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Functional biodiversity and plasticity of methanogenic biomass from a full-scale mesophilic anaerobic digester treating nitrogen-rich agricultural wastes

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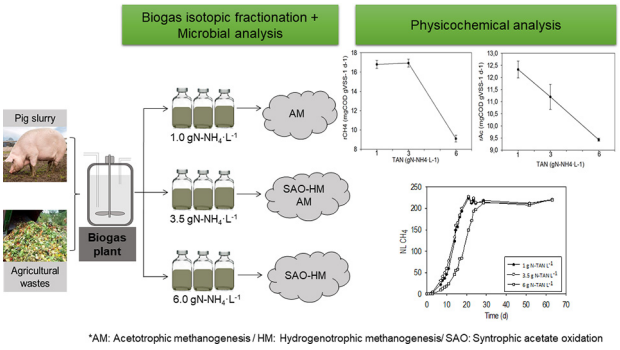
HIGHLIGHTS

- N-adaptation of biomass in a full-scale digester ensued robust reactor performance.
- NH_3 quickly shifted metabolism from acetotrophic to hydrogenotrophic methanogenesis.
- Gene expression in several syntrophic bacteria was stimulated under high NH_3 exposure.
- A novel archaeal *Methanoculleus*/*Methanomassiliicoccus* syntrophism is proposed.
- The functional biodiversity of the SAO process still remains rather unexplored.

GRAPHICAL ABSTRACT



*AM: Acetotrophic methanogenesis / HM: Hydrogenotrophic methanogenesis/ SAO: Syntrophic acetate oxidation

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ABSTRACT

The effect of ammonia on methanogenic biomass from a full-scale agricultural digester treating nitrogen-rich materials was characterized in batch activity assays subjected to increasing concentrations of total ammonia N. Acetotrophic and methanogenic profiles displayed prolonged lag phases and reduced specific activity rates at 6.0 gN-TAN L^{-1}, though identical methane yields were ultimately reached. These results agreed with the expression levels of selected genes from bacteria and methanogenic archaea (qPCR of 16S rRNA and *mrcA* cDNA transcripts). Compound-specific isotope analysis of biogas indicated that ammonia exposure was associated to a transition in methanogenic activity from acetotrophy at 1.0 gN-TAN L^{-1} to intermediate and complete hydrogenotrophy at 3.5 and 6.0 gN-TAN L^{-1}. Such pattern matched the results of 16S-Illumina sequencing of genes and transcripts in that predominant methanogens shifted, along with increasing ammonia, from the obligate acetotroph *Methanosaeta* to the hydrogenotrophic *Methanoculleus* and the poorly understood methylotrophic *Methanomassiliicoccus*. The underlying bacterial community structure remained rather stable but, at 6.0 gN-TAN L^{-1}, the expression level increased considerably for a number of ribotypes that are related to potentially syntrophic genera (e.g. *Clostridium*, *Bellilinea*, *Longilinea*, and *Bacteroides*). The predominance of hydrogenotrophy at high ammonia levels clearly points to the occurrence of the syntrophic acetate oxidation (SAO), but known SAO bacteria were only found in very low numbers. The potential role of the identified bacterial and archaeal taxa with a view on SAO and on stability of the anaerobic digestion process under ammonia stress has been discussed.

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1. Introduction

The anaerobic digestion (AD) of organic materials is a well-consolidated technology for the treatment and revalorization of organic waste into renewable energy (methane from biogas), and contributes significantly to the sustainability of several industrial processes (Lettinga, 2010). However, a significant proportion of the organic waste generated by the agrifood sector contains relatively large amounts of nitrogenated compounds (i.e. animal dejections, slaughterhouse by-products, and other protein-rich food-processing wastes). Organic nitrogen compounds are reduced to free ammonia (NH_3), often referred as free ammonia N (FAN), and its ionized counterpart ammonium (NH_4^+). In aqueous media, these two chemical species are in a pH and temperature-depending equilibrium. NH_3 is by far a stronger inhibitor of methanogenesis than NH_4^+ but, because of practical reasons, NH_3 and NH_4^+ are commonly measured together as total ammonia N (TAN) (Yenigün and Demirel, 2013). Such inhibitory effects might affect all microbial communities involved in the AD syntrophy, but the methanogenic archaea appear to be particularly sensitive to ammonia exposure (Demirel and Scherer, 2008). Yet, not all methanogens are affected equally; acetoclastic methanogenic archaea (AMA), which under common non-inhibitory conditions are responsible for most of the generated methane (CH_4), have been described to be vulnerable to relatively low concentrations of ammonia (circa 3.5 gN-TAN L^{-1}) (Banks et al., 2012; Schnürer and Nordberg, 2008). Conversely, the less sensitive hydrogenotrophic methanogenic archaea (HMA) are able to remain active at those concentrations, while reported ammonia inhibition thresholds are above 5 gN-TAN L^{-1} (Wang et al., 2015). Furthermore, AMA inhibition by ammonia might result in the accumulation of acetate up to inhibitory levels, thus contributing further to a negative feedback mechanism that eventually leads to complete reactor failure (Wang et al., 2015).

Under such high concentrations of ammonia and/or acetate, the so-called syntrophic acetate oxidizing bacteria (SAOB) are able to reverse the homoacetogenic pathway and convert acetate to carbon dioxide (CO_2) and hydrogen (H_2) (Schnürer et al., 1999). This process is thermodynamically favourable through the concomitant consumption of H_2 by HMA and, therefore, the syntrophic association between SAOB and HMA prevents the inhibition of methanogenesis during the AD of nitrogen-rich substrates (Petersen and Ahring, 1991). An increasing number of SAOB strains have been isolated in the recent years and their physiology and genetics have been characterized quite thoroughly, but information on the diversity, occurrence and role of SAOB in full-scale anaerobic digesters is still limited (Westerholm et al., 2016). In an earlier integrative study based on the metagenomic characterization of biomass and on the biogas isotopic profiling of different industrial anaerobic digesters, we pointed out at the predominance of both HMA communities and the hydrogenotrophic pathway in those digesters operated under relatively high nitrogen loads (Ruiz-Sánchez et al., 2018). These conditions are conducive to the enrichment of SAOB.

Here we aim at gaining a deeper insight into the microbial interactions, both of metabolically active bacterial and archaeal populations that are potentially involved in the SAO process. This new study focuses at the methanogenic biomass from an industrial anaerobic digester treating nitrogen-rich agricultural wastes with no previous records of process inhibition. A diversified research approach has been adopted for this purpose, which combined batch methanogenic activity assays under different ammonia contents, with Compound-Specific Isotope Analysis (CSIA) of $^{13}C/^{12}C$ natural isotopic fractionation of CH_4 and CO_2 in the generated biogas, and the in-depth characterization of present and metabolically active microbial populations by 16S-Illumina sequencing. The time-course expression of relevant genes from bacteria (16S rRNA) and methanogenic archaea (methyl coenzyme M reductase; *mcrA*) was quantified by qPCR.

2. Materials and methods

2.1. Batch experiments

Methanogenic biomass was collected from a 1500 m^3 full-scale completely stirred tank reactor (CSTR) for the anaerobic co-digestion of pig slurry and protein-rich agricultural wastes (Vilasana, Lleida, Spain). This digester was operated according to the following average parameters: total ammonia N (TAN) = 5.2 gN-TAN L^{-1}, chemical oxygen demand (COD) = 101.2 gO_2 L^{-1}, volatile suspended solids (VSS) = 61.2 g L^{-1}, pH = 8.3, acetate concentration = 0.0 gAc L^{-1}, hydraulic retention time (HRT) = 65 days, and temperature within the mesophilic regime. Experiments were conducted in triplicate batch cultures (120 mL total volume, 60 mL working volume, inoculated with 12.7 gVSS L^{-1}), containing 1.0, 3.5 or 6.0 gN-TAN L^{-1} by adding NH_4Cl and 2.36 gAc L^{-1} as sodium acetate. Anaerobic conditions were generated by flushing N_2 during 10 min. Cultures were incubated at 37 °C under rotatory shaking and a bicarbonate buffer solution was added to maintain a constant pH of 8 throughout the experiment. Control vials with neither acetate nor ammonia were included to assess the endogenous CH_4 production of the inoculum.

Specific rates of acetate consumption and CH_4 production were determined and expressed as gCOD $gVSS^{-1}$ d^{-1} (conversion factors: 2.857 mgCOD $mLCH_4^{-1}$; 1.067 gCOD gAc^{-1}). For this purpose, samples of the liquid phase from each batch replicate (1 mL) were collected after 0, 7, 11 and 17 days of incubation and directly centrifuged (4 °C, 20,000 rpm, 5 min). The supernatant (clarified fraction) was used for chemical analysis, while the pellets (sedimented fraction) were kept at −80 °C until further processing via molecular biology tools. Samples from the headspace of each culture were taken periodically for the characterization of the biogas composition during the experiment. CH_4 yield ($mLCH_4$ $gCOD^{-1}$), lag phase and specific CH_4 production rate (rCH_4; mgCOD $gVSS^{-1}$ d^{-1}) were calculated after fitting the experimental data to the modified Gompertz equation. Samples of the accumulated biogas at the end of the incubation were collected for analysing the natural $^{13}C/^{12}C$ isotopic fractionation of CH_4 and CO_2. Gas/liquid volume changes due to sampling were taken into account in the calculation of mass balances.

2.2. Analytical methods

Total Kjeldhal Nitrogen (TKN), TAN and pH were determined according to the Standard Methods (APHA, 2005). The biogas was monitored along the experiment by sampling 100 μL from headspace of each batch. Biogas composition (CH_4 and CO_2) and the concentration of individual volatile fatty acids (VFA) in the liquid media, including acetic (Ac), propionic, butyric, valeric and caproic acids, were measured in a gas chromatograph (Varian CP-3800). This instrument was equipped with a Varian Hayesep-Q 80–100 mesh capillary column and a TCD detector for the analysis of biogas, or a TRB-FFAP capillary column and a FID detector for the analysis of VFA.

CSIA of $^{13}C/^{12}C$ natural isotopic fractionation of biogas components was carried out by gas chromatography combustion–isotope ratio mass spectrometry (GC–IRMS). An Agilent 6890 gas chromatograph was fitted with a split/splitless injector and coupled to an isotope ratio mass spectrometer (Delta Plus Finnigan MAT) via a combustion interface (850 °C), consisting of a 60 cm quartz tube (0.65 mm ID) filled with copper oxide. A liquid nitrogen cold trap was used to remove water. Separation was achieved on a Cpsil5CB (Chrompack) fused silica capillary column (60 m × 0.32 mm; 0.12 μm film thickness) using He as carrier gas. The oven temperature was held at 40 °C for 1 min, and increased to 320 °C at a rate of 10 °C min^{-1}. This final temperature was maintained for 25 min. Squalene was used as internal standard. Each sample was run in triplicate to ensure reproducibility within ±0.2‰ (1σ), relative to the Vienna Pee Dee Belemnite (VPDB) standard. All carbon isotopic ratios were expressed as ‰ relative to the VPDB standard,