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Changes of the microbial population structure in an overloaded fed-batch biogas reactor digesting maize silage

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HIGHLIGHTS

- Shift of communities caused by overloading was detectable prior pH decrease.
- Overloading caused a decreased diversity of bacterial groups.
- *Methanosarcina thermophila*-related species appeared before overloading.
- Certain bacteria became dominant before and during acidification.
- Bacteria became dominant may be suitable as indicator organisms for acidification.

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ABSTRACT

Two parallel, stable operating biogas reactors were fed with increasing amounts of maize silage to monitor microbial community changes caused by overloading. Changes of microorganisms diversity revealed by SSCP (single strand conformation polymorphism) indicating an acidification before and during the pH-value decrease. The earliest indicator was the appearance of a *Methanosarcina thermophila*-related species. Diversity of dominant fermenting bacteria within *Bacteroidetes*, *Firmicutes* and other *Bacteria* decreased upon overloading. Some species became dominant directly before and during acidification and thus could be suitable as possible indicator organisms for detection of futurity acidification. Those bacteria were related to *Prolixibacter bellariivorans* and *Streptococcus infantarius* subsp. *infantarius*. An early detection of community shifts will allow better feeding management for optimal biogas production.

1. Introduction

The production of biogas has recently become one of the most promising technologies for overcoming future energy shortages which might occur when the availability of fossil fuels will decline. By the end of 2012, about 7515 biogas plants were being operated in Germany (German Biogas Assoziation, 2013). The anaerobic digestion leading to the production of biogas is influenced by different parameters like temperature, pH value, the concentrations of volatile fatty acids (VFA) and ammonia and the availability of macro- and micronutrients (Weiland, 2010). One of the most severe problems biogas plants confront is acidification of the reactors resulting in process instability (Burgstaler et al., 2010). Acidification of biogas reactors leads to lower biogas yields and a loss in biogas quality as trace gases like ammonia, nitrous oxide and hydrogen sulphide are produced. This causes severe technical problems in combined heat and power units when energy is recovered from gas mixtures (Burgstaler et al., 2011). It is therefore important to keep the pH value during anaerobic digestion in the range of 6.0–8.5 as the formation of methane by methanogenic *Archaea* is inhibited beyond those pH values (Weiland, 2010).

During the acidification the archaeal community is changing as previous study showed. Blume et al. (2010) investigated the methanogenic community in a mesophilic, continuously stirred tank reactor of 10 l volume inoculated with liquid pig manure and fed with maize silage. It was suggested that the absence of *Methanosaetaceae* might be an indicator for the instability of the biogas process since at lower organic loading rates (OLR) acetoclastic *Methanosaetaceae* dominated while at OLRs above 3.7 g dry organic

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mass l^{-1} d^{-1}, hydrogenotrophic *Methanobacteriales* were dominant. Hori et al. (2006) investigated the stability of microbial communities in a 1.4 l thermophilic bioreactor treating synthetic wastewater. It was observed that the archaeal community was affected by the concentration of volatile fatty acids (VFAs) while the bacterial community was impacted by the pH value. In a study published by Munk et al. (2010) six mesophilic biogas reactors of 28–30 l volume were operated with maize silage as sole substrate. The methanogenic community in these reactors consisted of *Methanosaetaceae* which were only found at low acetate concentrations and *Methanobacteriales* and *Methanosarcinaceae* which were dominating at higher acetate concentrations. Under acidified conditions, *Methanomicrobiales* like *Methanospirillum hungatei* or *Methanoculleus* sp. were most abundant while the overall number of methanogens had decreased (Munk et al., 2010). The study of Chen et al. (2012) evaluated the archaeal population of an animal waste biogas plant before and 350 days after overfeeding when biogas production had recovered by archaeal 16S RNA gene clone libraries. A higher population diversity which developed during the stressful time of overloading could be found. However, most of these studies focus on the methanogenic *Archaea* while the fermenting bacteria are mostly neglected.

To detect an impending acidification of the biogas reactors early enough to counteract the acidification process, it would be helpful to find indicator microorganisms showing the futurity acidification before chemical parameter like pH-value changed. If the pH-value and the biogas yield have already dropped the recovery of stable conditions and high biogas yields need much more time. To find those indicator microorganisms and to prove the hypothesis that a population shift of fermenting bacteria or methanogenic *Archaea* can be monitored upon overloading before acidification we conducted the present study. The development of the microbial population structure in mesophilic fed-batch biogas reactors which were overloaded with maize silage at on organic loading rate (OLR) of up to 16.8 g VS (volatile solids) l^{-1} d^{-1} was investigated via single strand conformation polymorphism (SSCP) analysis in order to identify the community shifts resulting from acidification. Primer pairs detecting methanogenic *Archaea*, *Firmicutes*, *Bacteroidetes* and the whole group *Bacteria* were used. *Firmicutes* and *Bacteroidetes* were chosen since they represent the majority of the fermenting bacteria. In contrast to other studies, 200 l pilot-scale reactors were used including frequent sampling for simultaneous logging of bacterial and methanogenic population and pH-values.

2. Methods

2.1. Reactor operation and sampling

Anaerobic reactor experiments were carried out at the Eichhof in Bad Hersfeld, Germany. The laboratory fed-batch biogas reactors were filled with the digestate from a biogas reactor with a volume of 600 m^3 and initially consisted of liquid cattle manure (70%) and liquid pig manure (30%) with a dry mass of 3–4%. This reactor was regularly fed with maize silage and bruised grain and was kept at a temperature of 36–38 °C. For the experiments, two parallel running laboratory scale fed-batch reactors of 200 l were used with anchor stirrers that reached the bottom of the tanks. Stirring was conducted for 15 min per hour. Temperature was maintained at 39 °C. Before starting the laboratory experiment, digestate from the 600 m^3 main reactor was incubated until no further gas formation could be detected. Then, maize silage was added to the digestate with following organic loading rates: At the beginning 2.55 g VS per l digestate and day was added, after 167 h 5.1 g VS l^{-1} d^{-1} and between 372 h and 507 h around 3.4 g VS l^{-1} d^{-1} was added until the specific methane yield per kilogram silage was stable. Starting with 537 h the organic loading rate was increased to 16.8 g VS l^{-1} d^{-1} (Fig. 1). Volatile solids were determined after the standard method and the loss of volatile compounds (alcohols, fatty acids) in the 105 °C step were corrected according to Pietschmann et al. (2012). At different time points (a–j) the samples for SSCP analysis were taken (Fig. 1). A third 200 l reactor was handled as the other but not fed with any substrate and served as control. Per time point one sample from each reactor was taken in sterile 50 ml plastic centrifugation tubes (Falcon, Greiner Bio-One, Frickenhausen, Germany) via a valve that was situated at half height of the tank reactor and immediately frozen at −20 °C. They were transported to Giessen, Germany, in a freezing box to prevent thawing and stored again at −20 °C.

Gas production was measured in a drum-type gas meter (Type 1/6, Ritter, Bochum, Germany) and gas was collected in bags. Methane contents were determined with a nondispersive infrared sensor (NDIR, Type GS IRM 100, GS Messtechnik GmbH, Ratingen, Germany). The NDIR analyser was placed in the gas flow between reactor and bags and the gas was measured once an hour. Concentrations of volatile fatty acids were determined by injection of 1 µl sample in an Agilent 6890N chromatograph (Agilent Technologies, Böblingen, Germany) with a flame ionization detector (300 °C), a Zebron ZB-WAX-Plus column (30 m × 0.25 mm × 0.25 µm; Phenomenex, Torrance, USA) and N_2 as carrier gas. The sample preparation was done by mixing 20 g homogenised digestate with 10 ml formic acid (96%) and filling up the volume to 100 ml with distilled water. The temperature program of the oven included following steps: 1. 75–130 °C heating up rate 10 °C min^{-1}, 2. 130–220 °C heating rate 80 °C min^{-1}, 3. 220 °C holding time 5.7 min and 4. 75 °C holding time 0.25 min.

2.2. Molecular analysis of manure samples

Manure samples were thawed and homogenized for one minute in a Stomacher®80 Biomaster lab homogenizer (Seward Laboratory Systems Inc., Bohemia, USA). From each sample, 200 mg were used for DNA extraction with the QIAamp Stool minikit (Qiagen, Hilden, Germany) as described previously (Kampmann et al., 2012b).

All primers used in this study are listed in Table 1 and were purchased from Eurofins MWG Operon (Ebersberg, Germany). PCR was performed according to Kampmann et al. (2012b). PCR with primers targeting the *mcrA* gene encoding the alpha subunit of the methyl coenzyme-M reductase which is unique to methanogenic *Archaea* started with an initial denaturation for 5 min at 95 °C, followed by 35 cycles that included denaturation for 45 s at 95 °C, annealing for 45 s at 50 °C and extension for 60 s at 72 °C. A final extension step of 30 min at 72 °C was added. As a migration marker for SSCP analysis, a mixture of single-stranded rRNA genes from different pure cultures was used and the PCR for generating the SSCP standards was carried out as described in (Kampmann et al., 2012b). For PCR products that were used for single strand digestion, phosphorylated reverse primers for the corresponding PCR reactions were used. PCR products were checked for quality and amount by agarose gel electrophoresis and measurement at 260 nm in a photometer (Thermo Scientific GENESYS 20™, Thermo Fisher Scientific Inc., Waltham, USA).

Purification of PCR products, single strand digestion, purification and denaturation of single stranded DNA was performed according to Kampmann et al. (2012b). For every sample, equal amounts (2000 ng) of DNA were applied to the polyacrylamide gel. Single strand conformation polymorphism (SSCP) analysis including electrophoresis, staining of the gels, preparation of migration markers and processing and normalization of gel scans were conducted as described previously (Kampmann et al., 2012b). Intensive DNA-bands that appeared or disappeared before or after acidification or were dominant during the entire incubation time were cut out