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Comparison of sampling techniques and different media for the enrichment and isolation of cellulolytic organisms from biogas fermenters☆

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ABSTRACT

Biogas plants achieve its highest yield on plant biomass only with the most efficient hydrolysis of cellulose. This is driven by highly specialized hydrolytic microorganisms, which we have analyzed by investigating enrichment strategies for the isolation of cellulolytic bacteria out of a lab-scale biogas fermenter. We compared three different cultivation media as well as two different inoculation materials: Enrichment on filter paper in nylon bags (*in sacco*) or raw digestate. Next generation sequencing of the V3/V4 region of the bacterial 16S rRNA of metagenomic DNA from six different enrichment cultures, each in biological triplicates, revealed an average richness of 48 different OTU's with an average evenness of 0.3 in each sample. β-Diversity of the bacterial community revealed significant differences between the two sampling techniques or the different media used. The isolation attempt of single cellulolytic organisms resulted in several clonal pure cultures. Regardless which medium or inoculation material, well-known cellulolytic key players such as *Clostridium cellulosi*, *Herbinix hemicellulosilytica* and *Hungateiclostridium thermocellum* were among the isolates. The inoculation material as well as the cultivation conditions are crucial to cultivate the representative cellulolytic organisms. Taking raw digestate as inoculation material and using the same material, filtered and sterilized, for supplementing media allowed to imitate the natural habitat. Pre-enrichment of cellulolytic organisms directly in their natural habitat led to significant advantages concerning high diversity and high abundance of unknown cellulolytic organisms, which is a key factor for the isolation of hitherto unknown species.

Introduction

The microbial community is the driving system of the biogas process [2]. The key organisms for biomass hydrolysis, acidogenesis, acetogenesis and methanogenesis of cellulosic biomass to methane have been extensively studied [21,24,27]. The introduction of microbial OMIC approaches such as metagenomics, metatranscriptomics, and metaproteomics enabled comprehensive studies of the microbial ecology [19]. These studies revealed a huge number of non-assignable sequences because of the discrepancy between culture-independent and culture-dependent studies, mainly due to the limited availability of reference strains and their corresponding genomes in public databases [19]. As known for a long time now, only a minority of microbes is cultivable under laboratory conditions [1]. Recent studies on the cultivation of anaerobic microorganisms in the human gut microbiota revealed that 35–65% of molecular species detected by sequencing have representative strains in culture [18]. As the biogas microbiome is not as extensively studied as the human gut microbiota, there is still further need for cultivation attempts. In this study, cellulolytic organisms in a lab-scale biogas fermenter fed with maize silage and operated at 55 °C were evaluated. Especially in thermophilically-operated biogas plants, the abundance of known hydrolytic bacteria within the whole biogas consortium is low [19]. This makes the cultivation setup to enrich cellulolytic organisms even more important. In this study, two different inoculation materials in combination with

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three different cultivation media to enrich and isolate cellulolytic organisms are compared by combining next generation sequencing of the bacterial 16S rRNA from metagenomic samples as well as isolation of clonal pure cultures.

Material and methods

Media

The media used in this study were autoclaved tap water with 20% of filtered and autoclaved digestate (GR20), GS2 medium and GS2 supplemented with 2.5% of filtered and autoclaved digestate (GS2.5GR) with 0.5–1% (w/v) filter paper (Whatman No.1) as carbon source. The digestate used for media preparation was centrifuged three-times for 20 min, 50.000 × *g* at 4 °C. The supernatant was stored at 4 °C. GS2 medium, originally developed by Johnson et al. [10] was used in modification as previously described [13] with the following composition per liter: 6 g yeast extract, 2.9 g K_2HPO_4, 2.1 g urea, 1.5 g KH_2PO_4, 2.9 g trisodiumcitrate dihydrate, 1.0 g cysteine hydrochloridemonohydrate, 10 g MOPS, 0.1 g $MgCl_2{\cdot}6H_2O$, 0.015 g $CaCl_2{\cdot}2H_2O$, 0.125 mg $FeSO_4{\cdot}7H_2O$, 0.01% (w/v) resazurin and filter paper as carbon source. Media were prepared in butyl-rubber-stoppered serum bottles under anaerobic conditions and autoclaved.

Sample collection

All samples, as well as the digestate for medium preparation, originated from two identically operated lab-scale biogas fermenters run at 55 °C and fed with maize silage. Either raw digestate or *in sacco* enrichment was used as sample for the inoculation of enrichment cultures. For both sampling techniques, samples of both fermenters were mixed half to half. The *in sacco* method, originally developed by 20 [20], was performed in modification as described previously [14]. In brief, nylon bags (ANKOM Technology) with a size of 5 × 10 cm and pores of 50 μm were filled with 2 g shredded filter paper (Whatman No. 1), fixed on a sampling device and directly inserted into the germ carrier of the lab-scale biogas fermenter. In each case, two biological replicates at two independent time points were incubated for 5 days to enrich cellulolytic bacteria in their natural environment. Each *in sacco* sample was separately washed with isotonic NaCl solution (0.9% (w/v)) and then mixed in equal parts before use as inocula for enrichment cultures.

Inoculation of enrichment cultures

Enrichment cultures, performed as biological triplicates, were inoculated with one spade point of digestate or 0.3 mg of the partly degraded and washed paper and were incubated at 55 °C for one or up to two weeks until at least 50% filter paper degradation was reached. Media used were either GR20, GS2.5GR or GS2 under anaerobic conditions.

DNA extraction from metagenomics samples

For the extraction of metagenomic DNA, 1 ml liquid culture was centrifuged (10.000 × *g*; 5 min) and the pellet was resuspended in 600 μl RNAlater (Sigma). DNA extraction and purification was performed as described previously [3]. In brief, 400 μL phenol/chloroform/isoamyl alcohol (25:24:1, by vol; Sigma-Aldrich) were added before mechanical cell lysis (3 × 6.5 m/s for 40 s) with 500 mg 0.1-mm glass beads (Roth) through the use of a bead-beater (MP Biomedicals) fitted with a cooling adapter. After heat treatment (95 °C, 8 min) and centrifugation (16.000 × *g*; 5 min; 4 °C), 150 μL supernatant was incubated with 15 μL ribonuclease (0.1 μg/mL; Amresco) at 37 °C and was purified with the NucleoSpin gDNA Clean-up Kit (Macherey-Nagel), following the manufacturer's instructions. Concentration and purity were determined with the NanoDrop system (Thermos Fisher Scientific). If not processed immediately, samples were stored at −20 °C.

Next generation sequencing

High-throughput sequencing of 16S rRNA V3-V4 gene region using an Illumina MiSeq system was performed at ZIEL - Core Facility Microbiome/NGS, TUM. Preparation of amplicon libraries and sequencing was performed as described in detail previously [17]. In brief, amplicon libraries (V3–V4 region) and sequencing was performed using a PCR cycler (25 cycles) from 24 ng of metagenomic DNA with the bacteria-specific primers 341 F and 785R [12] following a two-step procedure to limit amplification bias [4]. After purification with the AMPure XP system (Beckmann), sequencing was carried out in paired-end mode (PE275) with pooled samples containing 25% (v/v) PhiX standard library in a MiSeq system (Illumina Inc.) prepared in accordance with the manufacturer's instructions [3].

Data analysis

Analysis of the raw data was realized via the Integrated Microbial Next Generation Sequencing (IMNGS) platform developed by Lagkouvardos et al. [16] based on the UPARSE approach [7]. In brief, sequences were demultiplexed (demultiplexor_v3.pl; Unpublished Perl script), trimmed to the first base with a quality score of 3 and paired using USEARCH 8.0 [6]. Sequences with less than 350 or more than 550 nucleotides as well as paired reads with an expected error of 3 were excluded from the analysis. Remaining reads were trimmed by 10 nucleotides on each end to prevent analysis of the regions with distorted base composition observed at the start of sequences. The presence of chimeras was tested with UCHIME [8]. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity, and only those with a relative abundance of at least 0.1% in one sample were kept. Taxonomic classification was performed by the RDP classifier v.2.11 training set 15 [25]. Sequence alignment was done by MUSCLE [5] as well as treeing by Fasttree [22]. Further analysis, including statistics, was performed with Rhea in a R script environment developed by Lagkouvardos et al. [15]. All details of the analysis and the scripts are available online (https://lagkouvardos.github.io/Rhea/).

Isolation of pure cultures

To assess the influence of cultivation, as well as for isolation of hitherto unknown organisms, isolation experiments were performed besides 16S rRNA amplicon analysis. To this end, enrichment cultures with at least 50% filter paper degradation extent were serially diluted up to 10^{-6} and, to obtain single colonies, 100 μl of selected dilutions (10^{-4}; 10^{-5}; 10^{-6}) were spread on agar plates with the same medium containing 1.8% (w/v) agar and overlaid with top agar. For all samples, GS2 medium-based top agar with 0.8% (w/v) agar, 0.5% cellulose powder (MN301, Machery Nagel) and 0.08% cellobiose was used. Single colonies with cellulolytic activity, indicated by halo formation, were selected and re-inoculated in liquid media. All experimental steps were performed under anaerobic atmosphere (98% N_2, 2% H_2) in an anaerobic chamber (Coy). Agar plates were incubated anaerobically in AnaeroJars (Thermos Scientific) at 55 °C.

Identification of single isolates

Identification of single isolates was based on 16S rRNA gene sequence analysis after extraction of genomic DNA from 1 ml liquid

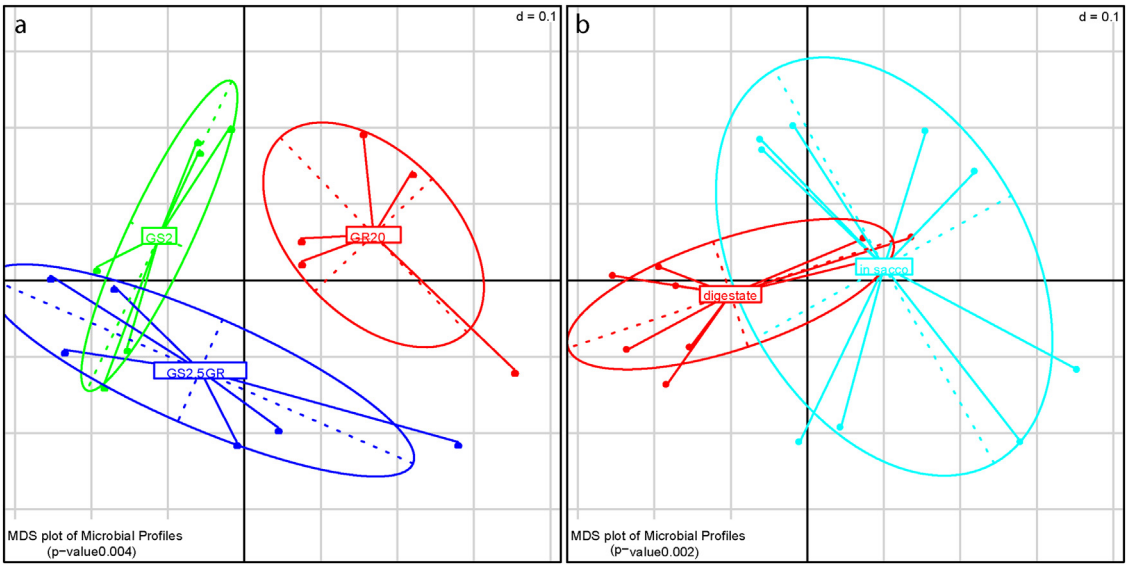

Fig. 1. β-Diversity comparing either the different media (a) or the two inoculation materials (b) used for enrichment of cellulolytic organisms. Analysis was performed with Rhea in a R script environment developed by Lagkouvardos et al. [15].

Table 1
α-Diversity calculated via Rhea [15] of all enrichment cultures.

Medium	Inoculum	r	Mean value (r)	e	Mean value (e)
	digestate1	34		0.35	
GR20	digestate2	27	34	0.11	0.27
	digestate3	42		0.36	
	digestate1	40		0.35	
GS2.5GR	digestate2	45	42	0.30	0.33
	digestate3	41		0.32	
	digestate1	53		0.40	
GS2	digestate2	47	48	0.39	0.37
	digestate3	45		0.34	
	InSacco1	30		0.33	
GR20	InSacco2	29	36	0.22	0.22
	InSacco3	48		0.12	
	InSacco1	65		0.32	
GS2.5GR	InSacco2	72	71	0.36	0.34
	InSacco3	75		0.34	
	InSacco1	63		0.36	
GS2	InSacco2	56	56	0.34	0.31
	InSacco3	49		0.25	

α-Diversity, meaning the diversity within one sample. Richness (r): quantity of detected OTUs in one sample. Evenness (e): distribution of single OTUs in the sample. Mean values were calculated for the biological triplicates of each inoculum in the same medium.

culture using the Soil DNA Extraction Kit (Roboklon, manufacturer's instructions). Gene amplification of the almost complete 16S rRNA gene sequences was performed with Phusion® DNA polymerase (Thermo Scientific, manufacturer's instructions) and the primer pair 616F-mod (AGAGTTTGATYMTGGCTC) and 630R (CAKAAAGGAGGTGATCC), originally developed by Juretschko et al. [11]. Sequencing of the purified PCR product (Quiagen PCR MiniElute; manufacturer's instructions) was done at Eurofins MWG. Results were compared to the NCBI database of 16S ribosomal RNA sequences from type material using the *Blastn* algorithm.

Results and discussion

Next generation sequencing of the V3/V4 region of the bacterial 16S rRNA gene in the metagenomic DNA of 6 different enrichment cultures, each in biological triplicates, revealed an average richness of 48 different OTUs with an average evenness of 0.31 (Table 1). Minimal Number of reads was 21,601. The rarefaction curve indicated a stagnation of the detection of novel OTUs in all samples (supplementary material). The highest diversity was observed using the *in sacco* sampling technique and GS2.5GR medium with 71 detected OTUs. The evenness was lowest when the GR20 medium was used, values from 0.11 to 0.36 with an overall mean of 0.29 were observed.

β-Diversity analysis, meaning the diversity of the bacterial community between different samples comparing either the different media or the different sampling techniques, showed significant differences. The bacterial community was, in comparison to the GR20 medium, highly shifted when a GS2-based medium, either GS2 or GS2.5GR, was used (Fig. 1). Furthermore, significant differences of the bacterial community enriched from the two different sampling techniques were observed. P-values were 0.004 and 0.002, respectively.

Taxonomic binning of OTU's at genus level (Fig. 2) revealed similarities within the bacterial communities among each of the biological triplicates. For example, the highly abundant genus *Clostridium* Cluster III (now classified as *Hungateiclostridium*) was detected in the same abundance in all three biological triplicates of one inoculation material and medium used. Nevertheless, certain differences, especially for less abundant genera, were observed. Sometimes also large differences occurred between the parallel cultures of triplicates that had been inoculated and incubated in the same way, indicating flexibility of relative growth characteristics within the microbial population. For example the genus *Defluviitalea* occurred in high abundance in the samples GR20-digestate3 (10%) and GS2.5GR-digestate3 (34%) as well as in GS2-digestate1, 2 and 3 (30%, 8%, 6%, respectively), but was only a minor taxon in GR20_digestate1 and 2 as well as in GS2.5GR_digestate1 and 2 with less than 0.01% of relative abundance. Remarkably, the genus *Tepidanaerobacter* was only observed in GS2-based media and not in GR20_digestate or GR20_InSacco. Species within this genus, *T. syntrophicus* [23] and *T. acetatoxydans* [26], were isolated from biogas related environments and described as syntrophic but non-cellulolytic organisms. The absence of this genus when GR20 medium was used indicates that the GR20 medium leads to certain restrictions and therefore higher accumulation of certain genera. Nevertheless, the accumulation of cellulolytic organisms, also keeping in mind the cultivation of hitherto unknown species, is of high interest in this study. The abundance of unknown *Clostridi-*

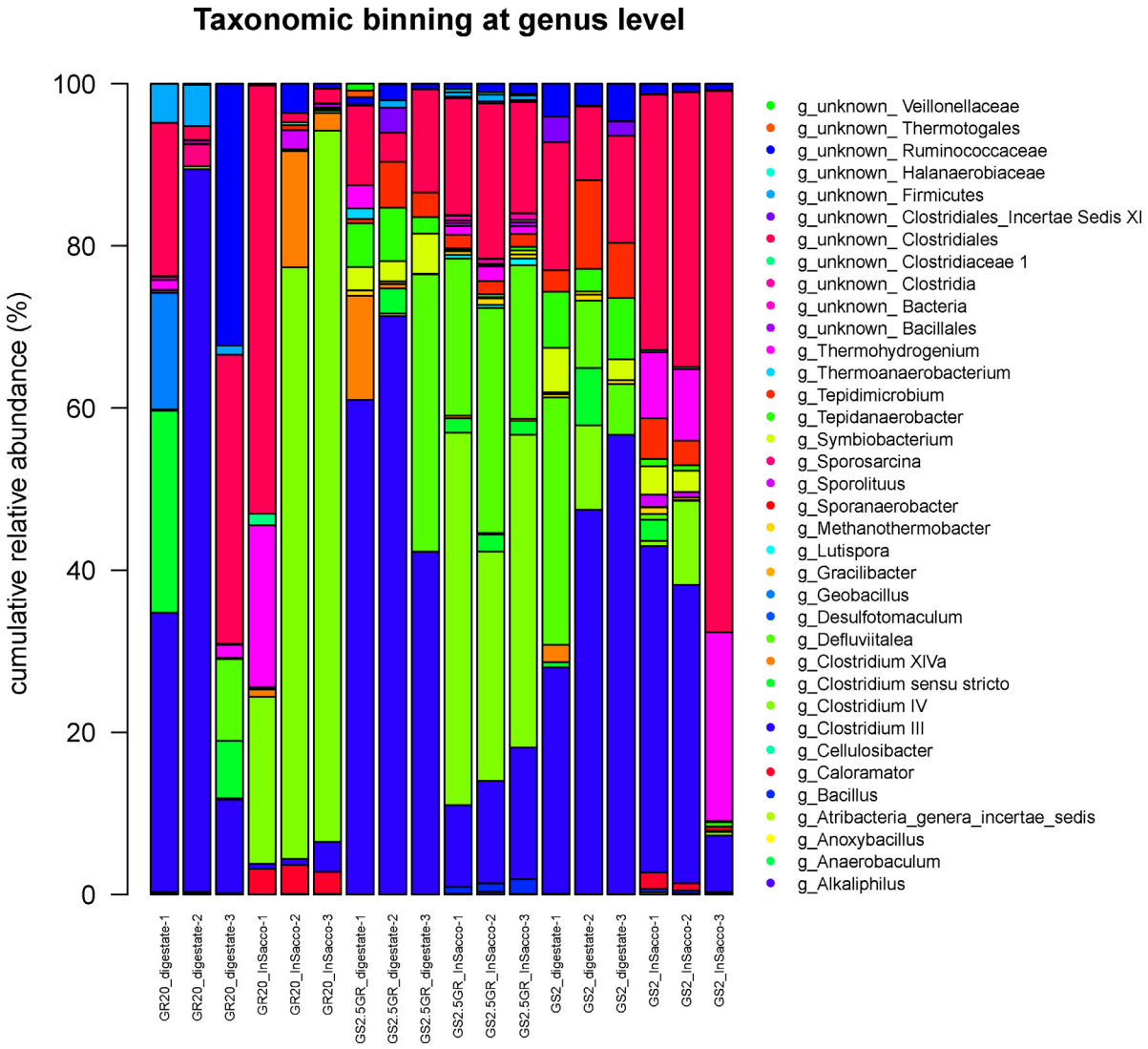

Fig. 2. Taxonomic binning of OTU's at genus level. Taxonomic classification was performed by the RDP classifier v.2.11 training set 15 [25].

Table 2
: Average relative abundance of unclassified taxa.

Medium	Inoculum	Average relative abundance of unclassified families	Average relative abundance of unclassified genera
GR20	digestate	21.5 %	33.6 %
GS2.5GR	digestate	5.4 %	11.9 %
GS2	digestate	6.8 %	18.2 %
GR20	*in sacco*	18.5 %	20.7 %
GS2.5GR	*in sacco*	7.9 %	19.2 %
GS2	*in sacco*	44.4 %	45.3 %

ales, which includes putative cellulolytic organisms, was higher in GR20_digestate1 and 3 (19 and 36%, respectively) than in the other digestate enrichment cultures. Remarkably, OTUs classified within this family were highly abundant when the *in sacco* sampling technique was applied, regardless which medium. Highest abundance of unknown *Clostridiales* was observed in GR20_InSacco1 (53%) and GS2-InSacco3 (67%). Remarkably, the relative abundance of OTUs classified within the class *Clostridia* laid between 79.8% (GR20_digestate1) and 99.8% (GR20_InSacco1; GS2.5GR_digestate3; GS2_InSacco3). Average relative abundances of unknown families and genera are summarized in Table 2. The GR20 medium (digestate and *in sacco*; 21.5 and 18.5%, respectively) as well as the GS2 medium (only *in sacco*; 44.4%) led to accumulation of unknown families and genera and have therefore high potential for the isolation of hitherto unknown organisms.

Isolation of cellulolytic organisms resulted in several clonal pure cultures as well as several mixed cultures. A selection of mixed cultures was analyzed in the same way as the enrichment cultures to assess the diversity as well as the relative abundance of single organisms within the mixed cultures. Results, showing the distribution of major OTU sequences of the next generation sequencing are summarized in Fig. 3. All raw sequencing data of the next generation sequencing of 16S rRNA genes were deposited at NCBI, accession no. PRJNA516126.

The mixed cultures consisted mainly of three to four different OTUs of which at least one is homologous to a well-known cellulolytic key player in biogas plants. Such species identified here were for example *Clostridium cellulosi*, *Herbinix hemicellulosilytica* and *Hungateiclostridium thermocellum*. Remarkably, the genera *Herbinix* (*Hx. Hemicellulosilytica*) and *Defluviitalea* (*D. raffinosedens* or *D. saccharophila*) appeared in five of six analyzed cultures.

Furthermore, isolation succeeded in following clonal pure isolates (Table 3), ordered by isolation medium. For all isolation media, clonal pure cultures of cellulolytic, or at least saccharolytic, organisms were obtained. As *Hc. thermocellum* and *Hx. hemicellulosilytica* were also dominant in mixed cultures (Fig. 3), these organisms were

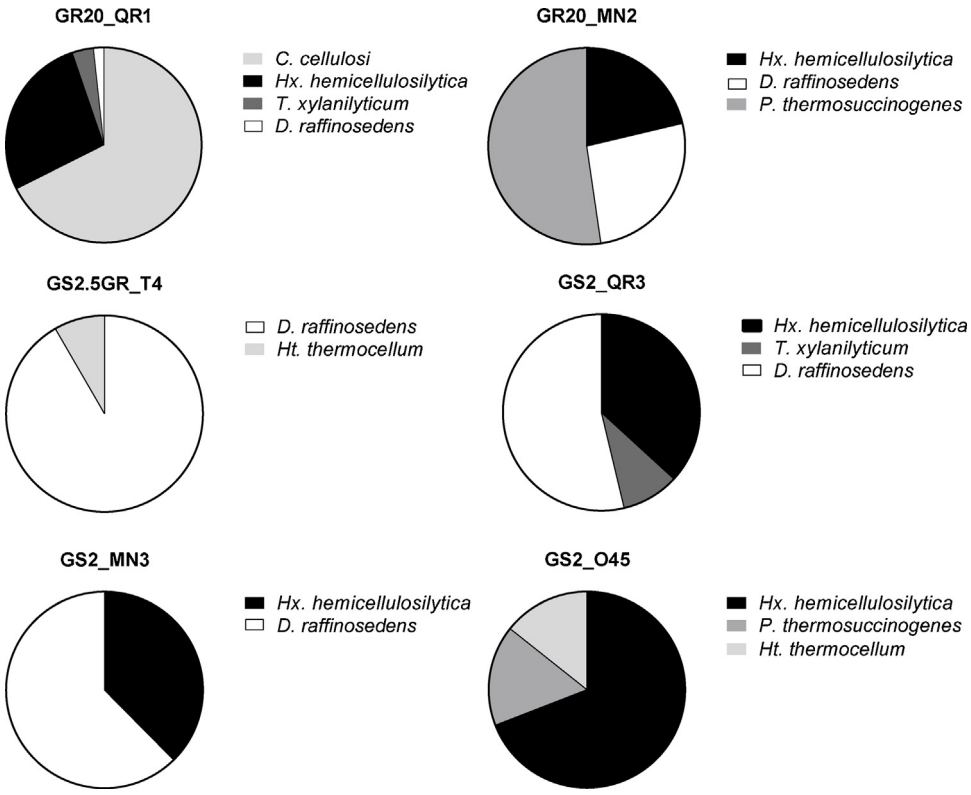

Fig. 3. Distribution of major OTU sequences of cellulolytic mixed cultures assessed via next-generation sequencing of bacterial 16S rRNA V3/V4 gene region. Taxonomic classification was conducted via Blastn analysis. All OTUs had 98–99% identity to the named organism.

Table 3
Clonal pure isolates ordered by isolation medium.

Medium	Organism	Isolate ID	I	degradation of filterpaper+/−
GR20	*Hc. thermocellum* JCM9322	T4-1	D	+
		T4-3	D	
		T3-3	D	
	Hx. hemicellulosilytica T3/55	S2T2-S3	IS	+
		S2T2-R4	IS	
		S2T2-R1	IS	
		S2T2-QR2	IS	
	D. raffinosedens A6	T1-2	D	–
			D	
	P. thermosuccinogenes DSM5807	T2-3	IS	–
GS2.5GR	*Hc. thermocellum* JCM9322	S2-O2	D	+
		S2-O3	D	
		S3T2-20	D	
		S3T2-21	D	
		S3-S31	D	
	C. cellulosi AS1.1777	S2T2-Q2	IS	+
		S2T2-Q3	IS	
	D. raffinosedens A6	3T1-1	IS	–
		3T1-3	IS	
GS2	*Hc. thermocellum* JCM9322	S3T2-15	IS	+
		S3T2-17	IS	
	Hx. hemicellulosilytica T3/55	S1-V5	IS	+
		O46	D	
		O49-1	D	
		O49-2	D	
	C. cellulosi AS1.1777	S2T2-ST1	IS	+
		S2T2-UV1	IS	
		O34	D	
		A3	D	
		A4	D	
	R. champanellensis JCM17042	Pa3	D	+/-

I: Inoculation material, D: raw digestate, IS: in sacco. Degradation of filterpaper: +, positive; +/−, positive at first isolation, but not reproducibly observed; −, negative.

isolated several times either with GR20 or GS2 medium. *C. cellulosi* was abundant in the mixed culture QR1 (GR20) and isolated in pure culture using either GS2.5GR or GS2 medium. *P. thermosuccinogenes* was only detected when the GR20 medium was used (isolates MN2 (Fig. 3) and T2-3 (Table 3)). Species of the genus *Defluviitalea* were detected in major or minor amounts in almost all mixed cultures, but could only be successfully separated as clonal pure culture with a medium containing digestate, GR20 or GS2.5GR (Table 3). With one exception, all isolates had a 16S rRNA gene sequence identity of 98.2–100 % to the named organism and therefore are considered to be presumably identified at the species level. Remarkably, isolate Pa3 had a very low 16S rRNA gene sequence identity (93.11% to *Ruminococcus champanellensis*): and could probably represents a hitherto uncharacterized species. In total, 30 cellulolytic or saccharolytic pure cultures were obtained, thereof 12 using raw digestate for the enrichment and 18 applying the *in sacco* method for pre-enrichment of cellulolytic organisms. The 16S rRNA gene sequences of all isolates were deposited at NCBI, accession no. MK431704-MK431733.

Conclusion

The first cultivation step has an immense impact on the microbial community structure of a culture inoculated from a complex environment. Cultivation methods that imitate the natural habitat are presumably best suited to preserve a given microbiota. In contrast, serial transfer of an enrichment culture leads to the accumulation of certain organisms that are favored by the applied cultivation method. Both, the preservation of a given microbial composition as well as the accumulation of organisms with desired properties, are of interest. In this study, taking raw digestate as inoculation material and using the same material, filtered and sterilized, for supplementing the media allowed to imitate the natural habitat best. Pre-enrichment of cellulolytic organisms directly in their natural habitat proved to have a significant advantage concerning high diversity and high abundance of unknown, cellulolytic organisms, which was a key factor for further isolation of hitherto unknown species. Nevertheless, well-known cellulolytic key players as for example *C. cellulosi* [9], *Hx. hemicellulosilytica* [13] and *Hc. thermocellum* [27] were isolated, regardless of medium or inoculation material, presumably due to their good laboratory cultivation.

Repositories

Raw sequencing reads of the 16S rRNA amplicon sequencing were deposited at NCBI with the BioProject accession no. PRJNA516126. The partial 16S rRNA sequences of the clonal pure isolates (Table 3) are available under following accession no. MK431704-MK431733.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2019.05.002.

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