Ammonia tolerant enriched methanogenic cultures as bioaugmentation inocula to alleviate ammonia inhibition in continuous anaerobic reactors

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Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Ammonia tolerant enriched methanogenic cultures as bioaugmentation inocula to alleviate ammonia inhibition in continuous anaerobic reactors

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Abstract
Ammonia is the most common inhibitor of anaerobic digestion (AD) process, resulting in suboptimal exploitation of the biogas potential of the feedstocks, causing significant economic losses to the biogas plants. Ammonia is mainly inhibiting the aceticlastic methanogens, while the hydrogenotrophic methanogens are more robust to ammonia toxicity effect. It has been shown that bioaugmentation of a pure strain of a hydrogenotrophic methanogen (i.e. Methanoculleus bourgensis) in an ammonia inhibited continuous anaerobic reactor can improve methane production more than 30%. Nevertheless, cultivation of a pure culture, to be used as bioaugmentation inoculum, poses technical difficulties due to the required sterile conditions and the special growing media. On the contrary acclimatized enrichment methanogenic cultures have lower requirements to sterility. In the present study, we used an enriched ammonia tolerant methanogenic culture as potential bioaugmentation inoculum in a continuous stirred tank reactor (CSTR) operating under “inhibited steady-state”, triggered by high ammonia levels (5 g NH₄⁺-N L⁻¹). The results of the current study established for the first time that bioaugmentation of an enriched ammonia tolerant methanogen in a CSTR reactor could completely alleviate the ammonia inhibitory effect. Furthermore, it was found that bioaugmentation with the enriched culture resulted in 25% higher methane production compared to when the bioaugmentation was achieved with pure methanogenic strains. The bioaugmentation was performed without pausing the continuous operation of the CSTR reactor and without excluding the ammonia-rich substrate from the feedstock. Thus, bioaugmentation with mixed methanogenic cultures could potentially support the development of an efficient and cost-effective biomethanation process of ammonia-rich organic waste in full-scale continuous reactors.

Keywords
Ammonia inhibition; Biogas; Biomethanation; CSTR; Methane.

INTRODUCTION
The agricultural and the food industrial sectors produce vast amounts of ammonia-rich organic wastes. One of the most promising and effective methods for treatment of these wastes is anaerobic digestion (AD) as it provides energy (methane) and a digestate with high nutrient levels (bio-fertilizer). However, ammonia-rich substrates are well known to inhibit AD process and it is estimated that many full-scale biogas reactors worldwide are seriously affected by ammonia toxicity leaving up to 1/3 of their methane potential unutilised (Fotidis, et al., 2013a). It is widely accepted that ammonia is mainly inhibiting the aceticlastic methanogenic pathway, while the syntrophic acetate oxidation pathway followed by hydrogenotrophic methanogenesis is more robust to ammonia toxicity (Westerholm, et al., 2011). It is therefore, logical to assume that use of ammonia tolerant hydrogenotrophic methanogenic consortia could provide a new solution to alleviate ammonia inhibition in AD process. Bioaugmentation has been proposed as a potential method to deliver and establish these ammonia tolerant consortia (Costa, et al., 2012) in continuous anaerobic reactors operating under high ammonia levels. Fotidis, et al. (2014) has shown for the first time that bioaugmentation of an ammonia tolerant methanogen (Methanoculleus bourgensis) in lab-scale continuous reactors, was possible with more than 30% increase in methane production. However,
The aim of the current study was to evaluate, via a complete control system, the complete ammonia oxidation rates of the MEC culture. Three distinct experimental periods were carried out in a continuous stirred tank reactor (CSTR) operating under “inhibited steady-state”, caused by high ammonia levels.

**MATERIAL AND METHODS**

**Methanogenic enriched culture**

The hydrogenotrophic methanogenic enriched culture (MEC) used in the bioaugmentation process derived from a mesophilic manure based full-scale reactor (Hashøj, Denmark). Before introduced to the CSTR reactor, MEC culture was acclimatised in stepwise increasing ammonia concentrations (up to 5 g NH₄⁺-N L⁻¹) in fed-batch reactors with bicarbonate buffered basal media-BM (Zehnder et al., 1980)(data not shown). Furthermore, the methanogenic pathway of the MEC culture at 5 g NH₄⁺-N L⁻¹ was determined using [2-¹⁴C] acetate as demonstrated before (Fotidis et al., 2013b).

**Inoculum and feedstock**

The inoculum used in the CSTR reactors was obtained from a full-scale mesophilic anaerobic reactor (Hashøj Biogas, Denmark). The feedstock used in the experiment was dairy slurry derived from Hashøj municipality (Denmark). The dairy slurry was sieved to remove coarse materials and kept at 4°C before it was introduced to the reactors (Table 1).

**Table 1. Characteristics of the inoculum and the dairy manure used as feedstock.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Inoculum Value ± SD</th>
<th>Feedstock Value ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS (g·L⁻¹)</td>
<td>29.9±0.2</td>
<td>56.1±0.0</td>
</tr>
<tr>
<td>VS (g·L⁻¹)</td>
<td>18.5±0.1</td>
<td>41.7±0.0</td>
</tr>
<tr>
<td>Total Kjeldahl nitrogen (g N·L⁻¹)</td>
<td>4.0±0.2</td>
<td>2.7±0.1</td>
</tr>
<tr>
<td>Ammonia (g NH₄⁺-N·L⁻¹)</td>
<td>3.2±0.2</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>pH</td>
<td>7.88</td>
<td>7.2</td>
</tr>
<tr>
<td>Total VFA (g·L⁻¹)</td>
<td>2.2±0.3</td>
<td>11.0±0.9</td>
</tr>
</tbody>
</table>

**Experimental setup**

The bioaugmentation experiment was carried out in two identical CSTR reactors (R_MEC: MEC culture bioaugmentation and R_Control: abiotic augmentation) with 2.3 L and 1.8 L total and working volume, respectively. Both reactors had organic loading rate (OLR) and hydraulic retention time (HRT) of 1.74 g VS L⁻¹·d⁻¹ and 24 days, respectively. Ammonium chloride was used as additional ammonia source. Each reactor’s setup had a feedstock tank, a feeding peristaltic pump, an effluent tank, two magnetic stirrers, a water-displacement gas meter and an electrical heating jacketed system. Both CSTR reactors started-up with an ammonia level of 1.65 g NH₄⁺-N L⁻¹ and methane production rate, at steady-state, of 457±12 mL CH₄ L⁻¹·d⁻¹. Ammonia levels were stepwise increased in the reactors to 5 g NH₄⁺-N L⁻¹ (data not shown). The complete bioaugmentation experiment was divided to three distinct experimental periods. One HRT after ammonia concentration in the reactors reached 5 g NH₄⁺-N L⁻¹, an induced “inhibited steady-state” was established (Period-I, days 1-12) for both reactors. The bioaugmentation of MEC in the R_MEC reactor took place twice on days 13 and 15 (Period-II) with a total of 200 mL of the MEC culture (OD₆₀₀=0.21-0.23 and μ_max=0.024 h⁻¹, under exponential growth phase) introduced into the reactor. At the same time, 200 mL of sterile BM medium with 5 g NH₄⁺-N L⁻¹, were also introduced in...
reactor $R_{\text{Control}}$, in order to replicate the same hydraulic effect (abiotic augmentation) that the volume of the MEC inoculum had on the $R_{\text{MEC}}$ reactor. Period-III (days 16-57) of the experiment was defined as the period after the bioaugmentation and abiotic augmentation processes took place. Throughout the duration of the experiment, both reactors were operated continuously and the ammonia concentration, the HRT and the OLR were kept stable.

**Analyses and calculations**
Methane content in CSTR reactors were measured with GC-TCD (MGC 82-12, Mikrolab a/s, Denmark). Volatile fatty acids (VFA) were determined using gas-chromatograph (HP 5890 series II) as described previously (Fotidis, et al., 2014). The pH was measured with PHM99 LAB pH meter. The maximum growth rate ($\mu_{\text{max}}$) of the MC culture was calculated as has been described before (Fotidis, et al., 2013a). The optical density at 600 nm (OD$_{600}$) was determined with a Spectronic 20D+ Spectrophotometer (Thermoscientific, Soeborg, Denmark). All the analyses were made in triplicate (n=3) and the averages with the standard deviations (± SD) are presented.

**RESULTS AND DISCUSSION**
During the initial “inhibited steady-state” (Period-I), both reactors were producing approximately 30% less methane, compared to the start-up period (Fig. 1a). Immediately after bioaugmentation (Period-III), the $R_{\text{MEC}}$ reactor demonstrated a significant improvement in methane production rate, which led to a new uninhibited steady-state (days 33-57). In this new steady-state, the $R_{\text{MEC}}$ reactor was operating continuously for more than one HRT with approximately 40% higher methane production rate compared to the initial “inhibited steady-state”. Surprisingly, the $R_{\text{MEC}}$ reactor regained the same methane production rate (~450 mL CH$_4$ L$^{-1}$·d$^{-1}$) it had before the introduction of the additional ammonia to the feedstock alleviating completely the ammonia inhibitory effect. Additionally, a previous study, performed under the same experimental conditions using a pure methanogenic strain *(Methanoculleus bourgensis MS2)$^T$ as bioaugmentation inoculum, demonstrated a 30% improvement of methane production (Fotidis, et al., 2014). The comparison between the two studies indicates that the enriched culture (MEC) alleviated ammonia toxicity more efficiently (by ~25%) than the pure culture (*M. bourgensis*). This finding strengthens the hypothesis that ammonia tolerant enriched cultures could potentially be the appropriate bioaugmentation inocula to alleviate ammonia toxicity in full-scale biogas reactors. Finally, bioaugmentation in $R_{\text{MEC}}$ did not affect the methane production rate of the reactor since the operational parameters were kept stable (e.g. HRT, OLR, temperature etc.). Thus, ammonia-rich feedstock was fed continuously in the reactor retaining the same high ammonia levels (5 g NH$_4^+$-N L$^{-1}$). This technical approach is in contrast to conventional methods (dilution, temperature lowering, etc.), used today to alleviate ammonia toxicity in AD reactors (Nielsen & Angelidaki, 2008).

![Figure 1.](image_url)

On contrary to $R_{\text{MEC}}$, $R_{\text{Control}}$ reactor remained in an ammonia induced “inhibited steady-state” in Period-III, increasing its methane production rate only by 5% compared to Period-I (Fig. 1a). This
statistically significant (p<0.05), but small improvement in methane production efficiency, could be attributed to a slow acclimation of the methanogenic populations to the high ammonia levels (Chen et al., 2008). A direct comparison between $R_{\text{MEC}}$ and $R_{\text{Control}}$ during the final steady-state (days 33-57), $R_{\text{MEC}}$ had an average of more than 36% higher methane production rate.

Finally, VFA accumulation and pH fluctuation after bioaugmentation process (Fig. 1b) of $R_{\text{MEC}}$ reactor, were kept stable and within the normal limits for AD of dairy slurry in CSTR reactors (Fang, et al., 2011). On contrary, VFA accumulation (Fig. 1b) of $R_{\text{Control}}$ reactor verified the ammonia “inhibited steady-state” period (days 33-57) at 5 g NH$_4$-N L$^{-1}$, with the VFA levels higher than the identify threshold (1500 mg HAc L$^{-1}$) for a healthy AD process in lab-scale CSTR reactors (Boe, et al., 2010). Despite the VFA accumulation, pH of the $R_{\text{Control}}$ remained inside acceptable pH range for the AD process due to high buffer capacity of the dairy slurry (Liu, et al., 2008).

CONCLUSIONS
In the current study was established that an enriched ammonia tolerant methanogenic culture was successfully bioaugmented in an ammonia inhibited CSTR reactor and could completely alleviate ammonia toxicity. This new method, is very promising, for development of an efficient and cost-effective biomethanation process of ammonia-rich organic waste in full-scale reactors.

ACKNOWLEDGEMENTS
This work was supported by Energinet.dk under the project framework ForskEL “MicrobStopNH$_3$-Innovative bioaugmentation strategies to tackle ammonia inhibition in anaerobic digestion process” (program no. 2015-12327).

REFERENCES


