Short communication

General Microbial Community Flexibility in Biochemical Methane Potential Assay is Highly Correlated to Initial Biogas Production Rates

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Abstract

Degradation of brewery spent grain as a novel test substrate was explored in routine biochemical methane potential assays (BMP) using three different inocula. Significant differences in the initial biogas production rates from spent grain, methane yield coefficients and final spent grain degradation were observed between inocula. Initial and developed communities degrading novel substrate showed significant differences in archaeal community fingerprints. Differences were observed irrespective of substrate identity (no substrate, glucose, spent grain) providing evidence of a significant general influence of BMP incubation on the microbial phylotypes. A linear relationship between microbial community flexibility in BMP assay and corresponding initial biogas production rates was identified as a novel parameter to diagnose anaerobic processes, particularly under dynamic conditions like start-up.

Keywords: Biogas; brewery spent grain; biochemical methane potential; T-RFLP; community flexibility

1. Introduction

Monitoring of anaerobic digestion is difficult and complex multivariate process.[1] Performance of such systems can be related to a number of different environmental parameters, but it is widely accepted that performance is tightly coupled to microbial community structure.[2,3,4] However, changes in community structure were shown also to occur at the time scale of days without detectable changes in performance.[3,4,5] Thus, the link between community structure and performance is unclear and more studies are needed.[6] In addition, very few studies investigating anaerobic microbial community structure have focused on simultaneously seeding replicate reactors, with few exceptions,[5,7,8] leaving thus little space for factorial experimentation. Despite these limitations, microbial diversity per se was shown to be of lesser importance for the biogas production than microbial community structure and its community flexibility.[3, 4]

Batch systems such as biochemical methane potential (BMP) or anaerobic toxicity assays (ATA)[9] have been routinely used as microbial model systems to determine optimal reaction conditions or compound toxicity due to the level of experimental control they offer in factorial experiments.[9,10,11] In attempt to link microbial communities and functional characteristics in ATA and BMP molecular analyses were conducted however, the structure of microbial communities present in negative and positive controls[12,13] and the starting community structures[12] have not been determined. To fill this gap, we analyzed and compared methanogen community shifts in association with routine process data under simulating batch start-up conditions using three different biomass inocula. Further, the relationship between the general community flexibility in BMP and initial biogas production rates was established as a novel parameter to diagnose anaerobic processes, particularly under dynamic conditions like start-up.

2. Experimental

2. 1. Inoculum and Substrate Collection

Inocula were obtained from three full scale digesters receiving different substrates: A – sewage sludge, B – urban organic waste and C – agricultural organic waste. Inoculum samples were collected aseptically in 20 L containers and immediately transferred to laboratory. None of the samples has treated brewery spent grain before.

The brewery spent grain was obtained from the brewery Pivovarna Laško, Slovenia. Substrate was collected in 20 L plastic containers and stored at 4 °C until use (< 5 days). TOC (106.8 g/kg) and TKN (13.511 g/kg) were determined according to the APHA methods (APHA, 2005) and SIS EN 25663 (1996), respectively. The C : N ratio was 7.9 and the NH_4^+ content 0.21 g/kg.

2. 2. Biochemical Methane Potential Assay (BMP)

The experiment was conducted essentially according to SIST EN ISO 11734:1999 standard and independently replicated twice. Oxitop® bottles (1170 mL) equipped with pressure sensors (WTW, Germany) were used as anaerobic batch reactors. Side neck sampling ports were sealed with butyl rubber stoppers to prevent gas leakage. The inoculum biomass concentration was adjusted to 2 g of volatile solids per liter (VS/L). Inoculum with no carbon substrate addition served as negative control of the residual metabolic activity. Positive control and experimental variants were supplied with 50 mg TOC of glucose or brewery spent grain per g VS inoculums (TOC/g $VS_{inoculum}$). The Oxitop® bottles were flushed with nitrogen gas for 15 minutes to ensure anaerobic conditions and were incubated at 37 °C and 120 rpm in a temperature-controlled incubator (Infors, Switzerland). Hourly measurements of total biogas produced during 14 days of incubation were recovered from pressure-sensor data loggers. The headspace gas composition was determined on day 0 and 14 by gas chromatograph (Shimadzu, GC14A-TCD). The gases were separated using 4 m long steel column with inner diameter of ¼ inches, filled with Porapak Q. Helium was used as a carrier gas. Injector and column temperature was 30 °C and the detector temperature was 80 °C. Calibration gas mixtures contained hydrogen, nitrogen, methane and carbon dioxide. Chromatographic signals were evaluated by integrator Chromatopack CR-4A (Shimadzu) based on an absolute calibration. The pH was measured on day 0 and day 14 using pH meter (Orion 520A).

2. 3. Microbial Community Structure

Genomic DNA was extracted using UltraClean Soil DNA Isolation kit (MOBIO) according to the manufacturer instructions for maximum DNA yield. The quality of DNA was checked by 1% agarose gel electrophoresis. PCR amplification was performed using MyCycler (BIO-RAD laboratories). The PCR primers 6-FAM labeled 109f (6-FAM, 5'-CAN GCT CAG TAA CRC GYR-3') and 691r (5'-CGA TTA CAR GAT TTC AC-3') were used to amplify the 16S rRNA gene of methanogenic archaeal community.[14] Three replicate PCR reactions were performed for each DNA extract and subsequently pooled as described before.[15] After the initial denaturation (5 min 94 °C), a total of 35 cycles, each including 60 s at 94 °C, 60 s at 53 °C, and 60 s at 72 °C, was followed by a final extension step of 10 min at 72 °C and PCR products were examined by electrophoresis on 1.5 % agarose gels. Residual primers were removed using High Pure PCR Product Purification Kit (Roche) according to the manufacturer instructions.

To provide a rationale in choosing gene-primers-enzyme combinations with highest resolution in T-RFLP analysis all restriction enzymes (http://rebase.neb.com/rebase/rebase.html) were tested on 120 000 archaeal sequences (Ribosomal Database Project II (http://rdp.cme.msu.edu/) Release10.05) using BESTRF software as described before.[16]

T-RFLP analysis was conducted as described before.[15] Digestion of 50 ng of PCR products was carried out in a 30 μL volume using 3 μL of 10x buffer and 0.5 μL of HhaI (10 U μL^{-1}, Fermentas Inc) in separate reactions. The volume was adjusted to 30 μL with nuclease-free water (Sigma-Aldrich) and incubated at 37 °C overnight. Digestions were inactivated for 15 min at 85 °C and purified by ethanol precipitation.[15,17] Separation, detection and basic GeneScan analysis of fluorescently labeled T-RFs were performed on an automated ABI PRISM 3100 DNA sequence analyzer (Applied Biosystems Inc.). Purified digested products were mixed with 0.5 μL internal-lane DNA standard (Genescan 500 ROX, Applied Biosystems Inc.) and 10 μL deionized formamide. Before analysis, DNA samples were denatured for 2 min at 95 °C and immediately placed on ice. T-RFLP patterns with total fluorescence exceeding the limit of 10.000 units were analyzed and peaks representing 0.5% or less of the total community signal were excluded from further analyses[15] using BioNumerics software (AppliedMaths, Sint-Martens-Latem, Belgium). Pearson's correlation coefficient with UPGMA clustering (unweighted pair-group method with arithmetic averages) was used to derive general similarity of community profiles and the significance of clusters was determined using cluster-cutoff tool.[15,17]

Variation partitioning was conducted in CANOCO 4.5 to extract the most important parameters explaining variation in microbial community data.[18] Detrended correspondence analysis was performed to determine whether unimodal or linear models fitted the community datasets better. Redundancy analysis (RDA) with forward selection was used to determine experimental parameters significantly associated with community data to eliminate co-linear variables. The significance of the final RDA was

determined by 999 Monte Carlo permutations. Data collected in BMP experiment served as explanatory variables. The association between flexibility of microbial community (defined as difference in Pearson's similarity between initial and developed microbial community fingerprints) and various BMP parameters was tested by multiple linear regression in STATISTICA 6 (StatSoft Inc.).

3. Results and Discussion

Initial methanogenic community in inoculum A differed significantly (54% dissimilarity) from inocula B and C. The latter shared a much larger fraction of communities (only 17% dissimilarity) (Figure 1) and formed one separate cluster from inoculum A community supported by cluster cut-off values. This general distinction between inoculum A and BC cluster was evident also after incubation in BMP as developed communities remained separated in two clusters (Figure 1). Significant starting differences in microbial communities were also recovered after incubation in BMP.

Developed inocula B and C were much more similar and branched closely to respective initial inocula communities (17% and 10% dissimilarity, respectively) (Figure 1). Developed microbial communities of inoculum A differed from initial community (35% dissimilarity), formed a separate cluster supported by cluster cut-off values and thus showed high response to novel conditions in BMP. It is important to note that microbial communities of either inoculum degrading novel substrate (spent grain) developed highly similar communities to respective glucose or no substrate BMP controls (> 91% similarity). Further, RDA identified incubation in BMP and inoculum as the key experimental parameters explaining 82.5% of the total variance in microbial community data ($P < 0.02$). These results show that for the correct distinction of effects of novel substrates in BMP or toxic compounds in ATA[9] from the general incubation effects observed in positive and negative controls, factorial design of experiments is needed. In published literature the start-up of unreplicated batch mode operated lab-scale reactors[4,5] are typically described (Supplementary material Figure S1).

To our knowledge, this may be one of the first studies which provided methanogen community dynamics in all routine BMP assay variants for three distinct inocula and statistically compared the community shift profiles. It also extends well to ATA, as reanalysis of published data reversed the outcome of previous studies[12] and showed that the effects of different substances on microbial communities could be detected (Supplementary material Figure S2).

Further, archaeal community flexibility was shown to correlate with stable biogas production in lab-scale operated reactors,[5,7,8] and during batch mode operated start-up of lab-scale reactors.[3,4] However, the relationship between community flexibility and routinely measured parameters in batch BMP has not been reported. In our study, biogas production rate from novel substrate spent grain (Table 1) and general community flexibility obser-

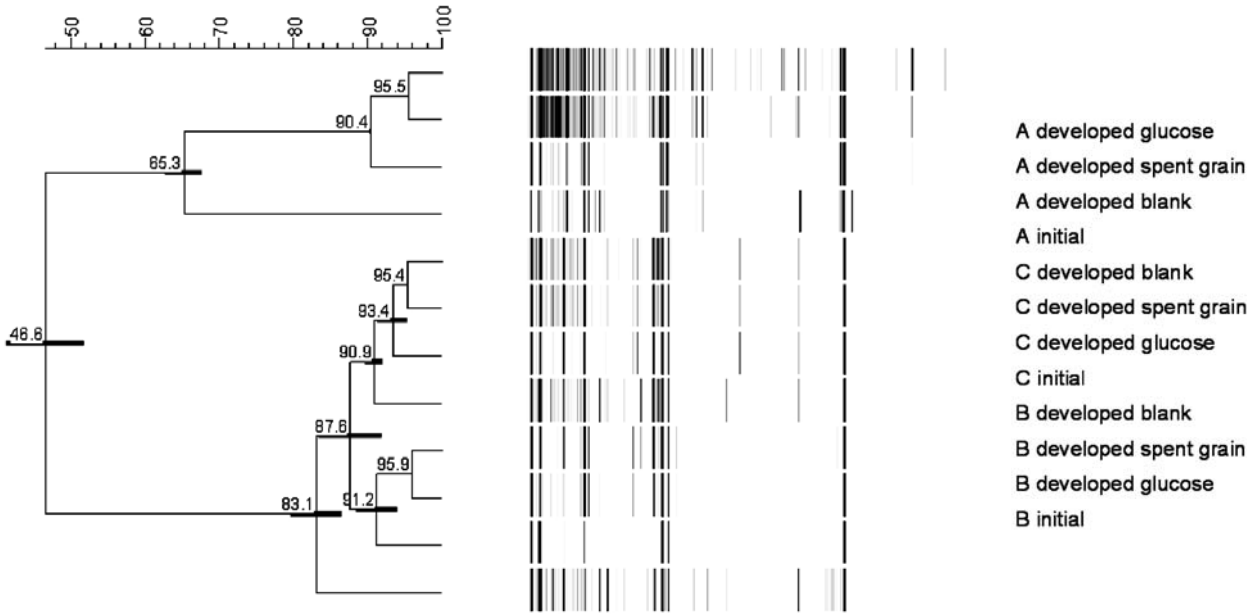

Figure 1: Pearson correlation dendrogram of T-RFLP fingerprints of methanogenic microbial communities originating from digesters treating sewage sludge (A), municipal organic waste (B) and agricultural waste (C). *initial* – inoculum at the onset of BMP assays; *blank, glucose, spent grain* – developed microbial communities without substrate or supplied with glucose or spent grain at 50 mg TOC/g $VS_{inoculum}$. The representative profiles are shown for simplicity. Gray lines delineate relevant clusters separated by the cluster-cut off method and similaritiy values are shown as numbers at each branch. Horizontal bars represent the reliability and internal consistency of the branch.

ved in BMP assays (Figure 1) showed a significant linear relationship ($P < 0.05$, $R^2 = 0.99$) (Figure 2).

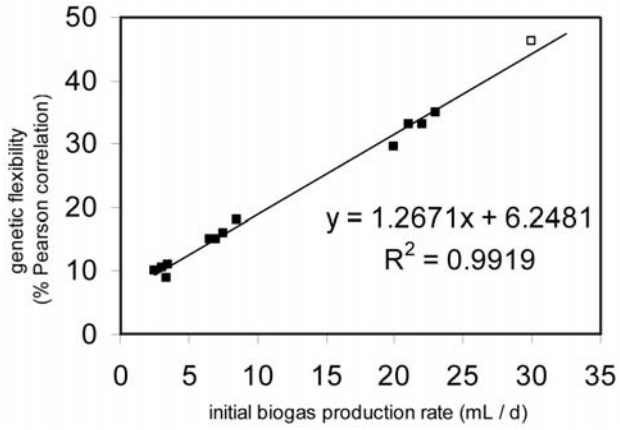

Figure 2: The relationship between initial biogas production rate and overall genetic flexibility observed in biochemical methane potential assays in experiments on three distinct inocula. □ – data re-calculated at day 13 of 42 day incubation after batch start-up (Figure S1).[4]

Table 1: Results of spent grain degradation in biochemical methane potential assay using microbial communities originating from digesters treating sewage sludge (A), municipal organic waste (B) and agricultural waste (C).

Parameter	A	B	C
initial biogas production rate (mL/d)	22.1 ± 1.5	7 ± 1.3	3 ± 1.2
adaptation period before maximum biogas production rates (d)	0.4	10.5	5
period before the start of stationary phase (d)	4	14	9
methane yield (mL CH_4/g TOC spent grain)	193 ± 4	215 ± 15.5	130 ± 12
spent grain degradation (%)	55 ± 4	65 ± 4	40 ± 3

Other routinely measured BMP parameters (Table 1) were not significantly related to general community flexibility in BMP assay. Thus the relationship between the general community flexibility and initial biogas production rates can be used as a novel parameter to diagnose anaerobic processes under dynamic conditions like start-up.

4. Conclusions

A general positive relationship between archaeal community flexibility and its initial biogas production rate were identified as a novel predictor of inoculum suitability in digestor start up.

5. Acnowledgments

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6. Contributions

DN and BS wrote the manuscript; RML and BS designed experiments; DN, IŠ and GO conducted the research; BS assisted in molecular analyses. All authors helped in the final editing of the manuscript.

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Povzetek

Za preizkus razgradljivosti pivskih tropin kot novega substrata smo uporabili rutinski test biokemijskega metanskega poten-ciala (BMP). Med tremi različnimi biomasami smo opazili statistično signifikantne razlike v začetni hitrosti proizvodnje biopli-na, deležu metana ter končni razgrajenosti pivskih tropin. Profili začetnih in končnih arhejskih mikrobnih združb so se medse-bojno razlikovali ne glede na razlike v vrsti substrata (brez substrata, glukoza, pivske tropine). To kaže na pomemben splošen vpliv testa BMP na mikrobne skupine. Ugotovili smo linearen odnos med fleksibilnostjo mikrobnih združb v testu BMP in začet-no hitrostjo proizvodnje bioplina, kar predstavlja nov parameter za diagnosticiranje anaerobnih procesov, predvsem v dinamič-nih pogojih, kot je zagon reaktorjev.

SUPPLEMENTARY MATERIAL

Methods

The DGGE profiles of the 16S rRNA gene PCR products generated from DNA extracted from biomass samples were enlarged from original publications [4,12] for maximum resolution and stored in *.jpg format. The data were then imported in BioNumerics (AppliedMaths) for trimming, gray-scale conversion, normalization, background subtraction and analysis using Perason's correlation coefficient with UPGMA clustering (unweighted pair-group method with arithmetic averages). The significance of clusters was determined using cluster-cutoff tool in Bionumerics.[15,17]

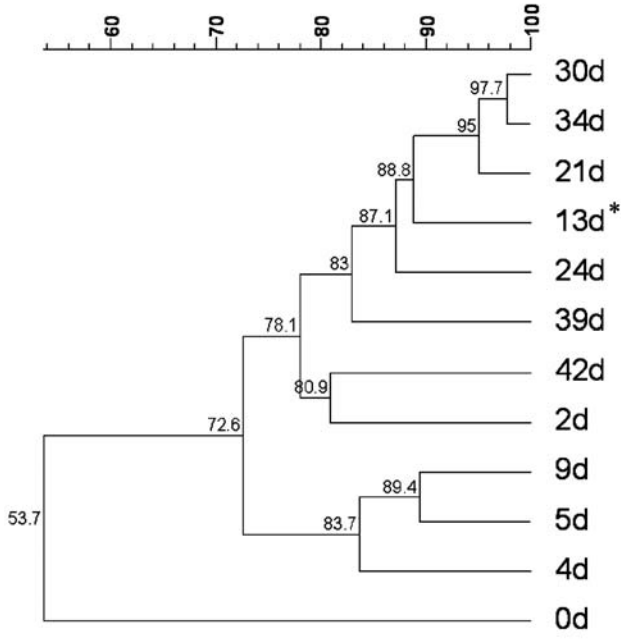

Figure S1: A reanalysis of archaeal microbial community dynamics during 42 day batch start-up of digester on a single substrate (Lee et al., 2010).
* – time of incubation comparable to 14 days used in routine biochemical methane potential assays. Three separate lineages could be delineated by cluster cut-off method (BioNumerics, AppliedMaths). For the exploration of microbial community dynamics the use of other substrates (or absence of any) was not reported.

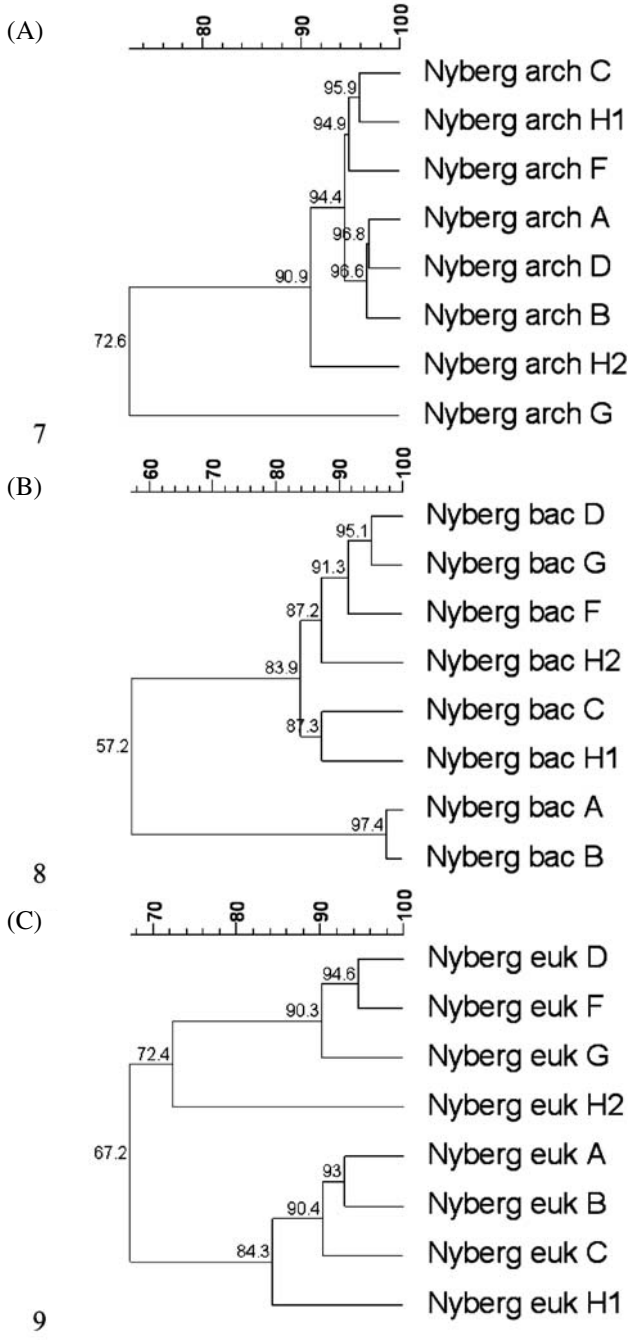

Figure S2: A reanalysis of the developed archaeal (A), bacterial (B) and eukaryotic (C) microbial communities at the end of degradation test different organic compounds in BMP (Nyberg et al., 2008). The data on the initial communities present in live or dried sludge were not provided.
In archaeal communities (A), only in treatment G (live sludge + dried sludge) significant differences could be detected, whereas all other developed communities were highly similar. It is important to note that only archaeal 16S rRNA genes were amplified using nested PCR design.
In bacterial communities (B), developed communities in treatments A (live sludge + fullerene dissolved in methanol) and B (live sludge + water solution of fullerene) developed significantly different communities from reference samples (H1 and H2).
In eukaryotic microbial communities (C), three separate clusters could be detected. First, all communities developed in the presence of fullerene formed a distinct cluster separated from those communities developed in the presence of dried sludge with or without toluene or xylene. In addition, both clusters were significantly different from reference H2, containing only live sludge.