	12.3	38.4
Sludge					
	Production	ton solids year^{-1}	58 560	5 468	8 198
	Costs	M€ year^{-1}	33.7	3.1	4.7
CO_2 emission		ton year^{-1}	102 480	81 984	23 424

Whey containing wastewater

Parameter		unit	Savings on aerobic treatment	Anaerobic treatment	
				CH_4	VFA
Energy					
	Energy con./prod.	10^6 kWh year^{-1}	5	34	0
	VFA production	ton VFA-COD year^{-1}	0	0	7 875
	Savings/benefits	M€ year^{-1}	0.5	1.3	3.9
Sludge					
	Production	ton solids year^{-1}	6 000	560	840
	Costs	M€ year^{-1}	3.5	0.3	0.5
CO_2 emission		ton year^{-1}	10 500	8 400	2 400

If 50% of the slaughterhouse and meat processing wastewaters would be treated anaerobically to CH_4 this can generate a total of 328 million kWh year^{-1} of energy (as heat and electricity), whereas the reduction in energy consumption for aeration of the activated sludge process aeration of the aerobic activated sludge process would be 50 million kWh year^{-1}. Also a considerable quantity of sludge reduction and associated costs can be achieved because anaerobic recovery, either as CH_4 or VFA, produces less solids than aerobic treatment. The profit from CH_4 production would be 12.3 M€ year^{-1}. However, from an economic perspective the savings on sludge treatment and disposal of excess activated sludge of 33.7 M€ year^{-1} are

more important. Anaerobic recovery of VFA generates a higher profit than CH_4 production, i.e. 38.4 M€ $year^{-1}$, simply because VFAs have a much higher value than biogas. VFA production also further reduces the CO_2-emission. For whey containing wastewater similar benefits can be demonstrated of anaerobic recovery compared to aerobic treatment, although in absolute numbers these benefits are lower.

It should be noted that in the estimation above the costs and energy consumption for VFA extraction were not included. Apart from this, although the revenues and environmental benefits from VFA production appear to be significantly higher than those from CH_4 production, this does not imply that VFA recovery always is the most attractive option. Whereas VFA production is not yet a mature technology, biogas production already is very common, even on local scale. The biogas can be applied directly for thermal energy use and electricity generation in CHP plants. It is also possible (in the future) to upgraded it to natural gas quality and introduce it into the gas grid, where applicable. Previous research (project number 01C-09/02-2013-2, funded by DOST) indicated a high demand of thermal and electricity recovered from biogas for steaming, heating water, hygienic purposes, cooking, etc. VFAs on the other hand, still are difficult to extract at high-grade quality and need to compete with petrochemical products, which are currently much cheaper than biotechnologically produced VFAs. In addition, a market for such VFA products, for example as a feedstock for bioplastic production, still needs to be developed.

6.5 Recommendations for future research

This thesis explored anaerobic conversion of proteins into CH_4 and VFA, with an emphasis on protein hydrolysis as the first step of this process. Generally, the results were promising and showed the potential of methane, VFA and possibly even amino acid recovery from protein rich wastewaters. Future research regarding this topic should focus on the following:

- The microbial composition of the (proteolytic) biomass that was used as the inoculum and that developed in the reactors was not analysed but could provide valuable information about the effect of pH and substrate composition.

- It may be useful to look for biomass from low pH/protein-rich environments to investigate if it has a lower sensitivity for (undissociated) VFA and therefore can yield higher protein hydrolysis rates.
- More (fundamental) knowledge is required about the mechanism of hydrolysis inhibition by VFA and amino acids.
- Results were generated for pH 5 and pH 7, but most likely the optimum pH that allows attractive VFA productivity and at the same time can prevent methanogenesis is somewhere between these two extremes and still needs to be identified.
- Protein rich wastewaters generally contain a large fraction of particulate proteins and the proteins are more diverse than the model protein gelatine that was used in this study. Therefore, reactors should be operated with realistic wastewater to verify if the same principles and design recommendations apply as were found in this study.
- Higher volumetric VFA productivities can only be achieved if excellent biomass retention can be achieved, for example when granules are formed. It is therefore crucial to investigate if granules can form at low pH on protein rich wastewaters.
- From an economic perspective VFA production appears to be more promising than biogas production. However, future research should be conducted regarding costs, selective VFA extraction technologies and market opportunities for VFA.

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Summary

Many industrial (agro-) wastes and wastewaters i.e. food waste, dairy, slaughterhouse wastewaters and beverage and food processing waste streams contain appreciable quantities of protein. Protein accounts for 20-75% of the chemical oxygen demand (COD) of meat and fish processing, slaughterhouse, and cheese whey wastewaters. These protein rich waste streams are ideal candidates for biological anaerobic treatment to generate energy-rich methane while simultaneously achieving the objective of pollution control. Alternatively, anaerobic conversion of protein-rich waste streams potentially enables the production of important intermediates, i.e. amino acids and volatile fatty acids (VFA). VFA are key platform chemicals suitable for producing high added-value bioproducts such as bioplastics. Serious problems related to incomplete degradation of proteins were reported, resulting in low organic removal efficiencies, low methane production, foaming, sludge flotation, deteriorating effluent quality and biomass washout. These problems hampered the application of anaerobic treatment of protein containing wastewaters. This thesis explored anaerobic conversion of proteins into methane (CH_4) and VFA under different environmental and process conditions, with an emphasis on protein hydrolysis as the first step of this process.

The introduction (**Chapter 1**) gives a brief overview of protein-rich wastes and wastewaters and a literature review on anaerobic conversion of proteinaceous wastewaters. Different environmental and microbial factors affecting the anaerobic protein hydrolysis process and subsequent formation of VFA and methane are discussed. Previous assessment of the kinetics and degradation performance of protein rich waste(water)s is generally based on the end products, i.e. ammonium and methane, based on the assumption that hydrolysis is always the rate limiting step. In **Chapter 2**, we explored anaerobic hydrolysis and amino acid fermentation of gelatine (as a model for dissolved proteins) at pH 7 and pH 5 at 35°C in batch experiments. In contrast with earlier findings, the hydrolysis of dissolved protein was significantly faster than acidification at pH 7, implying that not hydrolysis but amino acid fermentation is the rate limiting step in the dissolved protein fermentation. This was confirmed by temporary accumulation of amino acids at pH 7 under both methanogenic and non-methanogenic conditions. Interestingly, methanogenesis does not stimulate the rate of hydrolysis and acidification of gelatine at pH 7 and 35°C. Still the protein hydrolysis was suppressed at pH 5 and non-methanogenic conditions.

Proteins and carbohydrates are often present together in many types of wastewater and waste. In **Chapter 3**, we described the effect of the presence of carbohydrates on protein hydrolysis at neutral pH and 35°C. The results of batch experiments showed that the protein hydrolysis was not directly affected by the presence of starch under methanogenic and non-methanogenic conditions, both at 35°C. Gelatine hydrolysis rate constants ranged between 0.51±0.05 L g VSS^{-1} d^{-1} under methanogenic and 0.48±0.05 L g VSS^{-1} d^{-1} under non-methanogenic conditions at neutral pH. However, protein hydrolysis was strongly inhibited by a mixture of different VFA, which reduced the rate constants by 64±2% at a VFA to gelatine ratio of 5.9 (initial VFA concentration of 8.2 g COD L^{-1}) under non-methanogenic conditions and pH 7. This was not caused by the ionic strength exerted by VFA but directly by the VFA itself. Still, it cannot be excluded that during long-term operation of a continuous reactor on protein-rich wastewater the microbial population or the enzymatic machinery of the existing population may adapt to high concentrations of VFA. Methane production was slower at higher starch to gelatine ratios which can be explained by a higher VFA production, more in particular by the production of propionic acid. E.g. at a starch to gelatine ratio of 5.5, a maximum propionate concentration of 1.7 g COD L^{-1} was measured, which exceeds the inhibitory concentration of propionate to methanogenesis.

In **Chapter 4** we described the effect of the solid retention time (SRT) and pH on protein hydrolysis and amino acid fermentation for VFA production in continuous experiments. Two completely stirred-tank reactors (CSTR) were operated at pH 5 (SRTs of 12-30 days for 480 days) and at pH 7 (SRTs of 6-12 days for 600 days) under non-methanogenic conditions and 35°C. After a long-term exposure of the biomass to pH 5, the hydrolysis rate constant for protein at pH 5 of 0.05 L g^{-1}VSS day^{-1} was much lower than at pH 7 (0.62 L g^{-1}VSS day^{-1}). The difference most likely is caused by the inhibitory effect of undissociated volatile fatty acids (3.1-5.0 g COD L^{-1}) at pH 5. Hydrolysis was the rate-limiting step in gelatine degradation at pH 5 at SRTs of 12-30 days. VFA yield and VFA product spectra were not significantly affected by the SRT (12-30 days) at pH 5, but they are significantly affected by the SRT in the range of 6-12 days at pH 7. VFA production from gelatine at pH 7 is limited by either hydrolysis at SRT > 8 days or acidogenesis at SRT ≤ 8 days. The optimum volumetric VFA productivity was 2.3 g COD_{VFA} L^{-1} day^{-1} at SRT 10 days and pH 7. However, for a complete removal of protein a longer SRT is required.

In **Chapter 5**, we described the potential inhibitory effect of amino acids on protein hydrolysis by testing different models for the kinetics of hydrolysis: first-order kinetics, first-order kinetics including amino acid inhibition and Contois kinetics. The experimental data obtained during steady state operation of non-methanogenic CSTR at SRTs of 6-12 days, pH 7 and 35°C (Chapter 4) were used to test the hydrolysis models. The non-competitive amino acid inhibition model for protein hydrolysis gave the best fit for the experimental data. This suggests that hydrolysis is inhibited at elevated amino acid concentrations (1.7 to 3.3 g COD L^{-1}).

In **Chapter 6**, implications for the design and operation of anaerobic systems treating protein rich wastewaters and wastes for VFA or methane production are discussed as well as the anaerobic resource recovery potential. This study is relevant for Vietnam where food production is of significant economic importance, therefore the findings in this thesis are discussed for the treatment of Vietnamese slaughterhouse, meat processing or whey wastewater. Finally, recommendations for future research are given.

List of publications

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Thu Hang Duong, Katja Grolle, Nga Tran Thi Viet, Grietje Zeeman, Hardy Temmink, and Miriam van Eekert (2019). "Protein hydrolysis and fermentation under methanogenic and acidifying conditions". Biotechnology for Biofuels ISSN: 1754-6834. 12(1): 254.

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Thu Hang Duong, Miriam van Eekert, Katja Grolle, Nga Tran Thi Viet, Grietje Zeeman and Hardy Temmink (2020). "When co-digestion of protein-rich and carbohydrate-rich wastes become effective?" Oral presentation at (online) International Conference: Environmental Technology for Impact, Wageningen, The Netherlands, June 3-4, 2020.

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Thu Hang Duong, Miriam van Eekert, Katja Grolle, Nga Tran Thi Viet, Grietje Zeeman, and Hardy Temmink. "Volatile fatty acids or methane from wastewater portiens – effect of the presence of carbohydrates".

Thu Hang Duong, Miriam van Eekert, Katja Grolle, Nga Tran Thi Viet, Grietje Zeeman and Hardy Temmink. "Effect of solid retention time on protein hydrolysis and acidogenesis at pH 5 and pH 7".

Alberte Regueira[a], Thu Hang Duong[a], Miriam van Eekert, Grietje Zeeman, Marta Carballa, Miguel Mauricio-Iglesias and Hardy Temmink. "Anaerobic protein hydrolysis kinetics under non-methanogenic conditions" ([a]: contributed equally).

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Thu Hang Duong, August 2021.

About the author

Thu Hang Duong was born on the 13th of July, 1983 in Hanoi, Vietnam. In 2006, she graduated for her BSc in Environmental Engineering, specializing in Water Supply and Sanitation, and Water Environment at the National University of Civil Engineering, Vietnam (the Laureate Gradation) after which she has been working as a lecturer and research scientist at the same Department.

In 2007, she obtained a scholarship for a Master course of Environmental Sanitation at Ghent University, Belgium, where she researched different pre-treatments to enhance biomethanation of kitchen waste and sewage in the Laboratory of Microbial Ecology and Technology. She obtained her MSc diploma with the greatest distinction in 2009. In 2016, she started her sandwich PhD at Department of Environmental Technology, Wageningen University & Research, the Netherlands. She studied anaerobic conversion of proteins under methanogenic and acidifying conditions for production of volatile fatty acids and methane. Resource recovery from waste and wastewater is one of her research focusses.

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