Anaerobic degradation kinetics of a cholesteryl ester

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Abstract The most important components of wool scouring effluent grease are esters of sterols. Cholesteryl palmitate (CP) is the main ester in this grease. In this paper, the influence of the ester concentration in the anaerobic digestion and the relative rate of the different degradation steps, are studied. The experiment was carried out to measure methane production in the anaerobic degradation of acetate, palmitic acid (PA) and CP. A first-order kinetic model was assumed for hydrolysis and Monod models were assumed for both the methanogenic and acetogenic steps. Maximum hydrolysis rate was found to be around 20 times faster than the maximum methanogenic reaction rate during the experience. The lanolin emulsion drop size effect was also evaluated employing fine and coarse stock lanolin emulsions and no adapted sludge. Concentrations of 13.7 to 4.6 gCOD.l⁻¹ were employed. In a previous study, the effect of palmitic acid emulsion size was found important when similar sludge was tested. When esters are degraded, a significant effect of drop size on the degradation rate was not found. The difference between CP and PA emulsions behavior could be due to the fact that cholesterol produced during the ester degradation has a protective effect on the sludge.

Keywords Anaerobic degradation; cholesterol ester emulsion; kinetics; wool grease

Introduction
Emulsified wool grease is the most important component of wool scouring effluent. Wool grease is a complex mixture composed mainly of sterol esters of saturated long chain fatty acids (LCFA). To improve the yield of the anaerobic treatment, the anaerobic degradation of wool grease has been studied. Cholesteryl palmitate is the most representative wool grease ester, and it is used in this study as a model substrate. Previous results showed that in anaerobic conditions the fatty acid fraction of the esters is degraded but sterols remain adsorbed in the sludge without degradation (Gutiérrez et al., 1999; Peláez et al., 2001).

Some questions emerged from these results. One of them was whether the adsorbed sterols could affect the substrate mass transfer and LCFA degradation rate. A second question was whether the LCFA resulting from the hydrolysis process could produce inhibition, and whether the inhibition depends on the emulsion drop size. Anaerobic degradation results employing palmitic acid (PA) as substrate and anaerobic sludge without adsorbed sterols (non-adapted sludge), showed that 0.8 g.l⁻¹ of a PA fine emulsion was lethal for sludge, while the same concentration of a coarse emulsion was totally methanized (Gutiérrez and Viñas, 2001). Results presented in that paper also show that adsorbed sterols in the sludge avoid the inhibition caused by PA even when a fine emulsion was employed and that the fermentation rate and methane production was not reduced by the presence of such sterols.

The aim of this paper are 1: to set up a first, simplified model of CP degradation in order to compare the relative rates of different steps, and 2: to determine whether sterol ester degradation could depend on the emulsion size, as in the case of emulsions prepared with PA (Gutiérrez and Viñas, 2001).

Cholesteryl palmitate and lanolin were employed in order to carry out these experiments. Lanolin is purified wool grease, and its main components are sterol esters, CP is the most abundant of them.
Wool grease or sterol ester degradation kinetic studies were not found in the bibliography. In studies related with the kinetics of neutral grease degradation, experiences have been reported with triacylglycerides. Hanaki and Nagase (1981) and Petruy and Lettinga (1997) carried out studies with neutral fat of the whole milk. Sanders (2001) studied the hydrolysis rate of tripalmitin and other neutral lipids. In these studies the author evaluated the effect of the enzymatic activity and of physical aspects. The first order hydrolysis constant obtained was 0.030 to 0.040 d⁻¹ for tripalmitin in batch experiments. Masse et al. (2001) studied the effect of adding enzymes on the degradation rate of pork grease particles. The author found a first-order hydrolysis constant of 0.5 to 0.63 d⁻¹ when 2 g.l⁻¹ of pork grease was employed in a sequential batch reactor.

Concerning the β-oxidation of LCFA resulting from the ester hydrolysis, Novak and Carlson (1970) studied the degradation of oleic acid and Gutiérrez and Viñas (2001) the degradation of palmitic acid. In neither of them were shorter LCFA intermediates of β-oxidation reported in the supernatant, although Rinzena et al. (1994), found LCFA intermediates when capric acid was degraded in shock load conditions. Even when β-oxidation is a multi-step process, it is possible to set it up as a global reaction. Assuming that the hydrogen produced during β-oxidation is instantly oxidised by hydrogenotrophic methanogens (Rinzena et al., 1994) cholesteryl palmitate anaerobic degradation can be described as:

**a. Hydrolysis:**

![Cholesteryl palmitate degradation](image)

**b. β-oxidation and hydrogenotrophic methanogenesis**

$$
\begin{align*}
7 \text{HCO}_3^- + 7 \frac{1}{2} \text{H}_2\text{O} &\rightarrow 8 \text{CH}_4 + 7 \frac{1}{2} \text{CH}_4 \\
&\text{or} \\
8 \left( \frac{1}{2} \text{H}_2\text{O} \right) &\rightarrow k_m K_v \left( \text{CH}_4 + \text{HCO}_3^- + \text{H}^+ \right)
\end{align*}
$$

**c. Acetotrophic methanogenesis:**

The hydrolysis step is most commonly described by a first order relation, although more complicated and complete models have been stated, taking into account the fact that the hydrolysis mechanism is surface related (Sanders, 2001). Assuming a first-order kinetic model, Eq. (1) describes the degradation rate of cholesteryl palmitate:

$$
\frac{dC_{\text{CP}}}{dt} = - k_H C_{\text{CP}}
$$

(1)

with: $C_{\text{CP}}$: cholesteryl palmitate concentration (mol.L⁻¹), $t$: time (h) and $k_H$ (h⁻¹): the first-order hydrolysis constant. A Monod-kinetic expression can be used to describe the step corresponding to β-oxidation and hydrogenotrophic methanogenesis, and Eq. (2) describes the palmitic acid kinetic expression:

$$
\frac{dC_{\text{PA}}}{dt} = k_H C_{\text{CP}} - \frac{k_\beta X C_{\text{PA}}}{K_{\alpha\beta} + C_{\text{PA}}}
$$

(2)
with: $C_{PA}$ (mol.l$^{-1}$): actual palmitic acid concentration, $X$: biomass concentration (BM) (gBM.l$^{-1}$), $k_\beta$ (mol.gBM$^{-1}$.h$^{-1}$): maximum consumption rate of PA and $K_s$ (mol.l$^{-1}$): half-saturation constant for $\beta$-oxidation global reaction. Acetotrophic methanogenesis is also described with a Monod model. For acetate (AA) concentration Eq. (3) shows the variation in acetate concentration during CP degradation:

$$\frac{dC_{AA}}{dt} = \frac{8}{2} \frac{k_\beta X C_{AP}}{K_s + C_{AP}} - \frac{8}{2} \frac{k_m X C_{AA}}{K_s + C_{AA}}$$

with: $C_{AA}$ (mol.l$^{-1}$): actual acetate concentration, $k_m$ (mol.gBM$^{-1}$.h$^{-1}$) maximum specific acetate consumption rate, $K_s$ (mol.l$^{-1}$): half saturation constant for acetotrophic methanogenesis. As methane is produced from the b and c reactions, the methane generation rate: $1/V . d N_{met}/dt$, (mol.h$^{-1}$.l$^{-1}$) is:

$$\frac{1}{V} \frac{d N_{met}}{dt} = \frac{7}{2} \frac{k_\beta X C_{AP}}{K_s + C_{AP}} + \frac{8}{2} \frac{k_m X C_{AA}}{K_s + C_{AA}}$$

with: $N_{met}$: accumulated methane moles and $V$(l): liquid volume of the reaction vessel.

When employing PA as substrate, the corresponding global reactions are b and c. In this case, palmitic acid consumption rate is, instead Eq. (2):

$$\frac{dC_{PA}}{dt} = - \frac{k_\beta X C_{PA}}{K_s + C_{PA}}$$

and $C_{AA}$ and $N_{met}$ variations are described by the expressions (3) and (4).

When employing acetate as substrate, only reaction c takes place, and methane production can be described by equation (6). From the accumulated methane production, $k_m$ and $K_s$ can be obtained employing acetate as substrate by means of adjusting parameters in Eq. (6), with $C_{AAA}$, initial acetate concentration (mol.l$^{-1}$):

$$\frac{1}{V} \frac{d N_{met}}{dt} = \frac{k_m X (C_{AAA} - \frac{N_{met}}{V})}{K_s + (C_{AAA} - \frac{N_{met}}{V})}$$

with $k_m$ and $K_s$ and the methane production of PA , values of $k_\beta$ and $K_s$ can be obtained adjusting parameters of equations (3), (4) and (5), by means of numerical integration of AA and PA concentrations. Finally, with CP as substrate, and with $k_\beta$, $K_s$, $k_m$ and $K_s$, the hydrolysis constant $k_H$ is found as a result of adjusting the parameters of Eqs (2), (3) and (4).

**Methods**

**Experiments with different CPC initial concentration**

To evaluate the methane production rate from different concentrations of cholesteryl palmitate, stock coarse emulsion of 9.7 g.l$^{-1}$ was employed. Nonyl Phenol Ethoxylated (NPE) was used as emulsifying agent. Initial CP concentrations of 2, 4, 6 and 8 g.l$^{-1}$ were obtained by diluting the stock emulsion with detergent solution, so that NPE concentration was always 49.5 mg l$^{-1}$. In 150 ml vials (100 ml of liquid volume) inoculated with 5.0 g BM.l$^{-1}$ of sludge from a wool scouring anaerobic reactor, methane production was measured in duplicate. Control vials with 5.0 g BM l$^{-1}$ and with 5.0 BM l$^{-1}$ and 49.5 mg l$^{-1}$ of NPE were performed, and methane production from control detergent vials was discounted for calculation purposes.

Around 70% of volatile solids from the wool scouring anaerobic sludge consists of adsorbed sterols. For this reason, volatile, fixed solids and grease content were measured to
estimate a more realistic biomass (%BM) content. Assuming a 15% of grease content of a bacterial cell on a dry basis (Brock et al., 1987), the biomass in the sludge was estimated according to Eq. (7). % VS is the volatile solids percentage in the sludge and % G is the grease percentage in the same sludge:

\[
%BM = (\%SV - \%G) \times 1.15
\]

Comparing relative degradation rates
Reactors of 485 ml were inoculated with 5.0 g BM l⁻¹ of the same sludge as in the previous experiments. Acetate, palmitic acid emulsion and cholesteryl palmitate emulsion were employed as substrates to carry out anaerobic degradation experiments. The concentrations employed were \( C_{PC} \): 3.2 mmoles.l⁻¹ (2.0 g l⁻¹), \( C_{AP} \): 3.2 mmoles.l⁻¹ (0.82 g.l⁻¹) and \( C_{AA} \): 25.7 mmoles.l⁻¹ (1.54 g.l⁻¹), corresponding to a molar relation of \( C_{PC}:C_{AP}:C_{AA}=1:1:8 \). Both PC and PA emulsions were prepared at the same temperature, agitation and NPE conditions. Final NPE concentration in the test reactors was 49.5 mg.l⁻¹. A control vial with 5.0 g BM l⁻¹ and another with 5.0 BM l⁻¹ and 49.5 mgl⁻¹ of NPE were performed, and the methane production from detergent control vial was subtracted for calculation purposes.

Lanolin drop size distribution effect
Two lanolin (purified wool grease) stock emulsions of 13.7 g/l of COD were prepared, a coarse emulsion (CE) and a fine emulsion (FE). The emulsions were primarily characterised by filtration with Whatman filters 0.25–2.5 µ (Figure 1). The tested sludge was a flocculent sludge from an anaerobic lagoon treating wastewater of a yeast process, without adsorbed sterols (non-adapted sludge), as in Gutiérrez and Viñas, (2001).

Diluting these emulsions, 3 lanolin concentrations (13.7, 6.9 and 4.6 g COD l⁻¹) in coarse and fine emulsion (CE), (FE) were tested. 150 ml vials were inocculated with a sludge concentration of 5.8 g VS l⁻¹, with specific acetate methanogenic activity of 0.1 g COD gVS⁻¹.d⁻¹. The NPE final concentration was always 60 mg l⁻¹ Methane production was measured. Control vials with and without detergent were performed, and methane production from control vial detergent was discounted. All assays were made in duplicate.

Analytical methods
COD, total, volatile and fixed suspended solids, were determined according to Standard Methods for the Examination of Water and Wastewater (1995). Biogas production was daily measured with a differential pressure transmitter. Methane and CO₂ content in biogas was determined by GLC/TCD analysis. Grease extraction was performed with chloroform-methanol, as described in Christie, 1976. Ultra Turrax T-25 basic S25N-25G, (IKA Works, INC, USA) was employed to emulsify.

Figure 1 Drop size distribution of lanolin emulsions
Results and discussion

Experiments with different CPC initial concentration

Table 1 indicates the maximum methane production rate. It was calculated as the maximum slope of the methane production curves at different initial concentrations of cholesteryl palmitate.

From these results a substrate inhibition can be established when cholesteryl ester concentration is high. Substrate was lethal for sludge at 8.0 g.l\(^{-1}\) in this batch experience, although adapted sludge was tested. Hanaki and Nagase (1981) observed that the fatty acids produced by the hydrolysis of neutral fat (4.0 g.l\(^{-1}\) of whole milk) inhibited both β-oxidation and methane production, although neutral fat itself was not inhibitory. Also in Gutiérrez and Viñas (2001) when sterols were adsorbed in the sludge, 0.8 g.l\(^{-1}\) of palmitic acid (2 g.l\(^{-1}\) of \(C_{\text{CP}}\)) was methanized without inhibition. From these results, a diluted concentration of 2 g.l\(^{-1}\) of \(C_{\text{CP}}\) was selected to set up a first, simplified model of CP degradation in order to compare the relative rate of the different steps.

Comparing relative degradation rates

The parameters were adjusted by non linear least square employing Newton’s method. The experimental curves and models are shown in Figures 2, 3, and 4. Table 2 gives the global constants obtained with this model.

Maximum methane production rate obtained in these reactors fed with CP is 0.47 mmoles.gBM\(^{-1}\).d\(^{-1}\) (0.0304 g COD g BM\(^{-1}\).d\(^{-1}\)) (Figure 4), is comparable to the rate obtained with this concentration (2.0 g.l\(^{-1}\) in vials in Table 1.

The relative rate of the 3 steps: a, b, and c varies with actual intermediate (\(C_{\text{CP}}, C_{\text{PA}}, C_{\text{AA}}\)) concentration. In batch experiments with CP, maximum methanogenic rate (step c) appeared to be the slowest, and maximum hydrolysis rate was 20 times faster than maximum methanogenic rate.

\(k_\beta, K_s\beta, k_m\) and \(K_s\) values obtained from this simplified model are not applicable to other concentrations. Application is necessary to a broader model to mathematically incorporate inhibition effects and emulsion superficial characteristics.

Table 1 Maximum methane production rate

<table>
<thead>
<tr>
<th>Initial concentration ((C_{\text{CP}})) (g.l(^{-1}))</th>
<th>((g \text{ COD.gBM}^{-1}.\text{d}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.0298</td>
</tr>
<tr>
<td>4.0</td>
<td>0.0203</td>
</tr>
<tr>
<td>6.0</td>
<td>0.0187</td>
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<td>8.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2 Kinetic constants

<table>
<thead>
<tr>
<th>(k_\alpha) (d(^{-1}))</th>
<th>(k_{\beta}) (gCOD.gBM(^{-1}).d(^{-1}))</th>
<th>(K_s\beta) (mg COD.l(^{-1}))</th>
<th>(k_m) (gCOD.gBM(^{-1}).d(^{-1}))</th>
<th>(K_s) (mgCOD.l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07–0.12</td>
<td>0.08–0.12</td>
<td>7,500–9,000</td>
<td>0.08–0.13</td>
<td>1,700–2,800</td>
</tr>
</tbody>
</table>

Figure 2 Methane production from acetate

Figure 3 Methane production from PA

Figure 4 Methane production from PC
Comparing hydrolysis constant. From a laboratory anaerobic baffled reactor treating woolscouring effluent results described in Gutiérrez et al. (1999), the hydrolysis constant was determined by combining equation 1 with the reactor flow model. Measured neutral grease concentration was assimilated to CP. The flow model was determined with lithium chloride (unpublished data). The reactor has 48 l of liquid volume, and the model proved to be 5 CSRT in series with 20 l of death volume. A period of 2 months operation in which mean organic load, grease concentration and the biomass (BM) concentrations were 3.6 kg COD m$^{-3}$d$^{-1}$, 5 g.l$^{-1}$ and 4.7 g/l respectively, was employed. The experimental results of neutral grease (assimilated as PC) were adjusted by non linear least squares. The resulting constant was $0.15^{+0.07} d^{-1}$, which is in agreement with batch results.

Comparing the $\beta$-oxidation constants. In Gutiérrez and Viñas (2001) assays with palmitic acid (0.8 g.l$^{-1}$) and acetate (1.5, 2.2 and 2.9 g/l) as substrates and 4.3 g BM.l$^{-1}$ of sludge with sterols (to simulate woolscouring sludge) were made. The degradation was monitored by means of methane production and PA consumption. With this experimental data, the model (Eqs (3), (4) and (5)) was adjusted. The obtained $\beta$-oxidation constants were: $k_\beta = 0.076^{+0.081} g \text{COD g BM}^{-1} d^{-1}$, and $K_{s\beta} = 230^{+250} g \text{COD. l}^{-1}$. In this case $k_\beta$ is similar to that in the present batch assay. The affinity constant shows that the prepared sludge with adsorbed sterols (Gutiérrez and Viñas, 2001) has greater affinity than the sludge from woolscouring treatment reactor.

Lanolin drop size distribution effect

Maximum methane production rates of fine and coarse lanolin emulsions are shown in Figure 5. The experimental methane production data were adjusted by non linear least squares. From these experimental results it can be concluded that drop size is not a significant variable for lanolin emulsions of 4.6 to 13.7 gCOD.l$^{-1}$. For corresponding palmitic acid concentrations with similar non-adapted sludge, the FE completely inhibited biomass, but with sludge with adsorbed sterols, inhibition was not produced (Gutiérrez and Viñas, 2001). This different behaviour of palmitic acid and sterol ester can be attributed to a protective effect exerted by sterols resulting from the hydrolysis of PC. The model predicts that the emulsion size may be a significant variable for higher ester concentrations. For diluted emulsions, drop size has less influence and higher activities were found for fine emulsions.

Conclusions

In this paper, cholesteryl palmitate was employed as a model substrate of wool grease. From the evaluation of the methanization rate with different initial PC concentrations, substrate inhibition was observed at high concentrations. 8.0 g l$^{-1}$ appears to be lethal for sludge in this batch experience.

A diluted concentration of 2 g.l$^{-1}$ of cholesteryl palmitate was chosen to set up a first, simplified model of CP anaerobic degradation, in order to compare the relative rate of different steps. A first order kinetics model was adjusted for the hydrolysis step and the
Monod model was used for both methanogenic and β-oxidation steps. Resulting hydrolysis constants were 0.07–0.12 d⁻¹, which is in agreement with hydrolysis constant determined from neutral grease removal in a laboratory continuous anaerobic reactor treating woolscouring effluent.

The relative rate of the 3 steps: a, b, and c varies with actual intermediate (C_{CP}, C_{PA}, C_{AA}) concentrations. In this batch experiment with CP, maximum methanogenic rate (step c) was found the slowest, and maximum hydrolysis rate was 20 times faster than maximum methanogenic rate. k_β, K_{β}, k_m and K_s values obtained from this simplified model are not applicable to other concentrations. For a broader model application it is necessary to mathematically incorporate an inhibition effect and superficial characteristics of the substrate.

The effect of emulsion size on non-adapted sludge (sludge without adsorbed sterols) was evaluated, using fine and coarse lanolin emulsions. Drop size was not found as a significant variable for lanolin emulsions of 4.6 to 13.7 gCOD.L⁻¹. For corresponding palmitic acid concentrations with similar non-adapted sludge, the FE completely inhibited biomass, but with sludge with adsorbed sterols, inhibition was not produced (Gutiérrez and Viñas, 2001). This different behaviour of palmitic acid and sterol ester can be attributed to a protective effect exerted by sterols resulting from ester hydrolysis.

References