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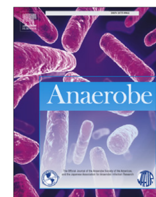

Molecular Biology, Genetics and Biotechnology

Towards molecular biomarkers for biogas production from lignocellulose-rich substrates

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ABSTRACT

Biogas production from lignocellulose-rich agricultural residues is gaining increasingly importance in sustainable energy production. Hydrolysis/acidogenesis (H/A) of lignocellulose as the initial rate-limiting step deserves particular optimization. A mixture of straw/hay was methanized applying two-phase digester systems with an initial H/A reactor and a one-stage system at different, meso- and thermophilic temperatures. H/A was intensified with increasing pH values and increasing temperature. H/A fermenters, however, were prone to switch to methanogenic systems at these conditions. Substrate turnover was accelerated in the bi-phasic process but did not reach the methanation efficiency of the single-stage digestion. There was no indication that two different cellulolytic inocula could establish in the given process.

Bacterial communities were analyzed applying conventional amplicon clone sequencing targeting the hypervariable 16S rRNA gene region V6–V8 and by metagenome analyses applying direct DNA pyrosequencing without a PCR step. Corresponding results suggested that PCR did not introduce a bias but offered better phylogenetic resolution. Certain *Clostridium* IV and *Prevotella* members were most abundant in the H/A system operated at 38 °C, certain *Clostridium* III and *Lachnospiraceae* bacteria in the 45 °C, and certain *Clostridium* IV and *Thermohydrogenium/Thermoanaerobacterium* members in the 55 °C H/A system. *Clostridium* III representatives, *Lachnospiraceae* and *Thermotogae* dominated in the thermophilic single-stage system, in which also a higher portion of known syntrophic acetate oxidizers was found.

Specific (RT-)qPCR systems were designed and applied for the most significant and abundant populations to assess their activity in the different digestion systems. The RT-qPCR results agreed with the DNA based community profiles obtained at the different temperatures. Up to 10^{12} 16S rRNA copies mL^{-1} were determined in H/A fermenters with prevalence of rRNA of a *Ruminococcaceae* subgroup. Besides, *Thermohydrogenium/Thermoanaerobacterium* rRNA prevailed at thermophilic and *Prevotellaceae* rRNA at mesophilic conditions. The developed (RT)-qPCR systems can be used as biomarkers to optimize biogas production from straw/hay and possibly other lignocellulosic substrates.

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1. Introduction

Mainly due to the German 'Renewable Energy Sources Act' (EEG) which was released first in 2004, agricultural biogas production has enormously increased in Germany in the last decade. The German Biogas Association predicts nearly 7900 biogas plants with an installed electric power of about 3750 MW in Germany for 2014 [1].

This development has additionally been fueled by the German 'national renewable energy action plan' [2] which stipulates that over 35% of the electricity consumption shall be supplied by renewable energy sources in the year 2020, and up to 80% in 2050. Wind and solar energy will contribute most to electricity

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generation in the German 'Energiewende' [3]. The contribution of bioenergy to electricity supply will be relatively small, but bioenergy is important for the 'Energiewende': beside electricity generation, biomass significantly contributes to heat supply, and biomethane is an emerging sustainable vehicle fuel. Biomass can be stored and biomethane can easily be distributed via the existing gas grid. Bioenergy can thus not only be used to provide some of the basal electricity supply, it can also be converted on demand, balancing energy gaps and overproduction emerging temporarily and seasonally in wind and solar energy generation.

Bioenergy will mainly be derived from agricultural biogas production, due to the outstanding advantages of methane in energy storage, the regionally regressing agricultural food and fodder production forcing farmers to switch to energy suppliers, and the limited availability of biowaste or other suitable input material e.g. from forestry. In Germany, the main substrates for biogas production are energy crop silages such as whole plant silages, maize, cereals and grains, grasses or beets, frequently along with liquid and/or solid manure. Energy crops are the most favored input materials due to their high potential methane yield, yet there has been a public debate on their competition with food production. Therefore, the utilization of 'hard-to-digest' lignocellulose-rich agricultural residues such as scrap material from landscape conservation, hay and straw is envisaged increasingly. More details on the boom of renewable energies and biogas particularly in the last decade can be found in a recent review [4].

In principle, the energy contained in methane of biogas produced from plant material is solar energy fixed by photosynthesis and converted by microorganisms to form biogas. About 70 gigatons of carbon are fixed annually by photosynthesis [5], mainly resulting in cellulose fibers of plant scaffold structures. For profitable methane production in biogas plants from lignocellulose-rich biomass, not only difficulties in substrate conditioning but also mechanical process engineering have to be overcome. It is essential to speed up the throughput, to provide optimum conditions for maximum activity of the process-relevant populations [4].

Ghosh proposed to separate the process physically in two phases [6], hydrolysis (technical term for the combined biological processes of hydrolysis, acidogenesis and partially acetogenesis, H/A, methane in biogas not more than 5–10%) and methanogenesis (technical term for the combination of some hydrolysis, acidogenesis and acetogenesis along with methanogenesis as the dominant process) because they are believed to run best at different conditions. Hydrolysis, which is initially the rate limiting stage, is reported to run optimally at pH 5.5–6.5 [7], while methanogenesis finds its optimum at pH 6.7–7.4 (higher pH values with more proteinaceous substrates possible). Although industrial two-phase systems are established to process biowaste with temporarily high variation in substrate composition, it is not known if a two-phase system does improve the digestion of agricultural lignocellulosic residues.

Along with the verification of characteristic biochemical values, knowledge on the composition of the biocenoses performing distinct sub-processes is essential if the process is to be optimized. However, there is only a limited number of reports on the microbial composition of technically and biologically different 'hydrolysis' (H/A) phases, and in the main these were targeting the 16S rRNA gene (16S rDNA, prokaryotic *rrs*), e.g. Refs. [8–10]. Additional uncertainty in the determination of the microbial community composition arises due to technical constraints. Several factors such as insufficient primer design, chimaera formation, preferential PCR or cloning artifacts have the potential to bias the results [11,12]. Since pyrosequencing directly from extracted DNA may confer the least bias [13], a comparison in the frame of a metagenome study should evaluate if the PCR/cloning approach does generate distortions.

We have therefore conducted a study on the microbial composition of H/A fermenters fed with a straw/hay mixture as a model substrate for lignocellulose-rich agricultural residues. The study should answer the following questions:

(i) Who is there, and does PCR/cloning bias results on the composition of the bacterial community?
(ii) At which concentration are specific relevant populations present?
(iii) Are the biocenoses of H/A phases different at different process temperatures?
(iv) Are there striking differences between the bacterial communities in the H/A phase and a single-stage system?
(v) Are the results on DNA and on RNA level different?
(vi) Can we derive process-specific biomarkers?

2. Material and methods

2.1. Digester management and sampling

Samples for molecular biology analyses were obtained from four different flow-through digester systems operated at the Bavarian State Research Center for Agriculture (LfL). Three of these were two-stage systems consisting of a horizontal tubular (40 L) and a vertical (72 L) continuously stirred tank reactor (Fig. 1).

The horizontal digesters were operated as hydrolytic/acidogenic (H/A) phases (<10% CH_4 in gas phase; HS1–HS3), HS1 at 38 °C, HS2 at 45 °C and HS3 at 55 °C, and the vertical digesters as methanogenic phases (MR) at 38 °C. The HS were fed daily with a mixture of dried hay and straw (50%/50%, weight/weight, Table 1) at an organic loading rate (OLR) of 3.5 $g_{VS}\ L_{FS}^{-1}\ d^{-1}$ (VS, volatile solids; FS, fermenter sludge) and recirculation liquid (0.4–1.5 $L_{FS}\ d^{-1}$) from the MRs to stabilize the pH values in the range pH 5.2–5.9. The MRs were fed daily after solid/liquid separation with the liquid phase which contained high amounts of fatty acids (Table 3). Samples for molecular biology were from equilibrated process stages. In order to provide representative samples, ca. 1 L fermenter sludge was recirculated twice from outlet to inlet and passed over for processing chemical and molecular biology analyses.

The fourth system (HS_{6shk}) was established with HS2 sludge that was adapted to 60 °C process temperature in a 6 L glass flask and operated as flow-through system in fill/draw mode at 60 °C. HS_{6shk} was initially intended to serve as a second HS in which a cellulolytic inoculum should be tested. The experiment started with adjustment to pH 6.8. Two days later, inoculation and HS_{6shk} feeding were started. Presscake of HS2 was fed until 2011-08-23 at an OLR of 4.8 $g_{VS}\ L_{FS}^{-1}\ d^{-1}$ with 9 d hydraulic retention time (HRT) and pH 6.8–7.0 in the process. After August 23, the OLR was decreased to 1.5 $g_{VS}\ L_{FS}^{-1}\ d^{-1}$. HS_{6shk} was inoculated twice weekly (11× in total) with 300 mL of a hydrolytic/acidogenic inoculum mainly consisting of *Clostridium thermocellum* (35%), *Clostridium stercorarium* (28%) and bacterium Aso3-CS349 (22%) [14]. Control reactor HS_{6k} which was inoculated with autoclaved inoculum was run in parallel (data not shown). However, since the CH_4 content increased from initially <10% after day 10 to over 45% in the gas phase in both HS_6 fermenters and measures to re-establish an H/A phase were not successful, HS_6 fermenters were regarded as single-stage methanogenic systems. During the experimental development, acetic acid equivalents decreased from ca. 1.5 g L_{FS}^{-1} to ca. 0.2 g L_{FS}^{-1}. Four samples were taken for molecular microbiology analyses, GF3_110726 (yymmdd, 2011-07-26) was taken immediately before initial feeding and inoculation, GF3_110728

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