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Effect of solid retention time (SRT) on protein hydrolysis and acidogenesis at pH 5 and pH 7 using gelatine as a model protein

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ABSTRACT

Anaerobic conversion 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 operated at 35 °C. At pH 5 and with an SRT of 12 and 30 days, hydrolysis was the rate-limiting step of protein degradation. At pH 7 and at SRT ≤ 8 days, the system shifted from being limited by hydrolysis to being limited by the conversion of amino acids to VFA. Even after a long-term exposure of the biomass to pH 5 (480 days), 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 difference between pH 5 and pH 7 is explained by the inhibitory effect of the large fraction of undissociated VFA at pH 5, which was confirmed in batch experiments. 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.

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 [1–3]. These protein-rich waste streams are attractive substrates for anaerobic treatment to generate energy-rich methane while simultaneously achieving the objective of pollution control [4–7]. 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 [8–11]. Anaerobic conversion of complex biowastes (containing mixtures of proteins, carbohydrates, fats and other compounds) has been extensively studied [5,7,12]. However, the necessary information on how to optimize the first steps in the conversion of proteins, i.e. hydrolysis and fermentation, is lacking.

Most hydrolytic acidogenic bacteria have an optimum pH between 5 and 7 [13,14]. Protein hydrolysis is inhibited at low pH, e.g. 4.6–5.5 [12,15], 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 [16]. 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

Abbreviations: SRT, solids retention time; VFA, volatile fatty acids; TS, total solids; VS, volatile solids; TSS, total suspended solids; VSS, volatile suspended solids; COD, chemical oxygen demand; TN, total nitrogen; BrES, 2-bromoethanesulfonate; CSTR, completely stirred tank reactor; 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.

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strategy [8]. The COD content in protein containing food-processing wastewaters can be as high as 30–45 g L^{-1} [17–19] and under non-methanogenic conditions this could result in inhibition of protein degradation by high concentrations of end products VFAs [20]. Yu and Fang [18] 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. [21] 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 approximately 5 times slower than hydrolysis of dissolved protein [15]. The hydrolysis rate constant and operational conditions, mainly the solid retention time (SRT) and pH, will affect protein conversion efficiency [22]. 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 short SRTs typically lower than 5 days, are economically more favorable, but will result in limited conversion [17,23]. This would also 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 [16,23–25]. 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.

2. Materials and methods

2.1. Inoculum and substrate characteristics

The seed sludge for the two CSTRs was obtained from a full-scale anaerobic reactor operating under methanogenic conditions and a temperature of 30 ± 3 °C treating brewery wastewater. 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. [26]. The feedstocks for each CSTR were kept in a water bath of 40 ± 2 °C to avoid gelation during feeding into the CSTR [27].

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). As gelatine concentrations in the CSTR were below 2%, gelation did not occur at the reactor temperature of 35 °C [27]. The pH of the feed was 5.3 ± 0.1. The pH in two CSTR were controlled at pH 5.0 ± 0.1 and pH 7.0 ± 0.1 with automated HCl (1 N) or NaOH (1 N) addition. The reactors were inoculated at an initial biomass concentration of 8.6 g VSS L^{-1}. In both reactors 2-bromoethanesulfonate (BrES, 20 mM) was added at day 0 to inhibit methanogenic activity and additional doses of BrES (10 mM) 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 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-minute cycle (1 min on and 4 min off) to set a 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.

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.

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 rpm for 240 h. The batch medium at pH 7 was adapted from Angelidaki et al. [26]. 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. BrES (20 mM) 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 (2 mL) and liquid samples (4 mL) 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. [15]. A lack of methane production in all bottles showed that methanogenesis was effectively inhibited (data not shown).

2.4. Analyses

Gas composition (CH_4, CO_2, H_2 and N_2) was quantified by injecting

Table 1
Operation strategies for CSTR at pH 5 and 7 and 35 °C.

	Period	0–150	151–290[a]	291–480
pH 5	SRT (day)	12	20	30

	Period	0–60	61–102	103–156	157–206	207–516[a]	517–600
pH 7	SRT (day)	12	10	8	6	8	10

[a] 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 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 [28]. Digestate (effluent) and sludge samples were analysed for total suspended solids (TSS), volatile suspended solids (VSS), and total chemical oxygen demand (COD) using the standard methods. The digestate samples were centrifuged (Eppendorf, Germany) at 10000 rpm for 10 min and filtered with pre-washed 0.45 μm cellulose acetate membrane filters (Sartorius, Germany). The soluble fraction was analysed 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 [29] at 660 nm using gelatine as standard. Total peptides were analysed in the supernatant samples as described by Cuchiaro and Laurens [30]. Volatile fatty acids (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. [31]. Amino acids were measured in the supernatant samples as described by Meussen et al. [32] via high-performance liquid chromatography (HPLC).

2.5. Calculations

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

$$\text{Degree of hydrolysis}: H = \frac{P_i - P}{P_i} \cdot 100\ (\%) \quad (1)$$

$$\text{Degree of acidification}: A = \frac{COD_{VFA}}{P_i} \cdot 100\ (\%) \quad (2)$$

$$\text{VFA yield}: Y_{VFA} = \frac{COD_{VFA}}{P_i - P}\ \left(g\ COD_{VFA}\ g^{-1}COD_{hydrolyzed\ protein}\right) \quad (3)$$

$$\text{Biomass yield}: Y_{sludge} = \frac{COD_{tot,eff} - COD_{sol,eff}}{P_i - P}\ \left(g\ COD_{sludge}\ g^{-1}COD_{hydrolyzed\ protein}\right) \quad (4)$$

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}).

Hydrolysis of biopolymers including proteins is generally described by a first-order model [33]. To account for wash-out of hydrolytic biomass a minimum SRT below which complete wash-out occurs was included in Eq. (5). Both the hydrolysis rate constant and minimum SRT were estimated using this equation using a least-squares method:

$$P = \frac{P_i}{1 + k_h \bullet X \bullet}(SRT - SRT_{min}) \quad (5)$$

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.

In the batch experiments, hydrolysis of protein could be described by a first-order model [33] with a linear dependency on the biomass concentration [34]. The first-order protein hydrolysis rate constants were estimated from the results of the batch experiments with the following equation:

$$P_{hydrolyzed\ prot}(t) = P_{added\ prot} \cdot (1 - exp(-k_{h-batch} \cdot X \cdot t)) \quad (6)$$

With $P_{hydrolyzed\ prot}$ (t) the (cumulative) concentration of hydrolyzed protein (g COD L^{-1}) after t time (days), $P_{added\ prot}$ 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.

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. 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 1), but recovered from this without having to take operational measures.

3.1. Protein hydrolysis and acidification at pH 5 and pH 7

The CSTRs (Fig. 1 and Table 2 for pH 5; Fig. 2 and Table 3 for pH 7) showed different patterns with respect to the degree of hydrolysis and acidification in response to SRT and pH. In general, as expected by the first-order kinetics (Eq. (5)), a longer SRT gave higher sludge

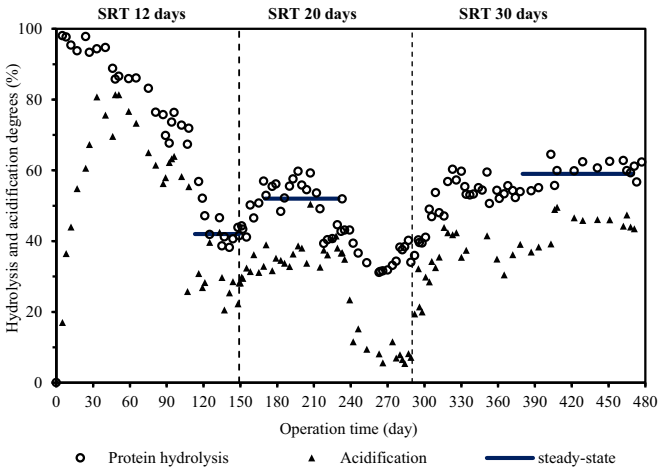

Fig. 1. Protein conversion at CSTR pH 5 and 35 °C.

Table 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 (Anova single-factor statistical analyses).

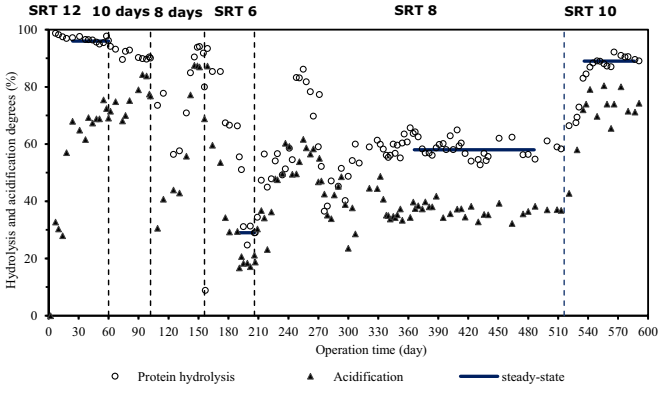

Fig. 2. Protein conversion at CSTR pH 7 and 35 °C. Data at SRT 10 days (day 60–102) and at SRT 8 days (day 102–155) varied more than 20%, therefore the latter periods (day 366–486 for SRT 8 days and day 536–583 for SRT 10 days) were used for calculation.

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 (Fig. 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} $g^{-1}COD_{hydrolyzed\ protein}$,

Table 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 (Anova single-factor statistical analyses).

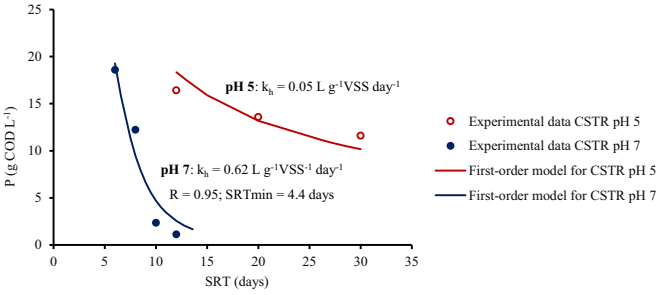

Fig. 3. Effluent protein concentrations (the mean) at different retention times and first-order model for protein hydrolysis in the CSTRs at pH 5 and pH 7.

which is in accordance with values reported by others [16,24,35].

For both CSTRs from the protein measurements a first-order hydrolysis rate constant k_h and a minimum SRT_{min} were estimated according to Eq. (5)) (Fig. 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.

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 (0.56 L g VSS^{-1} day^{-1}) [15]. 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}) [36]. 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 Supplementary Information. 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 [25].

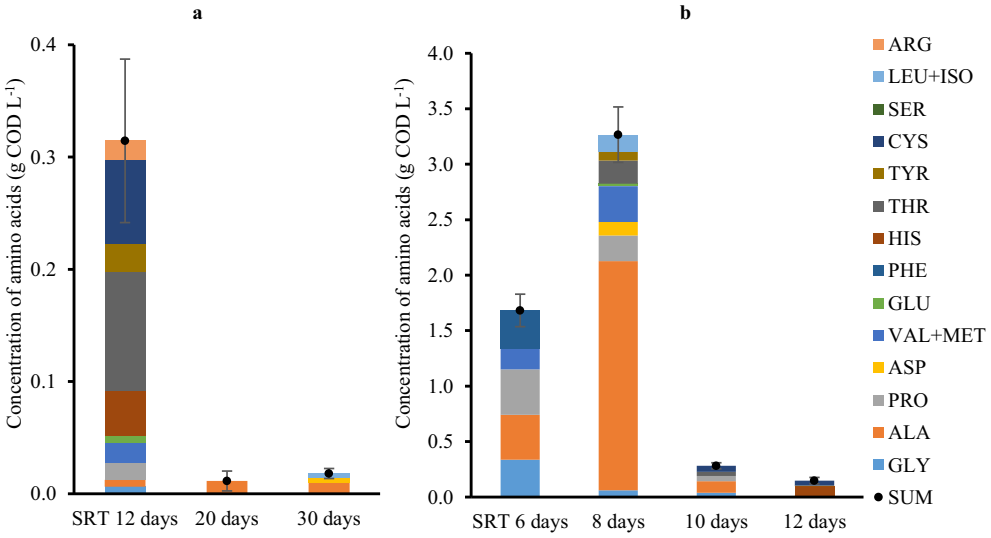

Fig. 4. Concentrations of amino acids at pH 5 (a) and pH 7 (b) at different solid retention times (SRTs). Data express the mean and standard deviation (only for the sum) during steady state at different SRTs. (GLY: Glycine; ALA: Alanine, PRO: Proline and Hydroproline, ASP: Aspartic acid; VAL + MET: Valine 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).

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

Fig. 4 shows the amino acid composition in the effluent during steady state conditions.

At pH 5 and an SRT of 12 days the total amino acid concentration was 0.31 g COD L^{-1} (Fig. 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. [15]. 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 (Fig. 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 Supplementary Information). 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, e.g. approximately 63% compared to 8% of alanine in COD composition in gelatine substrate (Fig. S2, SI). We also observed accumulation of alanine during previous batch tests [15] but do not have a mechanistic explanation for this.

3.3. VFA production and spectrum

VFA concentrations were measured to assess the VFA yield (Fig. 5).

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 (Fig. 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 remark that at pH 5 the concentration of undissociated VFA

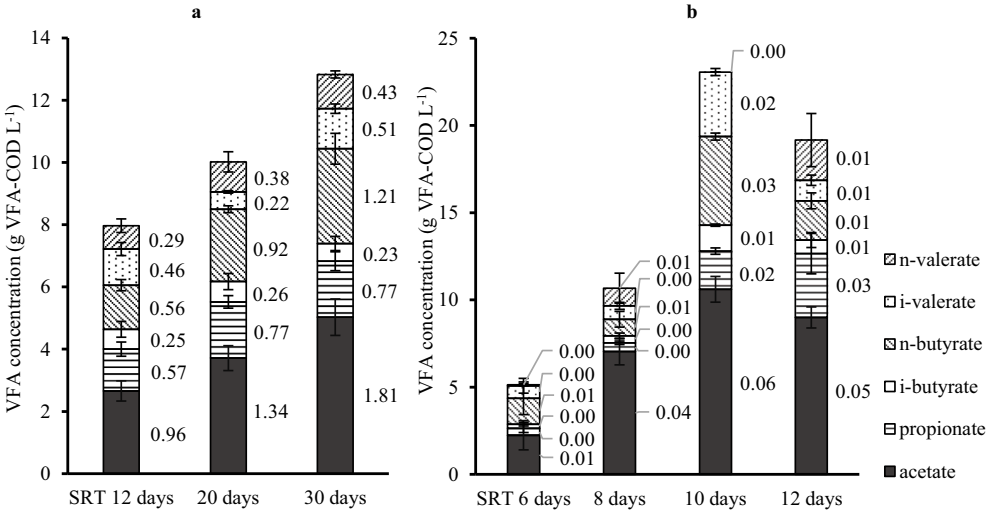

Fig. 5. VFA concentration and compositions at pH 5 (a) and pH 7 (b) at different SRT. Data expressed the mean and std. during steady state periods of different SRT phases. Number presents the average of undissociated VFA concentration of acetic, propionic, i-butyric, n-butyric, i-valeric and n-valeric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

was as high as 3.1–5.0 g COD L^{-1} (Fig. 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 [20], and 0.6 g COD L^{-1} for propionic acid and butyric acid [37]. 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 [38]. 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 (Fig. 5b). At this pH the concentration of undissociated VFA is much smaller 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 2.5.

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 (Fig. 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%). Although this was not further quantified, others have speculated that 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 [11,39].

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). The first-order model of Eq. (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 2.2.

The hydrolysis rate constants at pH 5 (0.14–0.34 L g^{-1}VSS day^{-1}, Table 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}, unpublished data), even at pH 7 can inhibit protein hydrolysis. Although the inhibition mechanisms remain unclear this may be due to the VFAs (i) directly affect the structure of gelatine or the structure or activity of existing protease, (ii) cause suppression of protease production, and/or (iii) give reduced growth of protease producing biomass as was reported by González et al. [20].

Table 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.

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, a 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 [25] and the use of (partly) already hydrolyzed proteins [16,24] (Supplement Information). 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 [25] 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 [10] 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. [40]. 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). 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. A 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. 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 a longer SRTs.

CRediT authorship contribution statement

Thu Hang Duong: Conceptualization, Methodology, Investigation, Resources, Formal analysis, Writing – original draft, Writing – review & editing. **Miriam van Eekert:** Conceptualization, Supervision, Formal analysis, Writing – original draft, Writing – review & editing. **Katja Grolle:** Methodology, Formal analysis, Writing – original draft. **Nga Tran Thi Viet:** Methodology, Supervision, Writing – original draft. **Grietje Zeeman:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Hardy Temmink:** Conceptualization, Funding acquisition, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors confirm that there are no known conflicts of interest associated with this publication and the financial support for this work has no influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2021.102398.

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