Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature

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ABSTRACT

In biogas processes, methane production from acetate proceeds by either aceticlastic methanogenesis or through syntrophic acetate oxidation (SAO). In the present study, the pathway for methane production from acetate was analysed; i) during a gradual increase of the ammonia concentration (final concentration 7 g NH_4^+ – N/L) in a semi-continuous lab-scale anaerobic digester (4.3 L), operating at mesophilic temperature (37°C) or ii) in diluted enrichment cultures (100 ml) experiencing a gradual increase in ammonia, sodium, potassium and propionic acid. The pathway for methane formation was determined by calculating the $^{14}CO_2/^{14}CH_4$ ratio after incubating samples with ^{14}C-2-acetate. In the anaerobic digester, as well as in the enrichment cultures, the $^{14}CO_2/^{14}CH4$ ratio clearly increased with increasing ammonium-nitrogen concentration, i.e. as the ammonia concentration increased, a shift from the aceticlastic mechanism to the syntrophic pathway occurred. The shift was very distinct and occurred as the NH_4^+ – N concentration rose above 3 g/l. No shift in pathway was seen during increasing concentrations of sodium, potassium or propionic acid. The shift to SAO in the biogas digester resulted in a twofold decrease in the specific gas and methane yield.

Key words | ammonia inhibition, anaerobic digestion, ^{14}C-labelling, mesophilic temperature, syntrophic acetate oxidation

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INTRODUCTION

Two different mechanisms for methane formation from acetate are known: In the aceticlastic pathway, used by *Methanosarcinaceae* sp. and *Methanosaetceae* sp., the methyl group of acetate is almost completely converted to methane and the carboxyl group to carbon dioxide (Jetten *et al.* 1992). The second mechanism is a syntrophic pathway, in which acetate is converted to hydrogen and carbon dioxide by one bacterium, followed by the subsequent reduction of carbon dioxide to methane by a hydrogen utilizing methanogen (Zinder & Koch 1984; Schnürer *et al.* 1994). Under methanogenic conditions, only three acetate-oxidizing bacteria have been isolated and described, two thermophilic (Lee & Zinder 1988; Hattori *et al.* 2000) and one mesophilic species (Schnürer *et al.* 1996).

The syntrophic pathway was first demonstrated in anaerobic digesters operating at thermophilic temperatures (Zinder & Koch 1984; Petersen & Ahring 1991; Ahring *et al.* 1993) but was later found also at mesophilic temperatures (Schnürer *et al.* 1994; Schnürer *et al.* 1999; Karakashev *et al.* 2006). Methane formation by syntrophic acetate oxidation has, in addition to biogas processes, also been suggested to occur in lake sediments (Nüsslein *et al.* 2001), oil reservoirs (Nazina *et al.* 2006) and soils (Chauhan & Ogram 2006). Different factors have been shown to have an impact on the degree of syntrophic acetate oxidation versus the aceticlastic mechanism; acetate concentration (Petersen & Ahring 1991; Ahring *et al.* 1993; Karakashev *et al.* 2006), dilution rate (Shigematsu *et al.* 2004) and prevailing methanogenic population (Karakashev *et al.* 2006). Furthermore, methane

doi: 10.2166/wst.2008.097

production through syntrophic acetate oxidation was in a previous study identified in digesters high in ammonia, sodium, potassium and VFA (Schnürer *et al.* 1999)

The aim of this study was to i) differentiate the influence of ammonia from salts (Na^+ and K^+) and propionic acid on the mechanism for methane production from acetate; ii) identify the critical concentration of ammonia at which the syntrophic mechanism becomes the dominating pathway for methane production from acetate. The pathway for methane production from acetate was investigated during a gradual increase of the ammonia concentration in a semi-continuous lab-scale biogas digester operating at mesophilic temperature (37°C) and in diluted enrichment cultures experiencing a gradual increase in ammonia, sodium, potassium or propionic acid. The pathway for methane formation was elucidated by incubating samples with ^{14}C-actetate (Schnürer *et al.* 1999) followed by measurements of produced $^{14}CH_4$ and $^{14}CO_2$.

METHODS

Anaerobic digesters

Two digesters (SAO1 and SAO3; 4.25 L active volume each) were started with inoculum from a 45 L digester operated at the same conditions for 6 years (Schnürer & Schnürer 2006). The two processes were run semi-continuously (feed once a day) at 37°C and at an OLR (organic loading rate) of 3 g VS L^{-1} day^{-1} (g volatile solids per L reactor volume and day) and at a HRT (hydraulic retention time) of 30 days. Both digesters were operated with source-sorted organic fraction of municipal solid waste (SS-OFMSW; Schnürer & Schnürer 2006). Before feeding, the waste was minced, diluted with water and hygienized at 70°C for one hour. The ammonium nitrogen content in SAO3 was increased after 70 days of operation by exchanging some of the municipal waste by egg albumin powder (Källbergs Industries, Sweden). Even though the substrate composition was changed, the influent concentration of VS in the substrate was kept constant at 9% over the whole experimental period. The amount of albumin added was based on an estimated net mineralization of total Kjeldahl nitrogen (TKN) of 50%, resulting in approximately 1.9, 3.3, 5.5 and 6.9 g $NH_4^+ - N$/L after 141, 225, 442 and 642 days of operation, respectively (Table 1).

Analyses

Total gas production, concentrations of methane and carbon dioxide, as well as pH, was determined according to Jarvis *et al.* (1995). TS, VS, and TKN were determined according to APHA's *Standard Methods* (1998). Volatile fatty acids (VFA) were analysed by HPLC (Agilent 1100; Agilent Technologies, Stockholm, Sweden). The acids were separated on an ion exchange column (Rezex-ROA-Organic Acid H^+; Skandinaviska Genetec AB, Västra Frölunda, Sweden) and detected with a refractive index detector. The eluent was 5 mM H_2SO_4 and the flow rate 0.6 ml $minute^{-1}$. The identity and the concentration of the organic acids was determined by comparing the retention times and peak areas of chromatograms with pure external standards of each component.

Cultivation of enrichment cultures

Enrichment cultures were started with the same inoculum used for start-up of the anaerobic digesters. At time zero, 10% (w/v) of inoculum were added during flushing (N_2/CO_2; 80/20) to sterile mineral medium (final volume 350 ml) in 500 ml anaerobic cultivation bottles. After the transfer, the bottles were closed with butyl rubber stoppers and aluminium caps. The medium used were the bicarbonate-buffered basal medium described by Zehnder *et al.* (1980), supplemented with acetic acid to give a final concentration of 50 mM and 1 ml of a solution containing 33.7 g $Na_2WO_4 2H_2O$ and 27.0 g $NaSeO_3 5H_2O$ in 200 ml 10 mM NaOH. In addition, one of either of the following components; KCl, NaCl, NH_4Cl or propionic acid was added at an initial concentration of salt or propionic acid of 3 g/l (K^+, Na^+ or $N - NH_4^+$) and 30 mM, respectively. Two parallel bottles were started for each component and two bottles without any salt or acid were started as controls. The pH of the medium was 7.3 (adjusted with Na_2CO_3 in the bottles with propionic acid) and the cultures were incubated at 37 * C without shaking. After degradation of in total 100 mM (corresponding to two additions of 50 mM) of acetic acid (no propionic acid was degraded), culture liquid

Table 1 | Results from the operation of the digesters SAO1 and SAO3

Day	NH_4^+-N (g/L)		VFA (g/L)		pH		CH4 (%)		Gas yield* (L/g VS)		Methane yield* (L/g VS)	
	SAO1	SAO3	SAO1	SAO3	SAO1	SAO3	SAO1	SAO3	SAO1	SAO3	SAO1	SAO3
70	0.78	0.82	<0.1	<0.1	7.2	7.2	61	61	0.66	0.63	0.40	0.38
141	0.65	1.94	<0.1	4.0	7.2	7.6	61	63	0.66	0.67	0.40	0.42
225	0.72	3.30	<0.1	17.8†	7.3	7.9	61	64	0.63	0.62	0.38	0.40
442	0.86	5.47	Nd	Nd	7.2	8.0	61	62	0.62	0.31	0.38	0.20
642	0.90	6.91	<0.1	29.7†	7.4	7.9	61	58	0.60	0.34	0.37	0.20

Nd = not determined.
*The yields corresponds to average values calculated from two weeks of analyses.
†Main acids produced were acetate, propionate and caproate.

(10% v/v) was transferred from all cultures to new anaerobic bottles containing fresh medium and new substrate (350 ml). At every transfer to new medium, the concentration of salt and propionate were increased by one 1 g/L and 10 mM, respectively. This procedure was repeated until the concentrations of Na^+, K^+, $N-NH_4^+$, and propionic acid as a final concentration reached 10, 13, 8 g/L and 200 mM, respectively.

Labelling experiments

20 ml aliquots of actively growing enrichment cultures or digestate from the digesters, were transferred during flushing (N_2/CO_2; 80/20) to sterile serum vials (118 ml). The bottles were closed with butyl rubber stoppers and aluminium caps, and the labelling studies were started by the addition (2-^{14}C)-acetate (Amersham, England) to a final concentration of 10 kBq/ml and, for the enrichment cultures, 20 mM of acetic acid. For the enrichment cultures, the labelling experiments were started at the same time as transfer to new medium was performed. For the digesters, labelling experiments were started just before a change in the substrate composition was performed for digester SAO3, i.e. after 70, 142, 225, 442 and 642 days of operation (Table 1 and Figure 1). A triplicate set of bottles were started for each digester and time. All cultures were incubated at 37°C and the degradation of (2-^{14}C)-acetate, the formation of $^{14}CH_4$ and $^{14}CO_2$, as well as ^{14}C label incorporated in biomass (for the enrichments cultures solely) was determined by scintillation counting according to Schnürer *et al.* 1994. The labelling pattern were analysed when approximately 90% of the labelled acetate had been converted. Recoveries of labelling varied between 83 and 104%.

RESULTS AND DISCUSSIONS

Anaerobic digesters

During the whole operation period (660 days), the control digester SAO1 had a ammonium-nitrogen concentration of 0.65–0.90 g/L and showed stable performance with a methane yield of 0.37–0.40 l/g VS, a concentration of volatile fatty acids (VFA) below 0.1 g/L and a pH between 7.2 and 7.4 (Table 1). In SAO3, the pH and VFA increased

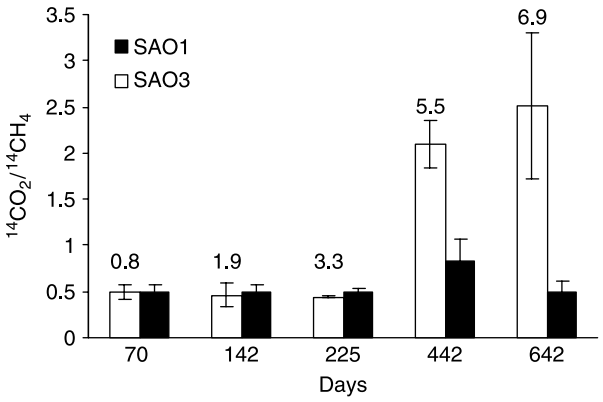

Figure 1 | Degree of acetate oxidation, measured as $^{14}CO_2/^{14}CH_4$, in the digesters SAO1 (control) and SAO3 (increasing ammonia) during the operational period of 642 days. Error bars show standard deviation, and figures above the bars show the ammonia nitrogen concentration (g/l) in SAO3. The ammonia-nitrogen concentration in the control digester SAO1 was <0.9 g/L over the whole operational period.

with increasing levels of ammonia-nitrogen, and at 3.3 g $NH_4^+ - N$/L the levels had reached values of approx. 18 g/L and 7.9, respectively (Table 1). At this point, the methane yield was still 0.40 l/g VS, i.e. same as SAO1. However, after running the process for several retention times at a concentration of 5.5 g/l $NH_4^+ - N$, the methane yield decreased to half of the previous level. This low methane yield cannot be explained by the increasing protein concentration in the substrate, as this theoretically should give a higher methane yield. This low yield was maintained also during operation at 6.9 g $NH_4^+ - N$/L. However, just two weeks after the final sampling occasion, a complete process failure occurred and the gas production stopped.

During the whole experimental period, the main product formed from (2-^{14}C)-acetate in the control digester SAO1 was methane, demonstrated by a $^{14}CO_2/^{14}CH_4$ ratio between 0.5 and 0.8 (Figure 1) These results clearly show that the main mechanism for methane formation from acetate in SAO1 was the aceticlastic pathway. In SAO3, the pathway used for methane formation from acetate changed over time (Figure 1). During the first 225 days of operation the aceticlastic mechanism dominated ($^{14}CO_2/^{14}CH_4$ was around 0.5). However, after raising the ammonium-nitrogen concentration above 3 g/L the mechanism clearly shifted to syntrophic acetate oxidation. This shift in pathways was illustrated by an increased production of $^{14}CO_2$ from labeled acetate giving a $^{14}CO_2/^{14}CH_4$ ratio above 2. This quota value is in the same range as found in a previous study in which acetate oxidation was found in Danish biogas digesters lacking *Methanosaetaceae* (Karrakshev 2006). The shift in the methane producing pathway likely explains the observed decrease in the gas and methane yield in SAO3, as these two events coincide in time. Syntrophic acetate oxidation was previously described to occur in biogas processes operating at high levels of salts and volatile fatty acids (Schnürer *et al.* 1999) but this is the fist study revealing a shift in the acetate degrading pathway as the concentration of ammonia (and VFA) increases.

Enrichment cultures

In the enrichment cultures with sodium, potassium, propionate or with no addition (except for the substrate acetic acid), the $^{14}CO_2/^{14}CH_4$ ratio never exceeded 0.2, suggesting that methane in these cultures were produced exclusively through the aceticlastic pathway (data not shown). The cultures were grown to a final concentration of 10 g/L, 13 g/L and 200 mM of sodium, potassium and propionic acid, respectively, but no shift in the methane producing pathway occurred. Higher concentrations of these compounds were not possible to obtain without a complete stop of the methane production, presumably caused by non-specific osmotic effects. In contrast, the enrichment cultures with addition of ammonium chloride demonstrated a clear shift in the pathway for methane formation from acetate as the concentration of ammonia increased. Below 5 g of ammonium nitrogen, the aceticlastic mechanism was the dominating pathway (Table 2). At 6 g ammonium nitrogen/l, the $^{14}CO_2/^{14}CH_4$ ratio had increased above 2 suggesting that the dominating mechanism now was syntrophic acetate oxidation (Table 2). At 7 g ammonium nitrogen/l, as much as 95% of the labeled acetate was oxidized to carbon dioxide. In a previous study, including

Table 2 | Fate of (2-^{14}C)-acetate* in methanogenic batch cultures amended with acetic acid (20 mM) and NH_4Cl. The results presented represent one of the duplicate bottles. The same pattern was obtained for the other duplicate

NH_4^+-N(g/L) (mg/L free ammonia†)	(2-^{14}C)-acetate‡ (10^6 dpm)	$^{14}CH_4$§ (10^6 dpm)	$^{14}CO_2$‖ (10^6 dpm)	$^{14}CO_2/^{14}CH_4$	Biomass (10^6 dpm)	Recovery (%)
5 (128)	0.24	4.8	0.94	0.20	0.043	101
6 (154)	0.58	1.8	3.7	2.1	Nd¶	92
7 (180)	0.053	0.24	5.7	24	0.040	101

*Total amount of (2-^{14}C)-acetate initially added to the batch culture was 10^6 dpm.
†Calculated acoording to the formula given by Hasimoto (1986).
‡Non degraded acetate still in the culture liquid.
§$^{14}CH_4$ in the gas phase.
‖$^{14}CO_2$ in the gas phase plus $H^{14}CO_3$ and $^{14}CO_2$ dissolved in the culture liquid.
¶Not determined.

different commercial anaerobic digesters, 40–85% of the labeled acetate was found in acetate (Schnürer *et al.* 1999). Possibly, at 6 g NH_4^+ – N/L both mechanisms were on-going but at 7 g NH_4^+ – N/L, the mechanism for methane production was solely syntrophic acetate oxidation.

CONCLUDING REMARKS

This study clearly shows that at mesophilic temperature, ammonia is a strong selective agent for syntrophic acetate oxidation. In the enrichment cultures, other salts or propionic acid did not by them self cause a shift in the acetate degrading mechanism. It is therefore likely that also the shift occurring in the anaerobic digester SAO3 was caused by ammonia and not by the increasing concentrations of volatile fatty acids.

Ammonia has a strong inhibitory effect on methanogens, particularly on the acetate-utilizing methanogenic population (Koster & Lettinga 1884; Sprott & Patel 1986). In non-adapted biogas systems, concentrations of 1.5–2.5 NH_4^+ – N/L has been shown to cause inhibition (van Velsen 1981; Hashimoto 1986). However, by adaptation of the biogas process, tolerance to ammonia concentrations high above this level has been reported (van Velsen A.F.M. 1981; Koster & Lettinga 1984; Hashimoto 1986, 1988; Hansen *et al.* 1998). The results from this study strongly suggest that the previously described adaptation corresponds to a shift in the methane producing population. As syntrophic acetate oxidation involves a hydrogenotrophic methanogen, tolerating higher levels of ammonia, methane production from acetate can proceed even though the acetetotrophic methanogens are inhibited. The free ammonia content has been suggested to be the active component causing the inhibition. Using the formula given by Hashimoto (1986) to calculate the level of free ammonia, it is clear that the shift in this study occurred at levels rising above 128–330 mg/l. These values are in the same range as previously reported to be inhibitory for methanogensis of un-adapted processes (Braun *et al.* 1981; De Beare *et al.* 1984).

Compared to the aceticlastic methane producing pathway, the syntrophic pathway is by thermodynamic necessity much slower. The doubling time of a mesophilic syntrophic acetate oxidation culture was calculated to be approx. 28 day (Schnürer *et al.* 1994), to be compared with the aceticlastic methanogens having doubling times around 2–12 day (Jetten *et al.* 1992). This suggests that biogas digesters to be run at high levels of ammonia should have a relatively long HRT, in order to avoid accumulation of volatile fatty acids and to retain a stable process. The HRT (30 days) used for the digesters in this study was apparently too short to retain a stable process. With increasing ammonia levels, the concentrations of VFA increased over time finally leading to a drop in pH and a complete process failure. However, a large-scale commercial anaerobic digester in Sweden demonstrates a god example of the possibility of retaining a stable process at high ammonia levels and with syntrophic acetate oxidation as the dominating methane producing pathway from acetate. This process have a $^{14}CO_2/^{14}CH_4$ ratio of 16 and have been in stable operation for several years with the following average operational parameters; HRT, 56 days; pH 8.0; NH_4^+-N, 5.3 g/L and VFA; 2,3 g/L (pers. comm. Lina Vallin, Svensk Biogas AB).

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