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Proteotyping of laboratory-scale biogas plants reveals multiple steady-states in community composition

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ABSTRACT

Complex microbial communities are the functional core of anaerobic digestion processes taking place in biogas plants (BGP). So far, however, a comprehensive characterization of the microbiomes involved in methane formation is technically challenging. As an alternative, enriched communities from laboratory-scale experiments can be investigated that have a reduced number of organisms and are easier to characterize by state of the art mass spectrometric-based (MS) metaproteomic workflows.

Six parallel laboratory digesters were inoculated with sludge from a full-scale BGP to study the development of enriched microbial communities under defined conditions. During the first three month of cultivation, all reactors (R1-R6) were functionally comparable regarding biogas productions (375–625 NL $L^{-1}_{reactor\ volume}\ d^{-1}$), methane yields (50–60%), pH values (7.1–7.3), and volatile fatty acids (VFA, <5 mM). Nevertheless, a clear impact of the temperature (R3, R4) and ammonia (R5, R6) shifts was observed for the respective reactors. In both reactors operated under thermophilic regime, acetic and propionic acid (10–20 mM) began to accumulate. While R4 recovered quickly from acidification, the levels of VFA remained to be high in R3 resulting in low pH values of 6.5–6.9. The digesters R5 and R6 operated under the high ammonia regime (>1 $gNH_3\ L^{-1}$) showed an increase to pH 7.5–8.0, accumulation of acetate (>10 mM), and decreasing biogas production (<125 NL $L^{-1}_{reactor\ volume}\ d^{-1}$).

Tandem MS (MS/MS)-based proteotyping allowed the identification of taxonomic abundances and biological processes. Although all reactors showed similar performances, proteotyping and terminal restriction fragment length polymorphisms (T-RFLP) fingerprinting revealed significant differences in the composition of individual microbial communities, indicating multiple steady-states. Furthermore, cellulolytic enzymes and cellulosomal proteins of *Clostridium thermocellum* were identified to be specific markers for the thermophilic reactors (R3, R4). Metaproteins found in R3 indicated hydrogenothrophic methanogenesis, whereas metaproteins of acetoclastic methanogenesis were identified in R4. This suggests not only an individual evolution of microbial communities even for the case that BGPs are started at the same initial conditions under well controlled environmental conditions, but also a high compositional variance of microbiomes under extreme conditions.

1. Introduction

Conversion of agricultural waste into biogas is a sustainable source of renewable energy. The so called anaerobic digestion (AD) is performed in large parallel or serial digester systems of different sizes and designs, commonly referred to as biogas plants (BGP). Additional classifications are made depending on the process temperature [1], the type (e.g. silage and/or manure and dung) and consistency (e.g. moisture content) of the used substrate [2,3], and

Abbreviations: 6-FAM, 6-carboxyfluorescein; AD, anaerobic digestion; ACN, acetonitrile; RT, room temperature (21 °C); bp, base pair; BGP, biogas plant; FA, formic acid; HRT, hydraulic retention time; Ino, inoculum sample; LC, liquid chromatography; M, mesophilic sample; MS, mass spectrometry/spectrometer; MS/MS, tandem MS; N/high-N, process with high load of nitrogen source; R, reactor; SAO, syntrophic acetate oxidation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SpC, spectral count; T, thermophilic sample; TRF, terminal restriction fragment; VFA, volatile fatty acid(s); xCGE-LIF, multiplexed capillary gel electrophoresis with laser induced fluorescence detection.

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the ammonium or ammonia concentrations [4]. Independent from these conditions, AD process is subdivided into the four steps hydrolysis, fermentation, acetogenesis and methanogenesis [3], which are executed by different groups of microorganisms forming complex microbial communities — the microbiome [5].

Laboratory-scale digester systems (ranging from a few hundred milliliters to several liters working volume) are commonly used as a scale-down model to investigate AD [6–8]. These systems benefit from a better control over the cultivation parameters, and allow well-directed disturbances without risking costly malfunction of full-scale BGP. In a highly controlled process, the substrate is fully defined and continuous stirring enables homogeneous mixing and representative sampling. This is in contrast to full-scale BGP with occasional dead zones or floating layers [3,9], and varying and non-sterile substrates [10]. However, microbial communities evolving in laboratory bioreactors operating under well-defined process conditions loose part of their complexity [11]. While this facilitates analytics, the question arises to what extent results can be transferred to the optimization of full-scale BGP.

Many different analytical methods are routinely applied to study microbial communities in BGP. Genomic approaches, like cloning and sequencing of microbial DNA [12] or fingerprinting of 16S rRNA genes (e.g. T-RFLP — terminal restriction fragment length polymorphisms [13]), allow to explore the diversity of *Archaea* and *Bacteria* of microbial communities. As a complementary approach, metaproteomics turned out to be well suited to capture the physiological state and functions of a microbial population [14]. State of the art methods rely on gel-free approaches [15] pushed by the rapid development of high resolving mass spectrometers (MS) for protein identification, and powerful tools for data analysis [16]. Their application revealed great potential for the characterization of mixed microbial communities, which was referred recently as proteotyping [17]. So far, this term was only used for the identification of single microorganisms by characteristic protein mass spectra derived from Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight MS analysis (MALDI-TOF-MS) [18]. In a recent review, however, the term proteotyping was extended to cover classification, characterization and identification of microorganisms as well as microbial communities by tandem MS and MS/MS-based shotgun proteomics [19]. The first comprehensive proteotyping of microbial communities in technical biocoenoses aimed at the correlation of biological processes of the microbiome in BGP with respective process parameters [17]. Applications of biostatistics and data mining tools (e.g. principal component analysis or clustering) allowed identifying correlations of taxonomies, functions and metaproteins with process parameters (e.g. temperature, substrate, reactor design or nitrogen content) from extensive lists of identified proteins — without laborious hit-by-hit evaluation.

In this study six parallel digesters were inoculated with sludge from a full-scale BGP to enrich microbial communities under defined conditions. After three month of cultivation, steady-state operation was achieved for all digesters. Subsequently, the temperature and the ammonia concentration were increased for two reactors each. Based on metaproteomics the following questions were addressed: how similar are stable microbial communities operating under exactly the same environmental conditions? Can marker species or functions be determined representing the different process regimes using proteotyping?

2. Material and methods

2.1. Reactor setup

For enrichment, a Sixfors multi bioreactor system (INFORS AG, Bottmingen, Switzerland) with six parallel 500 mL glass vessels was used (R1-R6; 400 mL working volume). Each reactor was equipped with an integrated Pt100 temperature probe, a pH electrode (Type 405-DPAS-SC-K8S, Mettler-Toledo GmbH, Gießen, Germany), and gastight tubing (Santoprene® LEZ-SAN, ID 1.6 mm, thickness 1.6 mm, Medorex, Nörten-Hardenberg, Germany) connected to a Luer/Lock sampling valve (Eppendorf AG, Hamburg, Germany). Agitation was performed by a magnetic propeller stirrer (INFORS AG, Bottmingen, Switzerland); an exhaust gas cooler supplied with 20 °C cold water removed any aqueous vapor from the produced biogas. All remaining reactor ports were closed by either plugs or bypassed with gastight tubing.

2.2. Sample origin and reactor inoculations

Approximately 5 L of fresh biomass were collected from a local BGP (Magdeburg, Germany, see Supplementary_Note 1 for details about setup, process and substrates) for reactor inoculations and directly transferred to the laboratory. Sludge was centrifuged (3000×*g*) in 50 mL vessels for 3 min at room temperature (RT) to remove crude fibers to obtain more homogenous suspensions in the digesters. For each of the six laboratory-scale reactors 190 mL supernatant were pooled and mixed in the reactor with 210 mL tap water preheated to 40 °C. First regular feeding/sampling was performed two days later along with the installation of the "GärOnA" gas analysis system, which will in the following be referred to as the starting point of the cultivation (0 d).

2.3. Medium and feeding

The basic composition of the medium was described by Bensmann et al. [20] with the following exception: meat extract, yeast extract, and peptone were replaced by 11.5 g L^{-1} glucose and 34.4 g L^{-1} cellulose as carbon source to exclude any undefined compounds. In addition 2.56, 5.13 or 15.38 g L^{-1} urea were used as a nitrogen source, depending on the process regime (see below). Finally, the vitamin solution was supplemented by 0.05 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} pantothenic acid, 0.02 mg L^{-1} biotin, 0.02 mg L^{-1} folic acid and 0.05 mg L^{-1} 4-aminobenzoic acid. The carbon, nitrogen and phosphor ratio (C:N:P) of the media was 105:7:1 (R1-R4) and 122:41:1 (R5-R6), respectively.

To allow adaption of microorganisms to the defined media, the substrate dosage was increased slowly from 5 mL per feeding during the first ten days to 7.5 mL from day 10–14 and finally 10 mL until the end of the experiment. Sampling was performed directly before feeding to keep the reactor volume constant, and to achieve quasi-continuous process conditions. During the main phase of cultivations (day 14–298), an average of 4.5 feedings per week was performed, which corresponds to a hydraulic retention time (HRT) of 62 d (Table 1).

Table 1
Medium and feeding characteristics for the different process regimes of R1-R6.

Process time [d]	Substrate [mL]	Urea in medium [g L^{-1}]		Molar C:N:P ratio of medium		HRT[a] [d]
		R1 to R4	R5, R6	R1 to R4	R5, R6	
0–10	5	2.56	2.56	105:7:1	105:7:1	200
10–14	7.5					71
14–93	10					62
93–200			5.13		108:14:1	
200–298			15.38		122:41:1	

[a] HRT: hydraulic retention time.