Influence of hydraulic retention time on the psychrophilic hydrolysis/acidogenesis of proteins

Paola Poirrier, María Cristina Schiappacasse, Marta Carballa and Juan M. Lema

ABSTRACT

The influence of the hydraulic retention time (HRT) on the anaerobic hydrolysis of complex substrates has been studied under psychrophilic conditions. For this purpose, a continuous stirred tank reactor was operated at 15 °C and neutral pH and gelatin was considered as a model protein. Three HRTs have been tested: 12, 21 and 36 h. Gelatin hydrolysis was greatly dependent on HRT, increasing from 40% at 12 h-HRT to a maximum of 65% at 36 h-HRT. Molecular size distribution analyses of the effluent showed that hydrolysis of compounds larger than 10 kDa was poor at 12 h-HRT, whereas the fraction of 1–10 kDa was completely transformed into compounds smaller than 1 kDa. Higher HRT (36 h) improved the degradation of the recalcitrant fraction (> 10 kDa), obtaining an effluent with around 95% of soluble molecules (< 1 kDa). In that way, the use of membrane bioreactors for the treatment of this type of macromolecules could improve the degradation efficiencies by enabling to increase the residence time of the non-hydrolyzed molecules, with what would be possible to achieve higher organic loading rate operation.

Key words | anaerobic process, hydrolysis-acidogenesis, particulate size, protein, psychrophilic

INTRODUCTION

Wastewaters composition is highly variable and very dependent on their origin. Among the different elements present, proteins are considered as one of the main components. Treating protein-rich wastewaters may lead to operational problems such as foaming or biomass bulking, thus limiting the efficiency and rate of the overall anaerobic treatment. This makes it difficult to apply this technology to certain types of wastewater such as the dairy and meat industry, so in these cases, the use of two-stage systems is recommended (Yu 2015). Moreover, an important fraction of proteins correspond to macromolecules with molecular sizes higher than 1 kDa (Logan & Jiang 1990; Fuchs et al. 2003), which are usually present in colloidal and sedimentable forms (Mahmoud et al. 2005). Although these macromolecules are soluble, they cannot enter directly into the cell, thus requiring the action of extracellular enzymes prior to be metabolized inside the cells. Moreover, particle size distribution affects not only the hydrolysis-acidification kinetics (Ginestet et al. 2002), but also the distribution of the resulting compounds (Confer & Logan 1997).

Under anaerobic conditions, proteinaceous substrates are first hydrolyzed and degraded by extracellular proteolytic enzymes named proteases into peptides and individual amino acids, which are subsequently fermented by acidogens into volatile fatty acids (VFAs), ammonium, carbon dioxide, hydrogen and reduced sulfur. The VFAs are further converted by acetogens into acetate and H₂/CO₂, both of which are lastly converted to methane by methanogens. The initial hydrolysis is the rate-limiting step in protein degradation (Fang & Yu 2002).

Hydrolysis-acidogenesis is a complex process greatly influenced by many factors, such as reactor configuration, hydraulic retention time (HRT), organic substrate composition, organic loading rate (OLR), temperature, pH and nutritional requirements. There are several studies in literature dealing with the anaerobic hydrolysis-acidogenesis of macromolecules (Elmitwalli et al. 2007; Lü et al. 2014), but only few of them are related to the hydrolysis/acidification of proteins, being most of the recent publications referred to methanogenic processes (Massé et al. 2010; Giard et al. 2013; Saady & Massé 2016). No clear results
were found about the influence of temperature on proteins hydrolysis/acidification, since some studies reported higher efficiencies in mesophilic compared to thermophilic conditions (Fang & Chung 1999), while others indicate the opposite (Guerrero et al. 1999; Yu & Fang 2003). Compared to temperature, pH is crucial to the acidogenesis efficiency, with increasing efficiencies at higher pH values up to 6.5, and product distribution (Yu & Fang 2003). HRT is a key parameter since it determines the solids solubilization efficiency and the degree of acidification of the influent. Slight positive influence of HRT ranging between 4 and 24 h was observed on the mesophilic acidification of proteins (Fang & Yu 2002), while the latter decreased with the increase of the influent organic concentration (Fang & Chung 1999; Fang & Yu 2002). Most of these studies were conducted at mesophilic and/or thermophilic temperatures. However, information at psychrophilic conditions on hydrolysis/acidification process is limited. Many wastewater treatments occur in the psychrophilic range (<20°C) and, at these conditions, the physico-chemical properties of wastewaters are significantly different from those at mesophilic range (35–37°C), such as the higher solubility of gases, the greater liquid viscosity, the lower microorganisms’ affinity for substrates (Nedwell 1999) and the slower biological reactions (Lettinga et al. 2003).

The objective of this work was to study the hydrolysis-acidification of proteins in a continuous reactor operated in the psychrophilic range. The influence of the HRT on the process efficiency and on the size distribution of the resulting products has been analyzed. Gelatin, a protein composed of 18 amino acids, with a molecular weight ranging from 20 to 150 kDa and widely used in the food processing industry, was used as model compound.

**Inoculum**

A non-methanogenic biomass adapted to the psychrophilic degradation of carbohydrates (starch) was used to inoculate the reactor (2 g VSS-L\(^{-1}\)). The specific hydrolytic and acidogenic activities of the inoculum determined at 15°C following the methodology proposed by Soto et al. (1993) were 12.8 g starch-g\(^{-1}\) VSS-d\(^{-1}\) and 0.9 g glucose-g\(^{-1}\) VSS-d\(^{-1}\), respectively.

**Proteolytic activity and hydrolysis-acidification kinetics of gelatine**

The proteolytic activity of the inoculum (g gelatin-g\(^{-1}\) VSS-d\(^{-1}\)) was evaluated in batch experiments at 15 and 37°C. For comparative purposes, a methanogenic inoculum coming from an anaerobic reactor of a brewery was also evaluated. Similarly, the proteolytic activity of the biomass adapted to gelatin hydrolysis (i.e. after the continuous experiment) was evaluated at 15°C. Furthermore, using the same batch experiments, it was determined the hydrolysis-acidification kinetics of gelatine for the inoculum.

The characteristics of the medium of these experiments were identical as those previously described for the synthetic wastewater (gelatin as sole carbon and nitrogen source + macro- and micro-nutrients + bicarbonate). The concentrations of gelatin, ammonium and VFA were monitored over time and the experiments were stopped when a stable minimum level of gelatin was reached.

**Continuous experiments**

A 2 L (H/D 1.5) continuous stirred upflow reactor (45 rpm) with recirculation (recycle:feed ratio of 14:1) was used (see Figure 1). The reactor was operated at 15°C by the recirculation of cold water through the external jacket of the
reactor. The feeding was stored at 4 °C and pumped to the reactor by means of a peristaltic pump (Masterflex, 77200-52). Three parameters were measured online: temperature (Desin Instruments, SR-RZH), pH (Desin Instruments, EPH-M12-FLAT) and stirring speed.

After a start up period of 90 days, the reactor was operated at a HRT of 12 h, 21 h and 36 h, corresponding to OLRs of 7, 4 and 2.3 kg COD·m⁻³·d⁻¹, respectively. Apart from the online measurements, the operation was monitored in terms of protein and ammonium concentrations, solids, total chemical oxygen demand (COD), and VFA twice or three times per week.

**Molecular size distribution**

The molecular size distribution of the resulting products was determined by using an AMICON ultrafiltration unit (Millipore) with regenerated cellulose membranes (Ym series, diameter: 25 mm) of 1 and 10 kDa. The fractioning method is based on a COD balance as described in Figure 2.

**Analytical techniques**

Protein and ammonium concentrations were measured by the Biuret method and the phenol-hypochlorite reaction respectively. VFAs were determined by gas chromatography (Hewlett Packard 5890A) with a flame ionization detector and dissolved Kjeldahl nitrogen by means of a Dohrmann total nitrogen analyzer DN-1900. Determinations of COD, total suspended solids (TSS) and volatile suspended solids (VSS) were carried out according to Standard Methods.

**Calculations**

**Protein hydrolysis percentage**

The protein hydrolysis percentage was determined as the difference between the gelatin concentrations in the influent and effluent divided by the gelatin concentrations in the influent (Equation (1)).

Hydrolysis percentage (%) = \[ \frac{\text{influent gelatin} - \text{effluent gelatin}}{\text{influent gelatin}} \times 100 \]  

(1)

**Ammonification percentage**

The ammonification percentage represents the fraction of ammonium nitrogen formed with respect to the dissolved...
Kjeldahl nitrogen in the effluent (Equation (2)).

Ammonification percentage (%)
\[
= \frac{\text{effluent } \text{N-}\text{NH}_4^+ - \text{influent } \text{N-}\text{NH}_4^+}{\text{effluent dissolved Kjeldahl nitrogen}} \times 100 \tag{2}
\]

Acidification percentage

The acidification percentage was determined as the fraction of COD associated to VFAs with respect to the total COD in the feeding, which remained equal to the total COD in the effluent during the whole experiment (Equation (3)).

\[
\text{Acidification percentage} = \frac{\text{COD}_{\text{VFA}}}{\text{COD}_{\text{feeding}}} \times 100 \tag{3}
\]

Solubilization percentage

The solubilization percentage was determined as the fraction of COD associated to the molecules with sizes below 1 kDa with respect to the total COD in the effluent (Equation (4)).

\[
\text{Solubilization percentage} = \frac{\text{COD}_{<1\text{kDa}}}{\text{COD}_{\text{effluent}}} \times 100 \tag{4}
\]

RESULTS AND DISCUSSION

The anaerobic hydrolysis-acidification of gelatin, representative of proteinaceous macromolecules, has been studied at different HRT under psychrophilic conditions (15 °C) and neutral pH (7.2 ± 0.1).

Proteolytic activity of the inoculum and gelatine hydrolysis-acidification kinetics

Figure 3(a) shows the hydrolysis-acidification kinetics of gelatin with the non-methanogenic inoculum at 15 °C. Gelatin was degraded by 85%, approximately, after 160 h with the concomitant production of ammonium (around 30 mg L\(^{-1}\)). In terms of nitrogen balance, gelatin is consumed and ammonium is produced, but the dissolved total Kjeldahl nitrogen (TKN) remained constant during the experiment (data not shown).

As shown in Figure 3(b), the protein hydrolysis percentage at 15 °C non-methanogenic inoculum (85%) was lower than the values obtained at 37 °C (90%), and at this condition, the proteolytic activity was 0.83 g gelatin·g\(^{-1}\) VSS·d\(^{-1}\) at 15 °C and 4.37 g gelatin·g\(^{-1}\) VSS·d\(^{-1}\), respectively. So it is possible to conclude that involved microorganisms are psychrotolerant (McHugh et al. 2006). Also, the proteolytic activity of the non-methanogenic inoculum used was determined and compared with a methanogenic one. When both values are compared at 37 °C, the latter showed higher proteolytic activity values (3.5 and 12 g gelatin·g\(^{-1}\) VSS·d\(^{-1}\), respectively) regardless of the temperature of operation, while the hydrolysis percentages were similar for both inoculums. The latter showed faster hydrolysis rates and higher hydrolysis percentages compared to the non-methanogenic inoculum, regardless of temperature.

Tarlera & Stams (1999) also observed a three-fold increase in the proteolytic activity when methanogenic microorganisms were present. This fact can be explained by the fermentation mechanisms of amino acids. Although Stickland’s mechanism (Ramsay & Pullammanappallil 2001) is the main mechanism involved in amino acids fermentation,
anaerobic oxidation (McInerney 1988) can also be necessary, and in this case, hydrogen-consuming microorganisms are required, which are absent in non-methanogenic biomass.

Most literature studies have been focused on the temperature effect on the overall anaerobic degradation process or methanogenesis, rather than acidogenesis. This work shows that although similar hydrolysis percentages were obtained at 15 and 37 °C, the hydrolysis kinetics were faster at 37 °C, as previously reported (Yu & Fang 2003). The specific gelatin degradation rate obtained in this study at 15 °C (0.94–1.71 g·g⁻¹·VSS·d⁻¹) was higher than the value reported by (Yu & Fang 2003), 0.370 g·g⁻¹·VSS·d⁻¹ at 20 °C and pH 5.5. The different results obtained by several studies could be due to the type of gelatin used as raw material, mainly related to its molecular size.

**Continuous experiments**

During the start up period (90 days), the biomass repeatedly floated and was washed out. Once these problems were overcome and a steady state was attained (effluent with 0.19 ± 0.03 g·VSS⁻¹·L⁻¹), three stages of operation with increasing OLR were performed. A summary of the reactor operation in each stage is shown in Table 1.

The reactor pH remained constant (7.2 ± 0.1) during the whole operation (234 days), and the biomass concentration increased up to 4 g·VSS·L⁻¹ by the end of the experiment. Ammonium production is influenced by the HRT operation, but does not have inhibitory values for the system; attached to the neutral pH, it is not converted to the more toxic form of ammonia.

Figure 4(a) shows the percentages of protein hydrolysis, ammonification and acidification achieved at the different HRTs. It can be observed that the higher the HRT, the higher the protein hydrolysis (38.7% at 12 h-HRT, 51.2% at 21 h-HRT and 64.7% at 36 h-HRT), ammonification (32% at 12 h-HRT, 45.5% at 21 h-HRT and 67.9% at 36 h-HRT) and acidification (32.5% at 12 h-HRT, 34.9% at 21 h-HRT and 60.9% at 36 h-HRT) percentages. In contrast, the VFA composition in the final effluent (Figure 4(b), calculated as a percentage of COD) was not affected by the different operational conditions, with acetic acid as the main component (around 55%), followed by propionic acid (28–36%), butyric acid (6–11%) and i-valeric acid (0–7%). Only at the highest HRT (36 h), the concentrations of propionate and butyrate increased slightly and i-valeric acid was fully metabolized. Moreover, the COD balance (Table 2) shows that, at 12 and 21 HRT, between 91–97% of the initial COD remained in the effluent, thus indicating that methanization did not take place. At 36 HRT the value reached is slightly lower.

The higher hydrolysis, ammonification and acidification percentages achieved at increasing HRTs, are in agreement

![Figure 4](https://iwaponline.com/wst/article-pdf/74/10/2399/456752/wst074102399.pdf)

**Table 1** | Summary of the hydrolytic-acidogenic reactor operation

<table>
<thead>
<tr>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
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<tbody>
<tr>
<td>HRT (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLR (g·COD·L⁻¹·d⁻¹)</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>pH</td>
<td>7.5 ± 0.1</td>
<td>7.03 ± 0.21</td>
</tr>
<tr>
<td>VSS (g·L⁻¹)</td>
<td>4.01 ± 0.63</td>
<td>4.05 ± 0.03</td>
</tr>
<tr>
<td>VFA (mg·L⁻¹)</td>
<td>800 ± 120</td>
<td>820 ± 220</td>
</tr>
<tr>
<td>NH₄⁺-N (mg·L⁻¹)</td>
<td>228 ± 16</td>
<td>340 ± 21</td>
</tr>
</tbody>
</table>

HRT: hydraulic retention time; OLR: organic loading rate; VSS: volatile suspended solids; VFA: volatile fatty acids.
with previously reported data in mesophilic and thermophilic conditions (Breure & van Andel 1984; Fang & Yu 2002; Yu & Fang 2003). Yet, these hydrolysis percentages were lower than those reported at mesophilic (78% at 4 h-HRT, 41% at 6 h-HRT, 77% at 24 h-HRT) and thermophilic (61% at 6 h-HRT) conditions (Breure & van Andel 1984; Guerrero et al. 1999). This influence of the HRT on protein hydrolysis can be explained by the fact that hydrolysis-acidogenesis is conducted by extracellular enzymes secreted by the microorganisms, and consequently, there should be a compromise between the enzymes production rate and the dilution rate, i.e. it is important to maintain an adequate biomass concentration in the reactor.

The acidification percentage did not change significantly between 12 h-HRT and 21 h-HRT; however, it almost doubled at 36 h-HRT. These results are in accordance with Maharaj & Elefsiniotis (2001), who observed the maximum VFA:COD ratio (acidification degree) at the highest HRT of 60 h. Comparing the hydrolysis and the acidification percentages, the former were greater than the latter at low HRT, while no significant differences were observed at 36 h-HRT. This fact indicates that larger HRTs facilitate not only the hydrolysis of proteins, but also the fermentation of amino acids to VFA. As ammonia levels achieved, these increase with operating HRT, as well as the percentage of ammonification, however are not inhibitory for biomass (ammonia-N between 228 and 380 mg/L) (Breure & van Andel 1984) in any case be higher than those obtained by Maharaj & Elefsiniotis (2001) when they are working with municipal an industrial wastewater between 20 h-HRT and 60 h-HRT and between 8 °C and 25 °C. The free ammonia concentrations remained below due to the ‘positive’ effect of temperature on the dissociation equilibrium. As long as the free ammonia concentration is kept below 0.8 g-N·L⁻¹, the process is running relatively undisturbed. However, when the free ammonia concentrations increase to over 0.8 g-N·L⁻¹, the process is inhibited (Angelidaki et al. 1999).

In contrast, the VFA composition (based on COD percentages) in the effluent was only slightly affected by the HRT (Breure & van Andel 1984). Acetic acid represented the major component, similar to other studies (Breure & van Andel 1984; Maharaj & Elefsiniotis 2001), followed by propionic acid, butyric acid and i-valeric acid. Fang & Yu (2002) observed also acetic acid as a major component, but at lower percentages (20–30%). The fraction of acetic acid was the same at all HRTs tested, while propionic and butyric acids increased and i-valeric disappeared at higher HRT. Fang & Yu (2002) propionic decreased and butyric increased at higher HRT. These results can be explained if taking into account the amino acids composition in the gelatin and the metabolism reactions described by Ramsay & Pullammanappillil (2001). Moreover, the molar acetate:propionate:butyrate:valerate:ammonia ratio fitted quite well with the model described by Angelidaki et al. (1999) for the acidogenesis of gelatin (Table 3), except for propionate and ammonia.

The largest proportion of ammonia present in the effluent may be related to the low growth of biomass obtained.

In Figure 5, gelatin hydrolyses rate and VGA production rate are presented and, it is observed that in both cases the rates are increased by increasing HRT operation.

In the first case, this may be linked to increased proteolytic activity as will be seen later, obtained at the end of reactor operation, after about 240 days of operation. The results may indicate adaptation of biomass to the substrate. In the case of VFA production rate the opposite effect is

<table>
<thead>
<tr>
<th>Table 2</th>
<th>COD balance at different HRT, for the process of anaerobic hydrolysis/acidogenesis of gelatine in psychrophilic conditions</th>
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</thead>
<tbody>
<tr>
<td>Feeding</td>
<td>COD (g l⁻¹)</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>Recovery %</td>
<td>3.5</td>
</tr>
<tr>
<td>COD (g l⁻¹)</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 5 | Gelatin hydrolysis rate and VFA production rate. [□] HRT 12 h; [●] HRT 21 h; [■] HRT 36 h.
obtained when reported by Fang & Yu (2002) and Maharaj & Elefsiniotis (2001), although the values obtained in this study are similar to those reported for the first, on the contrary they are significantly higher than those of Maharaj & Elefsiniotis (2001). Gavala & Lyberatos (2001) suggested that acidogenesis of the produced amino acids was the rate-limiting step of the overall metabolism of gelatin to fatty acids; this effect was not observed in this work, to obtain increases in both rates depending on the HRT operation. By contrast, in this work, the results indicate that hydrolysis would be the limiting step in the degradation of gelatin.

Molecular size distribution

Molecular size distribution analyses of the effluent obtained during each stage of operation constitutes an efficient tool to determine the influence of HRT on the hydrolysis of high molecular weight compounds. Figure 6 shows the molecular size fractioning of the feeding and the effluent from the reactor at each HRT.

It can be observed that about 40% of the feeding COD corresponded to molecules greater than 10 kDa and a similar fraction was obtained for molecules smaller than 1 kDa. During the first stage of operation (HRT of 12 h), only the intermediate fraction (1–10 kDa) was hydrolyzed, while the greater fraction (>10 kDa) remained unaltered. The increase of the HRT resulted in a decrease of the recalcitrant fraction, with a highly solubilized effluent (94% of COD < 1 kDa and 5% of COD >10 kDa) being achieved at 36 h-HRT.

The high solubilization obtained in this work is probably due to the low bloom value (80–100) of the gelatin used. The bloom value is a measurement of strength and gelatinous degree, so the higher the bloom value, the higher the molecular weight. Common bloom values for gelatin range between 50 and 250, corresponding the bloom range of 80–100 to molecular weights of around 20–25 kDa.

Analyzing the articles published to date, no information has been found with a similar study in order to compare the results obtained in this work. The fact that the fraction >10 kDa is the most difficult to degrade reinforces the idea that the hydrolysis step was limiting in the degradation of gelatin.

Proteolytic activity of the adapted biomass

At the end of reactor operation, the proteolytic activity of the adapted biomass to gelatin hydrolysis-acidification was determined at 15°C in order to evaluate the acclimation degree of the biomass to proteins’ degradation. The results obtained (1.83 g gelatin·g⁻¹ VSS·d⁻¹) indicated that during the continuous operation of the reactor, the biomass increased its proteins’ degradation capability at low temperature by 220%, approximately, which represents a sign of the successive adaptation of the microorganisms involved in the degradation process.

CONCLUSIONS

The anaerobic hydrolysis/acidogenesis of gelatin, representative of proteinaceous macromolecules, has been studied at different HRT under psychrophilic conditions (15°C) and neutral pH. The results obtained indicated that gelatine is partially hydrolyzed at 12 h-HRT (40%), increasing this value up to 65% when operating at 36 h-HRT. The molecular size distribution analysis showed that only the fraction between 1 and 10 kDa is hydrolyzed at 12 h-HRT, remaining unaltered the fraction greater than 10 kDa, whereas the effluent obtained at 36 h-HRT contains a 95% of molecules smaller than 1 kDa. Both the ammonification and acidification percentages increased when operating at higher HRT; however, the VFA composition in the final effluent remained constant for the different operational conditions, with acetic acid as the main component (55%).

To conclude, proteins degradation efficiency is strongly affected by HRT at psychrophilic temperature and, thus, there should be a compromise between the production rate of the extracellular enzymes required for the degradation, which is directly related to the biomass concentration in the system, and the process dilution rate. In that way, the use of membrane bioreactors for the treatment of this type of macromolecules could improve the
degradation efficiencies by enabling to increase the residence time of the non-hydrolyzed molecules, with what would be possible to achieve higher OLR operation.

ACKNOWLEDGEMENTS

This work was supported by the FONDECYT Project No. 11080245 and by a postdoctoral contract from the Xunta de Galicia (Isidro Parga Pondal program, IPP-08-37).

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First received 17 June 2016; accepted in revised form 23 August 2016. Available online 6 September 2016.