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Microbiome dynamics and adaptation of expression signatures during methane production failure and process recovery

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ABSTRACT

This study aimed to uncover microbial dynamics and transcriptional adaptations during mesophilic AD of maize silage and slurry. While one digester performed under optimal conditions, the investigations also evaluated the microbiome during a temperature drop mediated process failure accompanied by acidification and how it contributed to a process recovery. Composition and pathway activities were analyzed by *whole genome shotgun* (WGS) and metatranscriptome sequencing, respectively. A biodiversity of 112 species was observed with noticeable shifts over process time. Although four distinct groups of microbes could be identified with a correlating versatility according to substrate and to process disturbance, also tremendous effects on gene expression were monitored especially of the archaeal methane metabolism. Particularly, the expression of acetogenotrophic methanogenesis related genes was identified to be relevant for process regeneration.

1. Introduction

Biogas production is a very attractive ecological technology for the sustainable generation of renewable energy. The anaerobic digestion (AD) of organic matter generates methane which can substitute natural gas and in contrast to other renewable energies is easily storable (Weiland, 2010). It is expected that the need of balancing energies will increase in the future whereby methane from biogas has a promising economic growth potential (Tafarte et al., 2014). Another benefit of AD is the energetic use of agricultural and industrial waste. However, maize as a substrate in the bio-methane production is dominating other energy crops (Hutňan, 2016).

The process of AD can be divided into four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Batstone et al., 2002), each concerted by a complex interplay of primary fermenting bacteria, anaerobic fermenting bacteria, oxidizing bacteria and methanogenic archaea (Angelidaki et al., 2011). The balance within these distinct microbial groups is fundamental for a stable process and crucial for methane yield (Demirel & Yenigün, 2002). As these groups require significantly different environmental conditions it is challenging to ensure the balance during biogas production. Mostly, the process temperature is mesophilic (35–42 °C), while some plants are also producing methane under thermophilic conditions (45–60 °C) (Weiland, 2010). As one the key parameters, temperature is known to unbalance or inhibit the AD leading to an accumulation of organic acids and/or a decrease in methane yields (Chen et al., 2008). Currently, there is little known about the identity of the microorganisms, their dynamics, interplay and functions during the digestion process as well as in varying operating conditions. Biogas plants remain a “black box” for their operators with only few parameters to interfere in case of dysregulation. However, the identification of potential key players in the process and a better understanding of the role of different groups of microorganisms are mandatory for targeted interventions.

In fact, the microbiome’s complexity and the cross-species interplay make investigations challenging – simply due the vast lack of knowledge in species composition remaining mostly microbial dark matter. Traditional microbiological methods are limited in their ability to investigate comprehensively the microbial community and cannot go beyond the identification of some *in vitro* cultivable species most likely due to the complex interplay between the many dozens of microorganisms within the process (Campanaro et al., 2016). In contrast, molecular techniques like next-generation sequencing (NGS) provide an

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enormous volume of data and pave the way for complex metagenomic and -transcriptomic studies that were unimaginable before (Metzker, 2010). It allows the identification of unknown, non-cultivable microbial organisms and sheds a light on their function in the ecosystem of environmental or other complex samples. Especially approaches using *whole genome shotgun* (WGS) sequencing are promising for such poorly described populations as they allow for a *de novo* assembly which can then be used for a more accurate assignment to distinct taxons and beyond that for *in silico* protein annotation giving information about the functional properties of the population (Bremges et al., 2015; Schlüter et al., 2008; Wirth et al., 2012).

Metagenomic studies were performed within the last years investigating the microbial community according to different feed compositions and radical feedstock changes, which show a correlation between the type of the substrate and the microbial composition (De Francisci et al., 2015; Ziganshin et al., 2013). Further the effect of a temperature drop from 37 °C to 17 °C was shown to change the genera shares (Regueiro et al., 2014). Two other working groups increased the organic loading rate to initiate acidification also resulting in relevant shifts in the microbial composition (Goux et al., 2015; Kampmann et al., 2014).

Recently, as the publically available databases massively lack biogas related organisms, two recent studies focused on establishing a specific biogas microbiome reference database at species level resolution. Through implementing new binning strategies on the metagenomic assemblies the working groups published in total 236 nearly complete new genomes, each representing a novel species (Campanaro et al., 2016; Treu et al., 2016).

Indeed, population proportions and functional properties are deducible from such metagenomic information, but it does not reveal the activity of certain genes or pathways. Previous studies demonstrated that such activity can be inferred from metatranscriptomic data (Lin et al., 2016), but Zakrzewiski and colleagues showed that it is crucial for a meaningful analysis of the metatranscriptomic reads to generate a reference genome from the same environment (Zakrzewski et al., 2012). Thus, only an approach combining WGS sequencing with a *de novo* reference genome assembly and RNA-seq will allow the determination of the functional potential of versatile populations in an unbiased way. It can give comprehensive insights whether a single population is involved in multiple pathways or solely specializes on a certain step like Jiang and colleagues recently showed for five different environments (Jiang et al., 2016). Two working groups showed such combined approach for a mesophilic and a thermophilic agricultural biogas plant and reconstructed pathways showing high methane metabolism activity (Bremges et al., 2015; Maus et al., 2016).

The aim of our study was to uncover microbial dynamics as well as transcriptional adaptations during the whole digestion process starting from inoculation to a stable biogas production yield in mesophilic lab scale reactors. Further, this study evaluated the microbiome during a temperature drop mediated process failure accompanied by acidosis and how it contributed to the recovery of the process on a functional level.

2. Methods

2.1. Fermentation setup

Anaerobic Digestion Tests were carried out in a Continuous Biogas Test (CBT), among a series of trials on process stability and specific methane yield of maize silage variants in co-digestion with inoculated slurry and with inoculated slurry alone. The inoculum was taken from a full-scale research biogas plant fed on a mixture of approximately 35% slurry from cows, pig, chicken, sheep and horse and 65% renewable energy crops such as maize- and grass-silage as well as ground grain (Naegele et al., 2014). For this series three horizontal stainless steel digesters each with a gross volume of 20 L were used according to the VDI Guideline 4630 (VDI, 2006). The daily feeding amount was 150 g maize silage and 120 g separated digestate from the mentioned secondary digester of the full-scale research biogas plant. The substrates were fed into the tubular reactor, on the front side via an inlet and stirred with an electric driven reel agitator in intervals of 1 min agitations which was followed by a 3 min break (Haag et al., 2015). On the back side the digested substrate was taken out via an overflow. The process was heated to 41 °C by a water bath and circulation thermostat connected to a heating system surrounding the drum. The biogas was continuously released from the top end of the digester and flowed through a gas wash bottle into a storage bag. A gas measuring unit automatically analyzed the gas quantity (mass flow meter, Bronkhorst, Germany), as well as the content of CH_4 and CO_2 (D-AGM Plus, Sensors Europe, Germany) (Haag et al., 2015). The measurements were carried out once a day and corrected to standardized conditions of 1013 hPa and 273.15 K. As a control variation, one digester (R3) was fed with pure inoculation slurry, to get a correction factor to subtract the biogas and methane production of the inoculum from the samples being tested. Digester R1 and R2 were fed at an organic loading rate of 3 kg VS m^{-3} d^{-1} digester volume per day, resulting in a Hydraulic Retention Time (HRT) of 44 days. The ratio of inoculum to the maize silage was kept at 45/55% fresh matter (FM). The determination of Volatile Fatty Acids and the Volatile Organic Acids/Total Inorganic Carbon (VOA/TIC) value, as a measure for process stability, was carried out by High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC). The samples were treated according to a laboratory standard of the State Institute of Agricultural Engineering and Bioenergy (Haag et al., 2015).

2.2. Sampling and DNA/RNA extraction

From each reactor (R1, R2, R3), sample aliquots of 1 ml were taken and immediately frozen as beads by dropping into liquid nitrogen 7 d, 21 d, after starting point, plus one aliquot from inoculum at starting point 0 d (five sampling time points). To evaluate the stability and recovery of the populations, samples were also taken at 42 d and 84 d corresponding to the first and the second HRT, respectively. Cell disruption was carried out using a Mixer Mill MM 200 (RETSCH, Germany) with a shaking frequency of 30/s under cryo conditions. For DNA extraction half of the resulting powder was resuspended in 500 µl AL lysis buffer supplemented with 50 µl Proteinase K from Qiagen's QIAamp DNA Mini Kit (Qiagen, Germany) and incubated for 10 min at 56 °C while for RNA extraction the other half of the powder was resuspended in lysis buffer RLT plus supplemented with 0.01% v/v of ß-mercaptoethanol from Qiagen's RNeasy Plus Mini Kit (Qiagen, Germany). The following steps for both protocols were performed according to the manufacturer's instructions. For a higher purity an additional Agencourt XP Bead purification (Beckman Coulter, USA) step was performed for both nucleic acid types according to the manufacturer's instructions with a bead volume ratio of 1.8 × (AMPure XP beads for DNA and RNAClean XP beads for RNA purification). DNA and RNA quantification was carried out using the Qubit dsDNA HS Assay Kit (Thermo Fisher, USA) and the RNA 6000 Nano Kit on Agilent's Bioanalyzer 2100 (Agilent Technologies, USA), respectively. Simultaneously, RNA integrity was evaluated using the Bioanalyzer. DNA quality was checked on a 0.8% agarose gel.

2.3. Sample preparation and sequencing

Isolated DNA was prepared according Illumina's Nextera DNA Sample Prep Kit protocol with an input amount of 50 ng (Illumina, USA). Libraries were sequenced by a HiSeq2500 (Illumina, USA) with a length of 150 bases in paired-end mode and a depth of approximately 13 mio. read pairs per sample. To analyze the metatranscriptomes, RNA was prepared using Illumina's ScriptSeq Complete Kit for Bacteria according to its low input protocol (Illumina, USA). An amount of 100 ng