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Characterization of methanogenic biomass from a full-scale digester that might harbour SAO activity

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Abstract
Biomass from a full-scale anaerobic digester treating nitrogen-rich wastes was characterized by methanogenic batch activity assays at different ammonia concentrations. SAO was demonstrated by isotopic ^{13}C fractionation of biogas components, at 3 – 6 gN-TAN. Microbial inhibition was found at 6 gN-TAN both in the acetate consumption rate and by genetic expression studies. Several homoacetogenic eubacteria potentially related to the SAO process were detected by NGS microbiome analysis (5% – 10% in relative abundance), but *Methanoculleus* spp. was the predominant hydrogenotrophic syntrophic partner (58% of archaeal abundance).

Keywords
Ammonium; anaerobic-digestion; biogas-^{13}C-isotopic-fractionation; microbiome-characterization; syntrophic-acetate-oxidation

INTRODUCTION

The anaerobic digestion (AD) of organic materials is a well consolidated technology for the revalorization of organic waste into renewable energy (methane from biogas), and contributes significantly to the sustainability of several industrial processes. Approximately, 70% of methane in AD is produced from acetate as the main intermediate of the decomposition process by acetotrophic methanogenic archaea (AMA), while the remaining 30% is synthesised from hydrogen and carbon dioxyde by hydrogenotrophic methanogenic archaea (HMA). However, increased ammonia levels arising from the decomposition of protein-rich materials results in the inhibition of AMA and, thus, compromises the feasibility of the AD process (Angelidaki and Ahring, 1993). Alternatively, acetate can be oxidized to hydrogen and carbon dioxide by syntrophic acetate-oxidizing bacteria (SAOB). This process is thermodynamically favourable because of hydrogen consumption by concomitant HMA, which are comparatively more tolerant to ammonia than AMA. The fundamental role of SAOB in the methanogenesis under high ammonia conditions was already recognized in the late nineties (Schnüre et al. 1999) but relatively little is known about the diversity, physiology and biochemistry of these microorganisms (Westerholm et al. 2012).

This ongoing study is aimed at the better understanding of the microbial interactions in AD reactors when subjected to relatively high concentrations of acetate and ammonia from both macroscopic/applied and microscopic/fundamental perspectives. Methanogenic biomass from a full-scale digester treating protein-rich wastes under conditions that might favour the development of SAOB has been selected for this purpose. An interdisciplinary research approach was adopted, which combined batch methanogenic activity assays with the in-depth characterization of microbial populations by next-generation high throughput sequencing (NGS) and trough the quantification of the expression level of relevant genes by reverse transcription qPCR (RT-qPCR). Isotopic fractionation studies on ^{13}C-biogas components were implemented for depicting the predominant methanogenic pathway.

MATERIALS AND METHODS

The methanogenic biomass used as inoculum was collected from a 1,500 m^3 full-scale CSTR anaerobic digester treating protein-rich agricultural wastes (Vilasana, Lleida, Spain), and operated at a HRT of 65 days. A first laboratory experiments was carried out in two sets of duplicate batch cultures (7.5 gVSS-inoculum L^{-1}) prepared as follows: vials *1a* to *4a* were supplemented with 0.0, 1.0, 2.1 and 3.2 gAc L^{-1}, respectively, while vials *1b* to *4b* contained 0.7, 1.7, 2.0 and 2.4 gTAN L^{-1} and 3.2 gAc L^{-1}. Some of this ammonia concentration values fall within the inhibitory range for AMA of 1.5 – 3.0 gN-TAN L^{-1} (Angelidaki and Ahring, 1993). Three pulses of acetate were applied every 47 – 50 days in order to restore the initial substrate content. Biogas composition in the headspace and volatile fatty acids in the liquid were monitored chromatographically throughout the incubation time. Biomass samples from the inoculum and the incubated vials after the second pulse were taken and preserved at -20ºC for subsequent microbial characterization.

Ammonia concentration was raised in a second similar experiment. Triplicate batch cultures (12.7 gVSS-inoculum L^{-1}) were performed: vials *1c* to *3c* contained 1, 3, and 6 gTAN L^{-1} and 2.5 g L^{-1} of acetate. The isotopic $^{13}C/^{12}C$ fractionation ($\delta^{13}C$) of biogas was measured by GC-IRMS, and the $\alpha_C = (\delta^{13}C\text{-}CO_2+1000)/(^{13}C\text{-}CH_4+1000)$ was determined. Replicates vials (*2c** and *3c**) were also amended with methyl-labeled ^{13}C-acetate and the resulting ^{13}C-labeled biogas compounds were recovered and analyzed. Biomass samples from incubation vials were collected upon exponential methane production and kept at -80ºC for DNA/RNA extraction. All cultures were incubated at 37ºC under shaking conditions and a phosphate/carbonate buffer solution was added to keep the pH at 7 and 8 for the first and second experiments, respectively. Specific rates of acetate consumption (r_{Ac}) and methane production (r_{CH4}) were determined during the first days. All conversion rates were expressed in terms of gCOD $gVSS^{-1}$ d^{-1}.

Microbial community composition was characterized by Illumina MiSeq sequencing of eubacterial and archaeal 16S rRNA gene libraries. Community structure was represented in terms of the relative abundance of operational taxonomic units (OTU) defined at a 97% sequence homology cutoff. Expression of total *16S rRNA* from *Eubacteria* and *mcrA* from methanogenic *Archaea* genes were quantified by qPCR.

RESULTS

The r_{CH4} measured after every feeding pulse was directly correlated to the initial concentration of acetate, but tended to decrease in successive pulses (Figure 1A). A similar trend was observed on the r_{Ac}, phenomenon that is to be expected since r_{Ac} is substrate dependent.

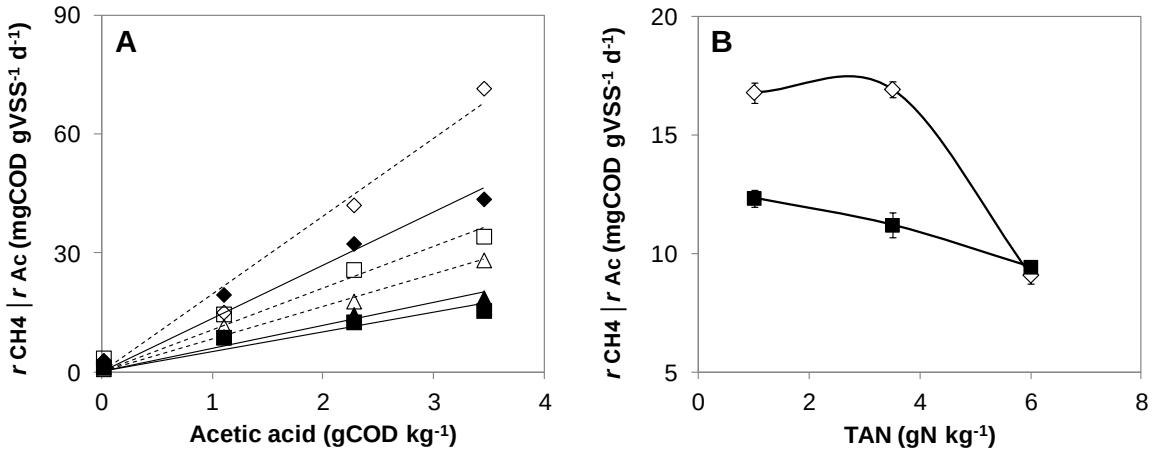

Figure 1. Influence of acetate (graph A: vials *1a – 4a*) and ammonia (graph B: vials *1c – 3c*) on r_{CH4} (solid symbols) and r_{Ac} (empty symbols). Rates in A correspond to pulses 1 (diamonds), 2 (triangles), and 3 (squares). Values in B correspond to the maximum rate (average and standard deviation of three independent vials).

The general time-course reduction in microbial activity rates could be attributed to the cultures approaching the stationary growth phase. Conversely, at equal amounts of acetate (3.2 gAc L^{-1}), r_{CH4} was rather similar regardless of the concentration of ammonia up to 2.4 gN-TAN L^{-1}, though it also decreased in successive pulses (*p1*: 35±4; *p2*: 18±1; *p3*: 15±5 gCOD $gVSS^{-1}$ d^{-1}). A certain degree of inhibition of the microbial activity rates was only observed at 6 gN-TAN L^{-1}, and affected more significantly to the r_{Ac} values (Figure 1B).

Specific patterns on the fractionation of carbon isotopes in relation to the concentration of ammonia were observed in biogas samples from ^{13}C-acetate-amended and unamended cultures (Figure 2). Biogenic CH_4 generated by CO_2 reduction tends to be more depleted in ^{13}C than that formed directly from acetate (Conrad, 2005). Hence, in natural conditions, α_C during methanogenesis is known to be greater for HMA (1.055 – 1.080) than for AMA (1.040 – 1.055). In the present study, reported αC values ranged from the predominance of acetotrophic methanogenesis in the inoculum, with no added ammonia (1.054±0.017), to the dominance of hydrogenotrophs at 3 gN-TAN L^{-1} (1.077±0.001) and even the exclusive hydrogenotrophic methanogenesis at 6 gN-TAN L^{-1} (1.080±0.000). In addition, CO_2 and CH_4 released from the AMA pathway arise from the carboxyl and methyl carbons of acetate, respectively, while the two carbon atoms contribute to both CO_2 and CH_4 by the SAOB/HMA-mediated methanogenesis. Hence, the observed accumulation of ^{13}C-CO_2 from cultures incubated with methyl-labeled ^{13}C-acetate demonstrates the occurrence of the SAO process at ammonia levels higher than 3 gN-TAN L^{-1} (Figure 2).

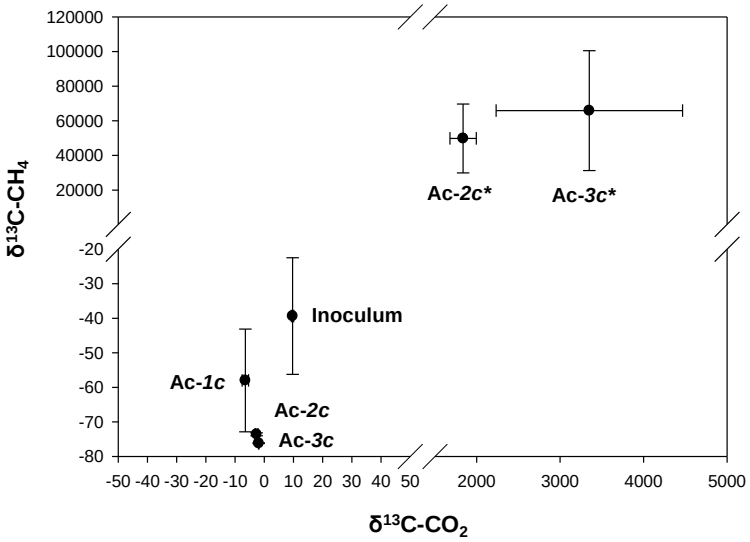

Figure 2. Biplot on the isotopic fractionation ($\delta^{13}C$) of ^{13}C-CO_2 and ^{13}C-CH_4 in biogas samples from batch cultures incubated at different ammonia concentrations: 1, 3 and 6 gN-TAN L^{-1} for *1c*, *2c*, and *3c*. Samples marked with an asterisk correspond to vials spiked with methyl-labeled ^{13}C-acetate. Depicted values and error bars correspond to the average and standard deviation of three independent vials.

Concerning the analysis of the microbial community structure, 438,503 high quality DNA reads (35,642 reads per sample) were obtained from NGS analysis of the *Eubacteria* domain. A predominance of several representatives of the phyla *Bacteroidetes*, *Chloroflexi* and *Firmicutes* (72% – 78% of OTU relative abundance) was observed. These groups encompass several anaerobic or facultative anaerobic heterotrophic bacterial species that are typical from digesters. About 5% – 10% of the total community accounted for the polyphyletic group of homoacetogenic bacteria potentially related to the SAO process, but changes in their abundance could not be linked to the concentration of ammonia. Regarding the *Archaea* domain (301,306 valid DNA reads, 25,086 reads per sample), its diversity was limited to only 6 genera accounting for more than 99% of the total population. Obligate HMA were predominant in the inoculum, particularly members of the genus *Methanoculleus* (58%), and were slightly more abundant (68%) in cultures incubated at high concentrations of ammonia. Previous studies have highlighted the role of *Methanoculleus* spp. as an important partner during SAO under mesophilic conditions (Westerholm et al. 2012). In contrast, the abundance of obligate AMA, such as representatives of the genus *Methanosaeta*, declined from 27% in the inoculum to just 4% in cultures with increased ammonia content.

The partial inhibition of microbial activity observed at the highest assayed TAN values (Figure 1B) was consistent with gene expression profiles on those same cultures (Figure 3). Eubacterial RNA transcripts from *16S rRNA* genes peaked after 11 days of incubation and were lower at 6 gN-TAN L^{-1} while expression levels were higher at 1 and 3 gN-TAN L^{-1}. This profile was similar in methanogenic archaea (*mcrA* genes), but a lag phase of about 7 days was observed in all cases and the expression level was significantly more inhibited at 6 gN-TAN L^{-1}.

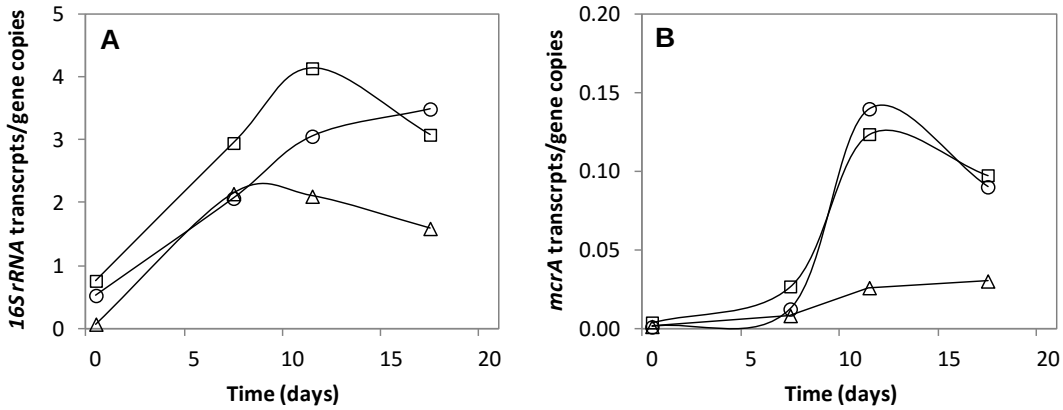

Figure 3. Genetic expression profiles of *16S rRNA* (*Eubacteria*) and *mcrA* (methanogenic *Archaea*) in vials *1c – 3c* incubated with 1, 3, and 6 gN-TAN L^{-1} (squares, circles, and triangles).

CONCLUSIONS

The integrated characterization of biomass specific activity rates, microbial community structure and genetic expression profiles, with the biogas isotopic fractionation provided strong evidence on the occurrence of SAO activity. Ammonia concentrations above 3 gN-TAN L^{-1} were needed for significant acetate oxidation to CO_2 and the process could be sustained even at 6 gN-TAN L^{-1}, though the first signs of microbial inhibition were then observed (lower activity rates and expression levels). The genus *Methanoculleus* appears to be a fundamental HMA syntrophic partner and could be used as a reliable bioindicator of SAO biomass, given the polyphyletic nature of SAOB. Our results also demonstrate that significant SAO can be enriched spontaneously in full-scale anaerobic digesters, provided that the right operational conditions are implemented.

Ongoing research focuses on developing specific molecular targets for monitoring SAOB and SAO activity at transcription level, and on extending the experimental design so that kinetic parameters can be identified univocally. These data are fundamental for updating the ADM1 model with the inclusion of the SAOB populations.

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