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Influence of nitrogen-rich substrates on biogas production and on the methanogenic community under mesophilic and thermophilic conditions

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ABSTRACT

Grass silage was evaluated as a possible substrate in anaerobic digestion for generation of biogas in mesophilic and thermophilic long-term operation. Furthermore, the molecular biological parameter Metabolic Quotient (MQ) was evaluated as early warning system to predict process disturbance. Since this substrate is rich in nitrogen, high ammonia concentration of up to 2.2 g * kg_{FM}^{-1} emerged. The high buffer capacity of the ammonium/ammonia system can disguise upcoming process acidification. At organic loading rates (OLR) below 1.0 $kg_{VS} * m^{-3} * d^{-1}$ (VS: volatile solids) for thermophilic and below 1.5 $kg_{VS} * m^{-3} * d^{-1}$ for mesophilic reactors, stable processes were established. With increasing OLR, the process was stressed until it broke down in the thermophilic reactors at an OLR of 3.5 $kg_{VS} * m^{-3} * d^{-1}$ or was stopped at an OLR of 4.5 $kg_{VS} * m^{-3} * d^{-1}$ in the mesophilic reactors. Mainly propionic acid accumulated in concentrations of up to 6.5 g * kg_{FM}^{-1}. Due to the high buffer capacity of the reactor sludge, the chemical parameter TVA/TIC (ratio of total volatile acids to total inorganic carbon) did not clearly indicate process disturbance in advance. In contrast, the MQ indicated metabolic stress of the methanogens before process breakdown and thus showed its potential as early warning system for process breakdown. During the whole experiment, hydrogenotrophic methanogens dominated. In the thermophilic reactors, *Methanoculleus* IIA-2 sp. 2 and *Methanothermobacter wolfeii* were dominant during stable process conditions and were displaced by *Methanobacterium* III sp. 4, a possible new bioindicator for disturbances at these conditions. In the mesophilic reactors, mainly *Methanobacterium* III sp. 4 was dominant at stable, stressed and acidified processes. A hitherto uncultivated genospecies, *Methanobacteriaceae* genus IV(B) sp. 3 was determined as possible new bioindicator for mesophilic process disturbance.

1. Introduction

Biogas production from renewable resources can reduce fossil energy consumption and mitigate greenhouse gas emissions. Due to increasing closure of dairy cattle farms, previously grazed grassland is available for anaerobic digestion to produce heat, fuel and electricity [1]. However, this lignocellulosic biomass is challenging for process engineering and for the microbial community conducting the anaerobic digestion. The high fiber content can cause abrasion of stirrers, blocking of pumps or sinking or floating layers. Grass silage can introduce high amounts of phenolic compounds, like coumarin, in the process which can inhibit the biogas production [2]. In addition, the high nitrogen content of grass silage causes a high ammonia concentration in the reactor sludge. Ammonia is a highly toxic compound which inhibits the energy gain of microorganisms. This can lead to inactivation or death of microorganisms and hence to process disturbance or in the worst case to process breakdown. Concerning methanogenic archaea, especially *Methanosaetaceae* are affected by high ammonia concentrations [3,4]. Hydrogenotrophic methanogens get dominant under these conditions. They interact with syntrophic bacteria which oxidize intermediate substrates and acetate [5]. The endergonic syntrophic reactions are only possible when hydrogenotrophic methanogens use the produced hydrogen and hence keep the hydrogen partial pressure low [6]. The ammonium/

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ammonia system has a high buffer capacity and can disguise upcoming process acidification. In this case, the well-established TVA/TIC (ratio of total volatile acids to total inorganic carbon) ratio does not reliably indicate process disturbance. This means that other parameters have to be used as early warning system to predict process disturbance. Previous studies used different chemical parameters [7] or stable isotope biogas probing [8–10]. A more direct approach is to analyze the workhorses of the biogas process, the microorganisms with molecular biological tools. They perform the chemical reactions in the process and are affected first before chemical or physical parameters indicate process disturbance. In a previous study, the metabolic quotient (MQ) was developed as a microbiology early warning system using a correlation between the concentration of methanogens and the methane productivity of the investigated reactor sludge [11]. This parameter has been based on mesophilic reactors operated with maize silage as the substrate. It was assumed that this parameter is also applicable for different substrates and conditions because methanogens can use only C_1-substrates like CO_2, acetate and methyl-groups to produce methane.

In this study, the feasibility was evaluated to operate anaerobic reactors with perennial rye grass silage as sole substrate at mesophilic and thermophilic reactors (38 °C, 55 °C) in a long term operation. The methanogenic community composition was analyzed to investigate the adverse conditions and how the community changes at stressed processes or at process breakdown. This should lead to development of methanogenic bioindicators for stable, stressed and acidified processes when grass silage is used as sole substrate in the biogas process. In addition, the parameter MQ was tested for its applicability on grass silage as substrate and on different temperatures.

2. Materials and methods

2.1. Biogas reactor management

Two mesophilic and two thermophilic continuously stirred lab-scale reactors (28 L) were fed with perennial ryegrass silage prepared without additives (see Table S1 for substrate composition). The lab-reactors were inoculated with sludge from a mesophilic pilot reactor which was fed with cattle manure and total mixed ration. For the thermophilic reactors the temperature was increased directly from 38 °C to 55 °C. After a short adaptation phase of ca. 2 weeks, the biocenosis adapted to the thermophilic conditions and the gas production started. The mesophilic reactors needed an adaptation phase of approximately one week to the new substrate. Firstly, the reactors were fed with a low organic loading rate (OLR) to establish a stable process (stable specific methane yield (SMY) > 350 L_{STP} CH_4 * kg_{VS}^{-1}, TVA/TIC < 0.5, VFAs < 2,000 mg * kg_{FM}^{-1}; STP: standard temperature and pressure; TVA/TIC: ratio of total volatile acids to total inorganic carbon; VFA: volatile fatty acids). Both, the mesophilic and the thermophilic biocenoses were subsequently stressed by increasing the OLR until process breakdown. Conventional chemical parameters, like pH, dry matter (DM), volatile solids (VS) content, volatile fatty acids (VFA) concentration and ammonium content were analyzed irregularly before feeding changes or indication of process disturbance as described earlier [11–13]. Physical process parameters such as biogas production and composition were monitored online regularly.

2.2. Extraction of nucleic acids, qPCR and RT-qPCR

Samples were taken from the reactor sludges at four different process stages (Fig. 1). DNA was extracted according to the protocol in Munk et al., 2010 [12]. In brief, 500 μL of digester sludge was washed twice in 0.85% potassium chloride (KCl) and 40 μL of washed sample was processed using the FastDNA Spin Kit for Soil (MP Biomedicals). The DNA was extracted according to the supplier's protocol, except for a second washing step with washing buffer SEWS-M. Finally, the DNA was eluted with 100 μL water. The DNA extraction efficiency was over 90%, as determined in previous spiking experiments where pre-quantified bacterial cell suspensions were added to the sample (free of these bacteria), and extraction was done as described [14,15]. The extraction efficiency is calculated by the fraction “recovered copy number”/“spiked copy number”.

Total RNA was extracted using the Fast RNA Pro Soil – Direct Kit (MP Biomedicals) according to the manufacturer's protocol except for pre-washing of the sludge sample (see DNA extraction) and a second washing step with 70% ethanol after isopropanol precipitation. The RNA was eluted with 100 μL nuclease-free water. The eluted RNA was treated with DNase (TURBO DNA-*free* kit, Ambion) to eliminate DNA contamination. 5 μL of DNA-free RNA was used for Reverse Transcription (RT) with AffinityScript Multi-Temp RT (Agilent) according to the manufacturer's protocol for gene-specific primers at 45 °C for 60 min. In a spiking experiment using extracted and pre-quantified (most probable number-qPCR) Enterovirus RNA the extraction efficiency was ca. 70% with the given RNA extraction kit [13,15]. This efficiency was taken into account for the calculation of the transcript concentration.

One μL of undiluted and of 10-fold diluted DNA or cDNA was used for quantitative Real-Time PCR (Mx3005P, Agilent) in triplicates using primers for the gene *mcr*A/*mrt*A or its transcripts (MeA-i 1046f: 5′-TAYATGWSIGGHGGIGTIGGITTYAC-3′ and MeA-i 1435r: 5′-TGRTCYTGIARRTCRWAICCRWAGAAICC-3′) as described earlier [13]. The comparison of the undiluted and diluted results gave indications if the PCR was inhibited by checking the expected difference of Cq-values of ca. −3.3. In addition, one μL of RNA treated with DNase was used as control if there was still DNA contamination in the sample. An external standard basing on gene inserts in the pCR®4 vector transformed in OneShot® TOP10 chemically competent cells (Invitrogen; see section Profiling of the methanogenic community) was used for quantification.

2.3. Profiling of the methanogenic community

In order to determine the methanogenic community composition in the reactors, a PCR cloning approach was conducted. After amplification of the *mcr*A/*mrt*A gene with primers MeA-i 1046f and MeA-i 1435r, the amplicons were checked for the correct length on an agarose gel, ligated into the pCR®4 vector and transformed into OneShot® TOP10 chemically competent cells using the TOPO-TA® cloning kit (Invitrogen). Clones were randomly picked and checked for the correct insert using M13 primers. Inserts with correct length were sequenced by Eurofins MWG Operon, Germany using the provided primer M13 uni (−43). The received sequences were included in dataset containing most of the *mcr*A/*mrt*A sequences from the NCBI nucleotide database and aligned using the software MEGA 6.06 [16].

Sequences obtained in this study were deposited in the NCBI GenBank database under accession numbers KY596700 to KY597462.

2.4. Metabolic quotient (MQ) and cDNA/DNA ratio

In previous studies the parameter Metabolic Quotient (MQ), was developed as early warning indicator of process disturbance [11]. The specific methanogenic activity (SMA) is determined at the sampling time point by dividing the methane productivity through