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Effect of carbohydrates on protein hydrolysis in anaerobic digestion

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ABSTRACT

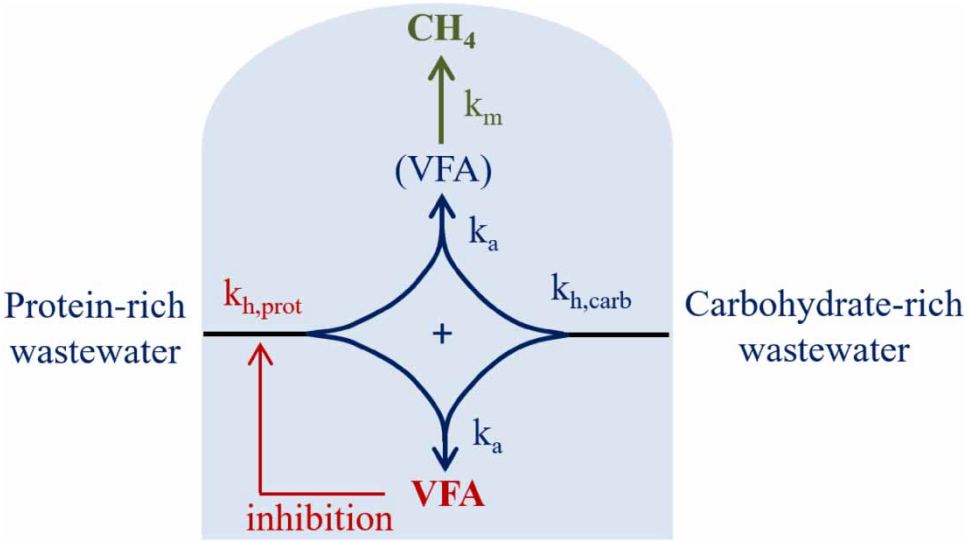
This study aimed to assess the effect of carbohydrates on protein hydrolysis and potential implications for the design of anaerobic reactors for treatment of protein-rich wastewaters. Batch experiments were carried out with dissolved starch (Sta) and gelatine (Gel) at different chemical oxygen demand (COD) ratios ranging from 0 to 5.5 under methanogenic conditions for methane production and up to 3.8 under non-methanogenic conditions for volatile fatty acids (VFA), 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 conditions, gelatine hydrolysis was inhibited by 64% when a spectrum of VFA was added at a VFA/Gel (COD) 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.

Key words: carbohydrates, methanogenic, non-methanogenic, proteins, volatile fatty acids

HIGHLIGHTS

- Carbohydrates do not directly affect protein hydrolysis and further degradation.
- Methane yield of 82-89% on COD basis when co-digesting proteins and carbohydrates.
- Build-up of VFA prevents complete protein hydrolysis in non-methanogenic conditions.
- VFA slows down protein hydrolysis rates.

GRAPHICAL ABSTRACT



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 & 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 fed with 3.5 g L^{-1} of gelatine at pH 7 under non-methanogenic conditions. Approximately 95% of the gelatine was hydrolysed and on a 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 & Benjakul 2015), and a misinterpretation of the results. Yu & 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 & 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 *et al.* 2001). As such the effect of carbohydrates on gelatine hydrolysis is unclear and results obtained so far by others warrant further research in the matter.

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 $0.33/(0.33 + \text{VFA})$, VFA as g acetate L^{-1}. 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). The model substrates were chosen because of their high solubility in water and because gelatine and starch are often used as a model protein and carbohydrate in wastewater treatment studies and literature references, as described above. This enables a comparison between our results and results obtained in literature. 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.

2. MATERIALS AND METHOD

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 1.

VFAs were applied as a mixture consisting of 58% acetate, 27% propionate, 9% butyrate and 6% valerate on a COD basis, which is representative for the VFA profile that was obtained after starch fermentation under non-methanogenic conditions at pH 7 (section 3.5). 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 n-butyric acid (Bu, CAS no.107-92-6) and 2.9 g n-valeric acid (Val, CAS no.109-52-4) diluted in demi-water and neutralized with 5M NaOH to pH 7.0 ± 0.2.

2.2. Inoculum and nutrient medium

The seed sludge was sampled from a full-scale anaerobic reactor that treated brewery wastewater. The reactor was operated at a temperature of 30 ± 3 °C. The characteristics of the sludge samples for all batch experiments 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},

Table 1 | Main characteristics of the substrates used in this experiment

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	–

Data are measured per gram and expressed in average ± standard deviation ($n = 10$).

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. 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.

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 were carried out: 'Sta/Gel - (for) CH_4', 'Sta/Gel - (for) VFA' and 'VFA/Gel – VFA' (Table 2) under different process conditions.

Table 2 | Substrate composition and concentrations of the substrate mixtures of batch bottles in the three experimental set-ups: 'Sta/Gel – CH_4', 'Sta/Gel – VFA' and 'VFA/Gel – VFA'

Mixture	Sludge	Gelatine	Starch
Sta/Gel – CH_4 experiment : Gelatine and varying starch concentrations under methanogenic conditions.			
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
Sta/Gel – VFA experiment: Gelatine and varying starch concentrations under non-methanogenic conditions.			
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
Mixture	Sludge	Gelatine	VFA
VFA/Gel – VFA experiment: Gelatine and varying VFA concentrations under non-methanogenic conditions.			
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

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}.

In the Sta/Gel – CH_4 experiment 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 $\pm$ 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. There was no decrease of pH (Figure S1, Supplementary Information (SI)) or production of VFA (Table S2, SI), and also no effect observed from a lower inoculum to substrate (I/S) ratio on hydrolysis in this experiment.

In the Sta/Gel – VFA experiment 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), which was confirmed by a lack of methane production through the experiments. Gelatine was added at a concentration of 1.4 $\pm$ 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 the Sta/Gel – CH_4 experiment, 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 $\pm$ 0.1 g VSS g^{-1} COD.

The VFA/Gel – VFA experiment 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 Sta/Gel – VFA, 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, the bottles were closed and flushed with N_2 gas for 20 minutes.

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 the Sta/Gel – CH_4 experimental set-up after 336 and 456 h to assess whether these bottles had reached complete methane production. 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). The sludge samples were centrifuged (Eppendorf, Germany) at 10,000 rpm for ten minutes and the supernatant was filtered with pre-washed 0.45 μm cellulose acetate membrane filters (Sartorius, Germany). The supernatant was analyzed for COD, total nitrogen and ammonium (NH_4-N), as described by Duong *et al.* (2019). Protein was determined using the Lowry method assay (Noble & 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 × 150 mm), 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. VFAs were quantified on a Trace gas chromatograph equipped with a Thermo TR-WAX column (30 m × ID 0.32 mm × thickness of 0.25 μm) connected to a FID detector as described by Sudmalis *et al.* (2018). 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).

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:

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

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:

$$P_{Acidified}(t) = P_{Acidified-end} \cdot (1 - \exp(-k_{acidogenesis} \cdot X \cdot t)) \quad (2)$$

$$P_{Methane}(t) = P_{Methane-end} \cdot (1 - \exp(-k_{methanogenesis} \cdot X \cdot t)) \quad (3)$$

with $P_{Acidified}(t)$ and $P_{Acidified\text{-}end}$ the produced sum of the concentration of VFA and methane at time t 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 $k_{acidogenesis}$ and $k_{methanogenesis}$ 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:

$$P_{Sta-hydrolyzed}(t) = k_{h,Sta} \cdot X \cdot t \text{ for } t < t_k \text{ and } P_{Sta-hydrolyzed-end} \text{ for } t \geq t_k \quad (4)$$

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 undefined COD was 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. RESULTS AND DISCUSSION

3.1. Effect of the Sta/Gel ratio on conversion of proteins to VFA and methane

Figure 1 shows COD mass-balances under methanogenic (Sta/Gel – CH_4 experiment at t = 0, t = 240 and also at t = 456 h at Sta/Gel of 4.6 or higher) and non-methanogenic conditions (Sta/Gel – VFA experiment 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 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%.

Similarly under non-methanogenic conditions, complete hydrolysis of starch was achieved in all test bottles. In the absence of starch, gelatine hydrolysis was also complete. However, 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 (Figure 1). 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 that a small fraction of the gelatine present at the start of the experiment 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 with accumulation of VFA. 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 lower (82–84%) at the higher Gel/Sta ratios, which is explained by the lower gelatine conversion in the presence of starch.

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

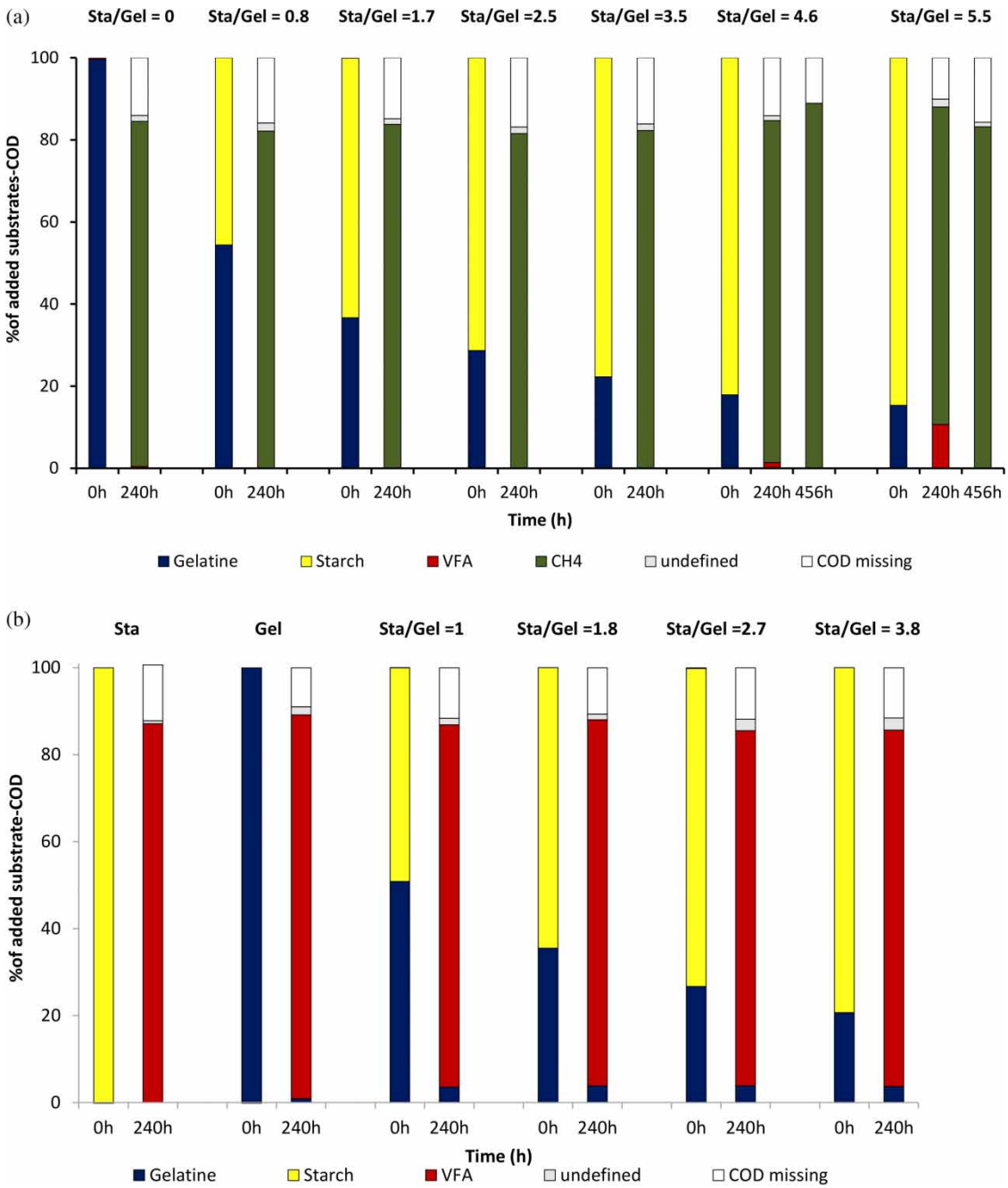


Figure 1 | COD mass balances in the Sta/Gel – CH_4 (upper graph a) and the Sta/Gel – VFA (lower graph b) experiments, respectively. The data present average values of triplicate bottles. Standard deviations can be found in Table S1 of SI.

3.2. Effect of Sta/Gel ratio on process rates

Rate constants were estimated for every single bottle and the regression coefficient always exceeded 0.98. Table 3 shows the average rate constants of triplicate bottles, together with their standard deviation ($n = 3$). Figures S2–3, S4–5 and Figure S6 of the supplementary information show average measured concentrations and concentrations that were estimated according Equations (1)–(4).

The pH in the different bottles varied between 6.5 and 7.5 (Figure S1, SI). Anova regression statistics did not show a correlation between the pH and the rate constants (p-value >0.1), which also agrees with results obtained by others that pH in the range of 6.5 to 7.5 does not affect hydrolysis of dissolved proteins (Yu & Fang 2003; Liu *et al.* 2012; Liu *et al.* 2015).

Table 3 | First- and zero-order (for starch) rate constants based on COD of the gelatine, starch, VFA mixtures and methane production

Sta/Gel – CH_4	$k_{h,Sta}$ g COD gVSS^{-1} day^{-1}	$k_{h,Gel}$ L g^{-1} VSS day^{-1}	$k_{acidogenesis}$ L g^{-1} VSS day^{-1}	$k_{methanogenesis}$ 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)
Sta/Gel – VFA	$k_{h,Sta}$ g COD gVSS^{-1} day^{-1}	$k_{h,Gel}$ L g^{-1} VSS day^{-1}	$k_{acidogenesis}$ L g^{-1} VSS day^{-1}	$k_{methanogenesis}$ 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)	–
VFA/Gel – VFA	$k_{h,Sta}$ g COD gVSS^{-1} day^{-1}	$k_{h,Gel}$ L g^{-1} VSS day^{-1}	$k_{acidogenesis}$ L g^{-1} VSS day^{-1}	$k_{methanogenesis}$ 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)	–

All data are expressed as average ± standard deviation ($n = 3$).

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.

Estimated zero-order rate constants for starch hydrolysis ($k_{h,Sta}$) in the Sta/Gel – CH_4 experiment and the Sta/Gel – VFA experiment varied between 0.78 and 1.37 COD g^{-1} VSS day^{-1} (Table 3). Starch hydrolysis was very fast, and it took less than 6–12 hours before the starch was completely hydrolysed (Figures S2 and S4). 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 (0.25 L g^{-1} VSS day^{-1}) was faster than acidification when only gelatine was added (0.15 L g^{-1} VSS day^{-1}) in the Sta/Gel – VFA experiment, 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 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 ($k_{h,Gel}$ of 0.5–0.6 L g^{-1} VSS day^{-1}, about 3.5 times higher than values of $k_{acidogenesis,Gel}$ and about 7.3 times

higher than values of $k_{methanogenesis,\ Gel}$) 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.

Under methanogenic conditions (Sta/Gel – CH_4 experiment), the 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 (Sta/Gel – VFA experiment) 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. 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. 0.56 L g VSS^{-1} day^{-1} under methanogenic as well as under non-methanogenic conditions (0.15 h^{-1} per equivalent 6.5 g VSS L^{-1} (Duong *et al.* 2019)) and 0.59 L g VSS^{-1} day^{-1} for dissolved (meat) peptone hydrolysis at mesophilic conditions (2.3 day^{-1} per 3.9 g VSS L^{-1} (González *et al.* 2005)).

Finally, in the Sta/Gel – CH_4 experiment methane production was slower at higher Sta/Gel ratios. Also this can be explained by VFA production, more in particular by the production of propionic acid (Angelidaki *et al.* 1999; Siegert & Banks 2005; Wang *et al.* 2009; Ma *et al.* 2011; Jojoa-Unigarro & González-Martínez 2022). 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), which exceeds propionate concentrations of 0.7–1.5 g COD L^{-1} that are reported to inhibit methanogenesis (Wang *et al.* 2009; Ma *et al.* 2011; Jojoa-Unigarro & González-Martínez 2022). This can be explained by the high concentration of propionic acid, which results in an increase of partial hydrogen pressure and subsequent inhibition of the overall acetogenesis and, consequently, the hydrogenotrophic methanization (Jojoa-Unigarro & González-Martínez 2022). The other acid concentrations, i.e acetate and butyrate, were well below inhibitory concentrations.

3.3. Effect of VFA/Gel ratio on 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 the Sta/Gel experiments (Table 3) did not indicate such inhibition, although under non-methanogenic conditions 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} (Figures S4–5, SI). In practice, in continuously operated reactors aiming to produce VFA, much higher VFA concentrations can be expected. For this reason non-methanogenic experiment was performed at different VFA/Gel ratios (VFA/Gel-VFA experiment, Table 2) to further investigate a potential negative impact of VFA on protein hydrolysis. Results in Table 3 (VFA/Gel-VFA experiment) show 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, corresponding VFA/Gel ratio = 0. In the Sta/Gel experiments 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. The COD mass-balance (Table S1) 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 (2)) and is the result of coupled protein hydrolysis and amino acid acidification. The highest concentrations of free amino acids were of about 2–4 mM after 8 h-incubation, which is similar to concentrations measured in the absence of VFA (data not shown). A negative effect of amino acids on hydrolysis can therefore be excluded.

3.4. Effect of ionic strength on protein hydrolysis

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 relatively unaffected by ionic strength (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 2 shows the gelatine hydrolysis rate constants as a function of the (calculated) ionic strength in the test bottles of the Sta/Gel – CH_4, Sta/Gel – VFA and VFA/Gel – VFA experiments 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 the VFA/Gel – VFA experiment already occurred starting at a much lower ionic strengths of 60–70 mM. For instance, at the VFA/Gel ratio of 2.2, the calculated ionic strength is 67 mM in which VFA accounted for 37 mM, equivalent to about 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. This is in accordance with Palenzuela (1999), who showed the protease activity remained unaffected at increasing NaCl concentration up to 20 g L^{-1}, equivalent to 340 mM. 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 need to be further investigated.

3.5. Effect of Sta/Gel on VFA production spectra

VFA product spectra of the tests in the Sta/Gel – VFA experiment are given in Figure 3. Varying starch concentrations changed the ultimate VFA product spectrum in a sense that the fraction of valerate decreased at high Sta/Gel ratios. Glucose is the only product of hydrolysis of starch and conversion from glucose to acetate and butyrate is the preferred energetic pathway (Regueira *et al.* 2020). Hydrolysis of proteins forms a mix of 20 different amino acids, in which glycine, proline, alanine, leucine and valine are the main components of gelatine. 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.* 2020). 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, which are among the most abundant amino acids in gelatine (22–25% w/w (Gelatin Handbook 2012)).

3.6. Consequence for design of anaerobic reactor systems for treatment of protein-rich wastewaters

The results in this study 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 (Table 3). For well-designed, continuously operating anaerobic reactors with methane as the desired end product this will not present a problem. Methanogenesis is much

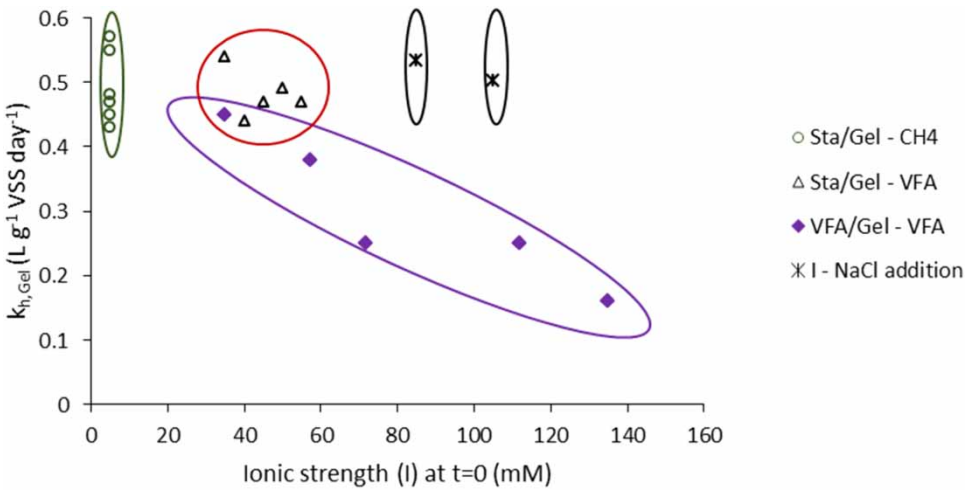


Figure 2 | Gelatine hydrolysis rate constants (the average values, $k_{h,\ Gel}$) as a function of ionic strength concentrations in the Sta/Gel – CH_4, Sta/Gel – VFA and VFA/Gel – VFA experiments and in the ionic strength tests excluding VFA (*). The green circles express the data under methanogenic conditions and the red ones under non-methanogenic conditions.

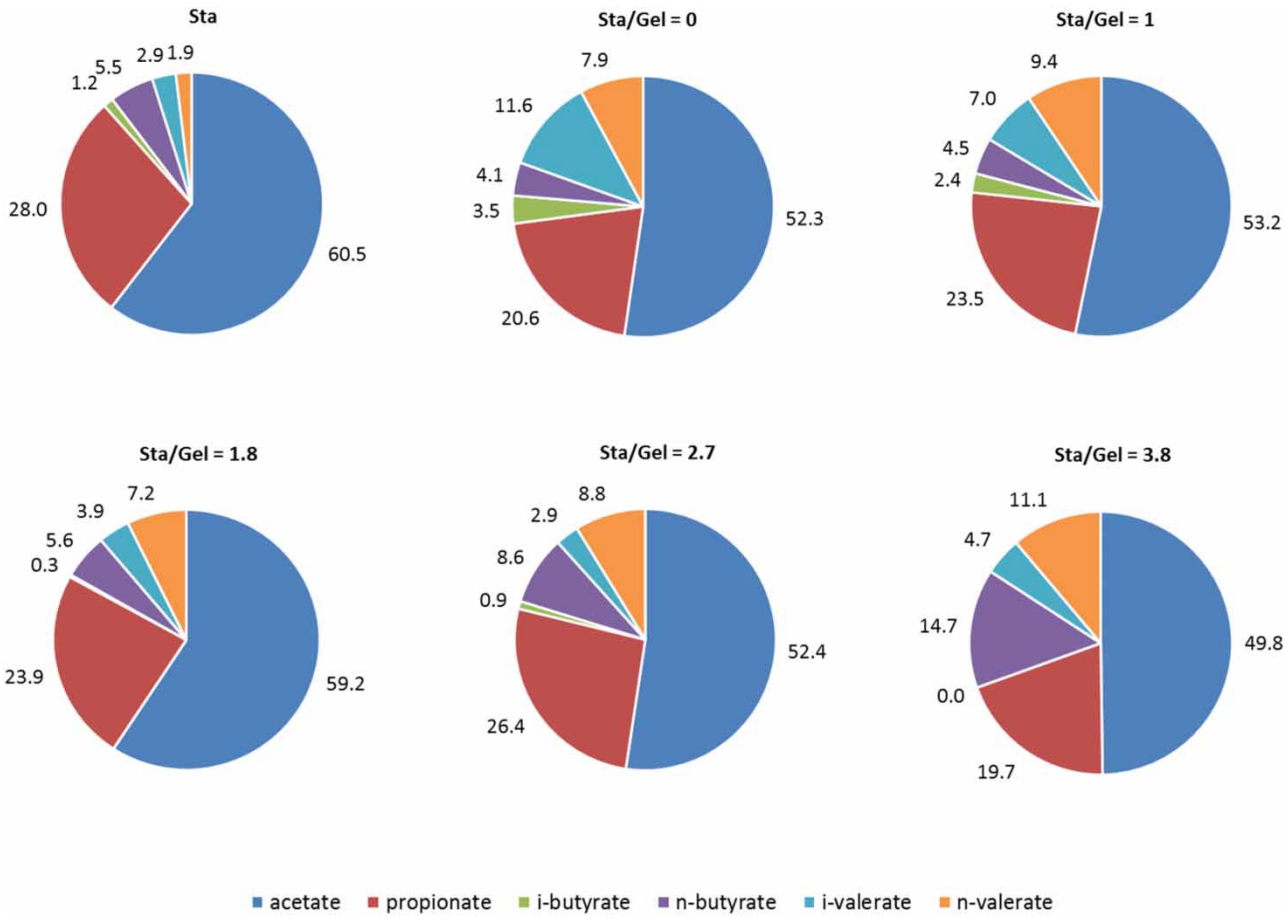


Figure 3 | VFA product spectra in Sta/Gel – VFA experiment.

slower than hydrolysis of dissolved proteins such as gelatine (Sta/Gel – CH_4 experiment) and dictates reactor design. At a solids retention time (SRT) high enough to avoid their wash-out, methanogens will keep the acetate and consequently 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} (VFA/Gel – VFA experiment) 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 VFAs 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 the Sta/Gel experiments also showed that the acidification rate is significantly slower 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.

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. Gelatine hydrolysis rate constants ranged between 0.51 ± 0.05 L g VSS^{-1} day^{-1} under methanogenic conditions and 0.48 ± 0.05 L g VSS^{-1} day^{-1} under non-methanogenic conditions at neutral pH. Acetate always was the most abundant VFA (49–58%), followed by propionate (21–26%) in the VFA profile under non-methanogenic conditions. 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%. However, protein hydrolysis was strongly inhibited by a mixture of different VFA, which reduced the rate constants by $64 \pm 2\%$ at a VFA to gelatine ratio of 5.9 under non-methanogenic conditions and pH 7. 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, and more knowledge is required about the mechanism of hydrolysis inhibition by VFA.

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AUTHOR CONTRIBUTIONS

Thu Hang Duong: Conceptualization, Methodology, Investigation, Sample processing, Data analysis, Writing – Original Draft, Writing – Review & Editing; Miriam van Eekert: Conceptualization, Supervision, Data analysis, Writing – Original Draft, Writing – Review & Editing; Katja Grolle: Methodology, Data analysis, Writing – Original Draft; Nga Tran Thi Viet: Methodology, Supervision, Writing – Original Draft; Grietje Zeeman: Conceptualization, Funding acquisition, Supervision, Writing – Original Draft; Hardy Temmink: Conceptualization, Funding acquisition, Data analysis, Supervision, Writing – Original Draft, Writing – Review & Editing.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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