Influence of multiple substrates on anaerobic protein degradation in a packed-bed bioreactor

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Abstract Data on the influence of substrate composition on the anaerobic degradation of bovine serum albumin (BSA) in a bench-scale packed-bed reactor are presented and discussed from the standpoint of substrate consumption kinetics. The experiments were carried out in a horizontal-flow anaerobic immobilized biomass (HAIB) reactor fed with BSA based substrates. BSA was the sole carbon source in the first one, while the others were composed of BSA, carbohydrates and lipids. In all the experiments, the HAIB reactor was operated at the hydraulic detention time of 4 hours. The reactor’s performance was evaluated based on physicochemical and chromatographic analyses and also on microscopy techniques. A kinetic model of irreversible first-order series-parallel reactions with two intermediate products was proposed, allowing evaluation of the microbial consortium’s affinity with the substrates and the metabolic compounds formed. As the first-order kinetic model adhered quite well to the experimental data, the initial protein degradation rates ($k$) were estimated. The presence of carbohydrates and lipids led the initial protein degradation rate to be reduced. However, the system fed with protein and carbohydrates showed higher process stability.

Keywords Anaerobic degradation; HAIB reactor; kinetic model; metabolic pathways; protein

Introduction
Wastewaters contain a wide variety of organic compounds that are biodegraded simultaneously, but there are relatively few studies about how the use of multiple substrates affects the degradation kinetics of a specific compound, such as protein, in a mixed anaerobic community. Some authors (Torres, 1992; Gadelha, 2000) concluded that proteins were the main compounds responsible for the residual COD in the effluent of anaerobic reactors treating domestic sewage.

Breure et al. (1986) and Martins et al. (1991) also studied the anaerobic degradation of protein molecules, stating that the process efficiency is closely linked with the separation of protein from the other organic compounds present in wastewater, such as carbohydrates and lipids. However, it is known that such a separation process can be very expensive or even impossible to obtain.

A better understanding of biological degradation processes can be achieved by adjusting kinetic models to the experimental data. They permit, for instance, prediction of the volatile fatty acids accumulation in the reactor and are an important tool for designing optimized anaerobic treatment systems. Many studies have been made using Monod kinetics, its particular cases (zero and first order), and Haldane kinetics for volatile fatty acids formation and consumption (Vavilin and Lokshina, 1996). Batstone et al. (2000) developed a structural anaerobic model describing a complex wastewater degradation process that includes protein degradation, in addition to fat and carbohydrate degradation. This model is structured into a system divided into physicochemical and biological reactions. The biological equations are based on Monod kinetics with hydrogen and pH inhibition, while the pathway of protein degradation is assumed to occur via coupled Stickland reactions.
In this context, experiments were performed in a packed-bed reactor and the biochemical reaction kinetics were used as a tool to evaluate the protein anaerobic degradation process. The influence of the carbohydrates and lipids on the protein degradation kinetics was evaluated. In addition, microscopic techniques were used to verify the occurrence of microbial stratification along the reactor’s length.

**Materials and methods**

A 1 m long, 5 cm diameter HAIB reactor (Figure 1) with a total volume of 2 litres was used in this study. Cubic polyurethane foam matrices (5 mm sides) were used as the support for biomass immobilization. The bed porosity was 40%, resulting in a liquid volume of 800 ml and a superficial velocity of 25.2 cm.h⁻¹. The reactor consisted of 9 equidistant sampling ports along its length and a gas collector at its surface. The inoculum was taken from a UASB reactor treating poultry slaughterhouse wastewater. The granules were macerated and immobilized in the matrices, according to the procedure described by Zaiat et al. (1994).

The reactor was operated with a 4 hour hydraulic detention time and subjected to four successive operating conditions using different carbon sources. Initially, the reactor was fed with synthetic wastewater containing only BSA as carbon source (COD ~ 400 mg.l⁻¹).

The second carbon source employed was BSA plus starch and glucose (COD ~ 550 mg.l⁻¹). The third experiment was carried out with synthetic substrate containing all the carbon sources previously used plus lipids (COD ~ 600 mg.l⁻¹), and finally, the reactor was again fed exclusively with BSA, but with the same COD as in the third wastewater combination. A solution of inorganic nutrients was used to nutritionally balance the substrates (Torres, 1992).

The substrate reservoir was maintained at a temperature below 5°C to minimize the occurrence of biochemical reactions outside the reactor and the substrate was heated to 30 ± 1°C before entering the reactor.

The reactor’s performance and stability were evaluated by analyzing the influent and effluent bicarbonate alkalinity and chemical oxygen demand (COD) (Standard Methods for Examination of Water and Wastewater, 1998).

Spatial profiles of protein (Lowry modified by Peterson, 1979) and carbohydrate concentrations (Dubois et al., 1956), and volatile acids concentration by gas chromatography (Moraes et al., 2000) were recorded under each experimental condition to assess the behavior of the protein degradation kinetics. Samples were taken at intermediate sampling ports located at length to diameter positions (L/D) of 2, 4, 6, 8, 10, 12, 14, 16 and 18.

A kinetic model of irreversible first-order series-parallel reactions with two inter-mediated products was proposed based on the spatial profiles. The model was according to the diagram shown in Figure 2, where \( k_1, k_2, k_3, k_4, k_5 \) and \( k_6 \) are the kinetic constants of the process described herein, which were estimated utilizing an optimization algorithm based on generalized reduced gradient technique (Microsoft EXCEL® 2000 – SOLVER). Although the concentration of lipids was not measurable due to its low value (50.1 mg.l⁻¹), the application of the model was unaffected by the lack of these values, enabling the verification of the effect of the presence of lipids on the kinetic parameters.

![Figure 1](https://iwaponline.com/wst/article-pdf/48/6/23/423660/23.pdf)  
**Figure 1** HAIB reactor scheme
In Eqs (1) to (4), which represent the consumption of protein and carbohydrates and the production and consumption of acetic and propionic acid, $D$ is the reactor’s diameter, $L$ is its length, $\varepsilon$ is the bed porosity, $v_s$ is the liquid superficial velocity, $C_{BSA}$ is the concentration of BSA, $C_{BSA_0}$ is the initial concentration of BSA, $C_{CH}$ is the concentration of carbohydrates, $C_{CH_0}$ is the initial concentration of carbohydrates, $C_{acetic}$ is the concentration of acetic acid and $C_{propionic}$ is the concentration of propionic acid. These equations were obtained from mass balances in the HAIB reactor, which was considered as a plug-flow reactor (de Nardi et al., 1999).

\[
C_{BSA} = C_{BSA_0} \exp \left( -\frac{(k_1 + k_3)D}{\varepsilon v_s} \cdot \frac{L}{D} \right) \tag{1}
\]

\[
C_{CH} = C_{CH_0} \exp \left( -\frac{(k_5 + k_6)D}{\varepsilon v_s} \cdot \frac{L}{D} \right) \tag{2}
\]

\[
C_{acetic} = C_{BSA_0} \left( \frac{k_1 + k_3 \cdot k_4}{k_4 - k_1 - k_3} \right) \left( \exp \left( -\frac{(k_1 + k_3)D}{\varepsilon v_s} \cdot \frac{L}{D} \right) - \exp \left( -\frac{k_2 D}{\varepsilon v_s} \cdot \frac{L}{D} \right) \right) + 
\]

\[
+ C_{BSA_0} \frac{k_3 \cdot k_4}{(k_4 - k_1 - k_3)(k_2 - k_3)} \left( \exp \left( -\frac{k_2 D}{\varepsilon v_s} \cdot \frac{L}{D} \right) - \exp \left( -\frac{k_4 D}{\varepsilon v_s} \cdot \frac{L}{D} \right) \right) + 
\]

\[
+ C_{CH_0} \left( \frac{k_5 + k_6}{k_5 - k_6} \right) \left( \exp \left( -\frac{(k_5 + k_6)D}{\varepsilon v_s} \cdot \frac{L}{D} \right) - \exp \left( -\frac{k_2 D}{\varepsilon v_s} \cdot \frac{L}{D} \right) \right) + 
\]

\[
+ C_{CH_0} \frac{k_6 \cdot k_4}{(k_4 - k_5 - k_6)(k_2 - k_6)} \left( \exp \left( -\frac{k_2 D}{\varepsilon v_s} \cdot \frac{L}{D} \right) - \exp \left( -\frac{k_4 D}{\varepsilon v_s} \cdot \frac{L}{D} \right) \right) \tag{3}
\]

\[
C_{propionic} = C_{BSA_0} \frac{k_3}{k_4 - k_1 - k_3} \left( \exp \left( -\frac{(k_1 + k_3)D}{\varepsilon v_s} \cdot \frac{L}{D} \right) - \exp \left( -\frac{k_4 D}{\varepsilon v_s} \cdot \frac{L}{D} \right) \right) + 
\]

\[
+ C_{CH_0} \frac{k_6}{k_4 - k_5 - k_6} \left( \exp \left( -\frac{(k_5 + k_6)D}{\varepsilon v_s} \cdot \frac{L}{D} \right) - \exp \left( -\frac{k_4 D}{\varepsilon v_s} \cdot \frac{L}{D} \right) \right) \tag{4}
\]

Spatial profiles of ammonium-nitrogen concentration (Standard Methods, 1998) were also taken along the HAIB reactor to confirm the protein degradation profiles.

Just after the conclusion of each experimental step, three polyurethane foam matrices containing biomass were collected from each intermediate sampling port and immediately

![Figure 2 Diagram of the kinetic process](https://iwaponline.com/wst/article-pdf/48/6/23/423660/23.pdf)
replaced with clean matrices to maintain the flow pattern. One particle from each port was subjected to the technique described by Nation (1983) and modified by Araújo (1994) for observation in a scanning electronic microscope to verify the biofilm structure and the characteristics. The biomass from the other matrices was detached and analyzed by optical microscopy (phase contrast and fluorescence), allowing for an evaluation of biomass stratification throughout the reactor.

**Results and discussion**

The acclimatization period lasted for 26 days of operation, after which spatial profiles were taken at 10-day intervals. After the acclimatization period, the reactor was considered under steady state regime, displaying stable values of organic matter removal efficiency (~90% as COD) and mean bicarbonate alkalinity production of 75 mg CaCO₃.l⁻¹.

Figure 3 presents the spatial profiles obtained for each experimental condition and the results obtained from the proposed kinetic model (irreversible first-order series-parallel reactions with two intermediate products). The kinetic parameters are shown in Table 1.

The acetoclastic production of methane, represented by the value of \( k_2 \), is in the same order of magnitude under all the operational conditions, though greater in the experiment with carbohydrates. Under this condition, the microbial consortium was probably able to develop a higher affinity with acetate due to the presence of carbohydrates, which provided a better balance of carbon-nitrogen than the conditions 1 and 4 (protein-based substrate).

<table>
<thead>
<tr>
<th>Operating conditions</th>
<th>( k_1 ) (h⁻¹)</th>
<th>( k_2 ) (h⁻¹)</th>
<th>( k_3 ) (h⁻¹)</th>
<th>( k_4 ) (h⁻¹)</th>
<th>( k_5 ) (h⁻¹)</th>
<th>( k_6 ) (h⁻¹)</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.15</td>
<td>0.23</td>
<td>0.03</td>
<td>0.43</td>
<td>–</td>
<td>–</td>
<td>0.986</td>
</tr>
<tr>
<td>BSA + carbohydrates</td>
<td>0.17</td>
<td>1.34</td>
<td>4.16.10⁻⁵</td>
<td>1.19</td>
<td>1.04.10⁻⁵</td>
<td>0.278</td>
<td>0.983</td>
</tr>
<tr>
<td>BSA + carbohydrates + lipids</td>
<td>0.06</td>
<td>0.85</td>
<td>0.05</td>
<td>0.67</td>
<td>0.23</td>
<td>5.91.10⁻³</td>
<td>0.984</td>
</tr>
</tbody>
</table>

**Figure 3**  Spatial profiles of substrate consumption and metabolic production along the HAIB reactor: SAB (●); carbohydrates (♦); acetic acid (○); propionic acid (△) and model (—), A: BSA 400 mgDQO.l⁻¹, B: BSA + carbohydrates, C: BSA + carbohydrates + lipids and D: BSA 600 mgDQO.l⁻¹
The presence of lipids may have been responsible for the lower value of $k_2$ compared to that of condition 2. This issue will be discussed in detail further on.

A comparison of the values of $k_1$ and $k_4$ for all the experimental conditions indicates that propionic acid was the main precursor in the formation of acetic acid.

The analysis of the values of $k_1$ for all conditions reveals that the acetate production deriving from protein degradation was similar in the systems fed solely with protein and in that fed with protein and carbohydrates. However, the $k_1$ value obtained for condition 3 (with lipids) was approximately 65% lower than the values obtained under the other conditions, indicating a possible change in the acetate production pathway. Acetate production via carbohydrates improved after the addition of lipids, as indicated by the $k_5$ values. Therefore, acetate was formed mainly via protein in the second condition (protein plus carbohydrate) and via carbohydrate when lipid was added (condition 3).

Based on the values of $k_3$ and $k_6$ in the second condition (protein plus carbohydrates), it is possible to conclude that propionic acid was produced mainly via carbohydrate, while protein was the main precursor for propionic acid production when lipids were added (condition 3).

The acidogenic process from the propionic acid route, represented by $k_4$, can be related to the stability of the process, since this reaction is the most thermodynamically unfavorable in the anaerobic process. Therefore, comparing the values of $k_4$ in the four conditions, one can conclude that the process is a little more stable in the presence of proteins and carbohydrates, probably due to the aforementioned carbon-nitrogen relation.

Figure 4 presents the ammonium production under each experimental condition, while Table 2 lists the initial protein degradation rates.

The efficiencies of organic matter degradation, calculated from the influent and effluent COD values, were 91% for the first condition (protein – COD ~ 400 mg l$^{-1}$), 93% for the second (protein plus carbohydrates), 76% for the third (protein, carbohydrates and lipids) and 83% for the fourth (protein – COD ~ 600 mg l$^{-1}$). These results indicate that the presence of lipids in wastewater with high protein concentrations made the organic matter degradation efficiency decrease. Although the presence of carbohydrates reduced the

Table 2  Initial protein degradation rates

<table>
<thead>
<tr>
<th>Operating condition</th>
<th>Carbon source</th>
<th>Initial protein degradation rate (mg S.A.B.l$^{-1}$.h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BSA (COD = 400 mg l$^{-1}$)</td>
<td>72.8</td>
</tr>
<tr>
<td>2</td>
<td>BSA + Carbohydrates</td>
<td>66.4</td>
</tr>
<tr>
<td>3</td>
<td>BSA + Carbohydrates + Lipids</td>
<td>44.6</td>
</tr>
<tr>
<td>4</td>
<td>BSA (COD = 600 mg l$^{-1}$)</td>
<td>109.1</td>
</tr>
</tbody>
</table>

Figure 4  Spatial profile along the HAIB reactor – ammonium-nitrogen concentration
initial protein degradation rate, the overall efficiency was similar in both experiments, indicating that the 4-hour hydraulic detention time sufficed to provide suitable organic matter degradation even in the system with carbohydrates. In fact, the similarity of the efficiency rates demonstrates that, under the operating conditions, these organic fractions do not have to be separated to maintain good anaerobic protein degradation. COD removal efficiencies of 87%, 90%, 70% and 79% were observed under the first, second, third and fourth conditions, respectively.

The highest effluent residual fatty acid concentrations were observed when the reactor was fed with substrate whose composition contained lipids. The presence of lipid molecules can retard protein degradation and can also reduce the degradation rate of organic matter. This fact can be ascribed to the low availability of lipids due to their low solubility. Vidal et al. (2000) reported that the presence of lipids in wastewater whose composition included carbohydrates and protein caused the organic matter degradation rate to decrease, preventing the accumulation of fatty acids in batch assays performed in closed glass flasks with a total volume of 500 ml. In contrast, in the present study, the highest concentrations of volatile fatty acids were found when the reactor operated with synthetic substrate containing lipids.

The initial protein degradation rate decreased when another carbon source was added to the synthetic wastewater. Martins et al. (1991) came up with a similar finding in their study of the anaerobic degradation of albumin and casein, as did Breure et al. (1986) in their study of gelatin anaerobic degradation. The lipid-containing system presented the lowest reaction rate.

The ammonium-nitrogen concentrations found in the effluent for the first, second and third operating conditions were very similar. These results were congruent with the protein degradation efficiencies, since the three operating conditions had the same initial protein concentration value. It is worth noting that ammonium production in the presence of lipids occurred at a lower rate than under the other conditions. Because ammonium is a by-product of protein hydrolysis and amino acid consumption, this confirms that the presence of lipids has a negative effect on these processes. The higher ammonium concentrations found in the effluent under the fourth operating condition were attributed to the higher initial protein concentrations.

The gas composition was analyzed throughout the operating phase using gas chromatography techniques. However, methane and carbon dioxide volumes could not be measured due to problems in the configuration of the HAIB reactor, which did not expel a constant amount of biogas. The high percentages of methane gas (up to 72%) found after the start-up period is a reliable indication of the operating stability of the system and the rapid evolution of the anaerobic degradation process.

The SEM analysis revealed that the immobilization patterns were similar to those previously described by Varesche et al. (1997) and Tommaso et al. (2002) found in polyurethane foam matrices taken from a fixed-film reactor treating glucose-based substrate and from a differential reactor treating protein-based substrate, respectively.

The optical microscopy analyses showed several major morphologies, which varied according to the operating conditions and the position along the length of the HAIB reactor. The morphologies predominating throughout the operation time were Methanosaeta-like morphologies, hydrogenothrophic rods, filaments and non-fluorescent rods. Fluorescent cocci, Methanosarcina-like, and non-fluorescent cocci, spirochaete and curved rods were also present.

Figure 5 shows the predominance of microbial morphologies along the HAIB reactor. As can be seen, after 26 days of operation under the first condition, the reactor actually reached the steady-state regime. The figure reveals the predominance of nonfluorescent
rods from the front to the middle of the reactor, related probably to hydrolytic acidogenic microorganisms in association with hydrogenotrophic archaea at the beginning of the reactor. The action of the hydrogenotrophic archaea probably involved the removal of the hydrogen present in the bulk liquid, allowing for the subsequent consumption of propionic acid. After a sufficient quantity of acetate was produced, *Methanosaeta* sp. was found to predominate from the middle to the end of the HAIB reactor.

After the addition of carbohydrates, the *Methanosaeta* sp. were found to reallocate to the beginning of the reactor, a fact that was likely due to the slightly higher acid acetic concentration in this segment (Figure 3-B). Hydrogenotrophic archaea rods were more predominant in the middle of the reactor, while filaments were found to predominate from the middle to the back. A predominance of non-fluorescent cocci, sometimes in chains, was also observed at the back of the reactor.

With the addition of lipids, the *Methanosaeta* sp. predominated again from the middle to the end of the reactor, probably due to the negative effect of this addition at the beginning of the reactor. The predominance of filaments was very evident under this operating condition. A predominance of curved rods resembling *Desulfovibrio* sp., which can ferment this type of substrate in the presence of lipids and the absence of sulfate (Voordouw, 1995), was also found. Lastly, it was found that *Methanosaeta* sp. no longer predominated when carbohydrates and lipids were removed from the bulk liquid (operating

<table>
<thead>
<tr>
<th>Condition</th>
<th>Predominant Morphologies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beginning (sample analyses 1, 2 and 3)</strong></td>
<td>Fluorescent and non-fluorescent rods</td>
</tr>
<tr>
<td><strong>Intermediate (sample analyses 4, 5 and 6)</strong></td>
<td><em>Methanosaeta</em> sp.-like morphology</td>
</tr>
<tr>
<td><strong>End (sample analyses 7, 8 and 9)</strong></td>
<td>Filaments and curved rods</td>
</tr>
</tbody>
</table>

**Figure 5** Predominant morphologies found along the length of the HAIB reactor. (N.B. Scale bars on white background are 10 µm; scale bars on dark background are 2 µm.)
condition 4). Possibly, this kind of microorganism might appear after longer operating times, since the well formed consortium observed at the beginning of the reactor was similar to that found in the first operating condition.

Conclusions
The initial protein degradation rates were negatively affected by the presence of other organic compounds such as carbohydrates and lipids, but the system that was fed with the moisture protein plus carbohydrates showed the higher tendency for stability. The proposed kinetic model of irreversible first-order series-parallel reactions with two intermediate products adhered well to the experimental data. The kinetic parameters obtained permitted us to conclude that the pathway of protein degradation changed when lipid was added to the substrate, seeming to indicate that the acetic acid was mainly formed from propionic acid in such conditions. Biomass stratification along the HAIB reactor was clearly observed and the microorganism morphologies varied for the four operating conditions. Nevertheless, the predominating morphologies throughout the operating time were Methanosaeta-like archaea, hydrogenothophic rods, filaments and non-fluorescent rods.

Acknowledgements
The authors gratefully acknowledge the entire anaerobic process research group for its relevant discussions and helpful cooperation. Thanks are also due to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for its financial support of this research and for the grants awarded to R. Ribeiro and G. Tommaso.

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