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Bioaugmentation of the thermophilic anaerobic biodegradation of cellulose and corn stover

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ABSTRACT

Two stable, thermophilic mixed cellulolytic consortia were enriched from an industrial scale biogas fermenter. The two consortia, marked as AD1 and AD2, were used for bioaugmentation in laboratory scale batch reactors. They enhanced the methane yield by 22–24%. Next generation sequencing method revealed the main orders being *Thermoanaerobacterales* and *Clostridiales* and the predominant strains were *Thermoanaerobacterium thermosaccharolyticum*, *Caldanaerobacter subterraneus*, *Thermoanaerobacter pseudethanolicus* and *Clostridium cellulolyticum*. The effect of these strains, cultivated in pure cultures, was investigated with the aim of reconstructing the defined cellulolytic consortium. The addition of the four bacterial strains and their mixture to the biogas fermenters enhanced the methane yield by 10–11% but it was not as efficient as the original communities indicating the significant contribution by members of the enriched communities present in low abundance.

1. Introduction

Future economies should be increasingly based on renewable resources and non-fossil carbon from biomass. Alternative energy carriers should be implemented in large scale because of both economic and environmental considerations. Biogas production is one of the sustainable technologies with the considerable benefit of being able to generate useful energy carrier from various raw materials of biomass origin including plants and plant residues. Biogas is formed anaerobically and it mainly consists of methane (55–70%) and carbon-dioxide (30–45%) [1]. Plants provide the most abundant biomass on Earth by harvesting sunlight and CO_2 and converting it to carbon-neutral renewable resource, therefore it carries great potential for bioenergy production. Various lignocellulose-containing biomasses can be used for biogas generation, such as energy crops, agricultural and forestry residues, municipal and industrial wastes [2]. Among the possible substrates grass silage [3–5], giant reed [6], boreal herbaceous grasses [7], *Silphium perfoliatum* L. [8], tall fescue, cocksfoot and reed canary grass [9], paragrass [10,11], *Spartina alterniflora* (Smooth cord-grass), which can become an invasive species [12], napiergrass [13], wheat straw [14], tropical biomass wastes [15], oil palm mesocarp fibre [16], water hyacinth [17], bamboo waste [18], aquatic plants [19], sunflower stalks [20], corn stover [21] (Kakuk et al., 2017 personal communication), cotton wastes [22] has been studied in detail.

The main component of plant cell wall is lignocellulose. Lignocellulose is comprised of three types of biopolymers, i.e. cellulose, hemicellulose and lignin [23]. The average composition of the lignocellulosic biomass is 35–50% cellulose, 20–35% hemicellulose and 5–30% lignin on the basis of plant dry weight [24]. Cellulose is composed of β-D-glucose monomers. Hemicellulose contains pentose and hexose sugars such as glucose, mannose, xylose and arabinose [25]. Hemicellulose associates with cellulose microfibrils, creating a cross-linked matrix [26]. Lignin is a complex phenyl propane polymer, its major structural components are *p*-coumaryl

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alcohol, coniferyl alcohol and sinapyl alcohol, which link with ester bonds [25].

A family of various hydrolytic enzymes are needed for the complete decomposition of cellulose. These have been compiled into three major groups: endoglucanases (EC 3.2.1.4), exoglucanases, e.g. cellodextrinases (EC 3.2.1.74) and cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) [24]. For the efficient utilization of substrates having high cellulose content by anaerobic digestion (AD), the biogas producing microbial community should include cellulose degrading microbes with sufficient biological activity [23,27]. Because of its recalcitrance, pretreatment techniques are frequently applied.

In the AD process, a complex microbial community consisting of bacteria and methanogenic archaea converts organic substances to biogas in four main metabolic steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Some steps can be rate limiting particularly hydrolysis and methanogenesis. The microbial communities involved in AD are considered to be one of the phylogenetically and functionally most diverse among engineered microbiotas. In the first conversion step, complex organic matter, including carbohydrates, proteins and lipids, is converted to soluble monomers and oligomers. Hydrolysis is generally considered to be the rate limiting step in AD for particulate and lignocellulosic biomasses. Among the microorganisms involved in hydrolysis, the phyla Actinobacteria and Firmicutes present most cellulolytic capacity.

Pretreatment methods can be physical (grinding, steam-explosion, liquid hot water, extrusion, irradiation), chemical (alkaline or acid pretreatment, wet oxidation, ozonolysis, oxidation by peroxides, ionic liquids), biological (fungal, microbial consortium, enzymatic) or combined pretreatment [25,28]. Bioaugmentation is a promising method to introduce microorganisms into the biogas producing system and facilitate the target substrate degradation. Pure cultures [29–33] or consortia [34–37] have been tested. Hydrolysis of the complex organic matter is generally considered to be a major rate limiting step of AD. Therefore, our primary goal was to facilitate the breakdown of lignocellulosic material using the bioaugmentation approach.

In the present study thermophilic cellulose degrading bacterial consortia have been enriched on α-cellulose. Thermophilic (55 °C) temperature was selected for the enrichment because, as a general rule, at higher temperature the degradation of biomass is more efficient and the biogas yield is higher [1]. Our goals included the isolation of thermophilic cellulose hydrolyzing stable microbial communities, their characterization and utilization in the decomposition of the lignocellulosic substrate corn stover, which has huge practical potential as substrate for biogas production. The reproducibility of the enrichment approach was tested by isolating two communities separately from the same starting community. In addition, we wanted to test if the most abundant "key player" strains in the enriched community, are sufficient for optimal lignocellulose degradation. The composition and relative abundances of the bacterial strains comprising the enrichment cultures were determined by Ion Torrent™ whole genome DNA sequencing and the consortia and their most abundant components were tested for their bioaugmentation potential in AD fermentations.

2. Materials and methods

2.1. Strains and media

The strains purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and their recommended growth media are listed in Table 1.

The medium used cultivate cellulose degrading enrichments had the following composition: 2.7 g KH_2PO_4, 3.5 g K_2HPO_4, 0.53 g NH_4Cl, 0.08 g $CaCl_2$ x $2H_2O$, 0.1 g $MgCl_2$ x $6H_2O$, 0.2 g $MgSO_4$ x $7H_2O$, 0.01 g KI, 1 mg resazurin, 0.75 g L-cysteine-HCl x H_2O, 1 mL Trace element solution SL-10, 1 g D-cellobiose (Sigma), 2 g α-cellulose (Sigma), 2 g carboxymethyl cellulose (Sigma) in 1000 mL distilled water, and pH was adjusted to 7.2. After several subculturing (see Section 2.2.1.), a stable mixed cellulolytic consortium developed. In order to test the reproducibility of the enrichment procedure it has been repeated and the two separately isolated consortia were marked AD1 and AD2. The cultures were stored at −80 °C with 50% (v/v) glycerol for further use and retained their efficiency and composition even after 2 years.

Inoculum from a thermophilic biogas plant (Bátortrade Ltd., Nyírbátor, Hungary), fed with various plant biomasses, chicken manure and pasteurized slaughterhouse waste was used in the enrichment procedure. Sludge from a mesophilic biogas plant utilizing pig slurry and maize silage (Zöldforrás Ltd., Szeged, Hungary) was adjusted to thermophilic temperature before by incubation at 55 °C for 2 weeks. This community was used as inoculum in the bioaugmentation experiments.

2.2. Experimental setup

2.2.1. Enrichment

The first enrichment step was carried out in 500 mL glass vessels. Each vessel was sealed with a butyl rubber stopper and an aluminum cap and was flushed with nitrogen gas for 5 min to establish anaerobic environment. The vessels were filled with 400 mL sludge from a full-scale biogas plant operating at thermophilic temperature (55 °C) and were fed with α-cellulose (Sigma, cat. number: C8002) or glucose (Reanal, cat. number: 40056) as the sole carbon source. The initial 1 g/L weekly substrate supply was increased gradually to 10 g/L in 10 weeks and then set at 6 g/L because of the elevated volatile fatty acid concentration (Fig. 1). In the second enrichment phase the culture from the first phase was used as inoculum to start new fermentations. In addition to continue the enrichment process in the second phase AD experiments using *Caldicellulosiruptor saccharolyticus* were also included to compare the performance of the enrichments with that of a pure culture known for its thermophilic bioaugmentation capability [30]. In this set of experiments, the vessels contained 190 mL of inoculum and 10 mL of distilled water or *C. saccharolyticus* culture (1.8×10^7 CFU/mL), respectively. A second addition of *C. saccharolyticus* culture was accomplished on week 7. The weekly substrate addition was 4 g/L of α-cellulose. The enrichment steps were done in triplicates at 55 °C and the vessels were stirred manually once a day. The first enrichment step took 16 weeks and the second enrichment phase lasted for 18 weeks. A 4 weeks resting period was inserted between the two enrichments to decrease the accumulated volatile fatty acids. After the second phase, samples were taken from the enrichments fed with α-cellulose from the beginning and were maintained in the culture medium specified in section 2.1.

2.2.2. Bioaugmentation

The bioaugmentation effect of the enriched consortia (AD1 and AD2) and the pure culture of the four cellulolytic strains (Section 3.3.) (2.6×10^8 cells/mL) were assessed next. In the "mixture" sample presented in Fig. 7 the four cultures were mixed in a ratio of their relative abundances in AD1 and AD2 as shown in Fig. 6. In 125 mL serum vials the volume of the liquid phase was 60 mL comprising 50 mL inoculum sludge from the digestate of the industrial scale mesophilic reactor, which was acclimated at 55 °C, and 10 mL of the enriched consortium (4.5×10^8 cells/mL) or sterile distilled water. Acclimation for 2 weeks selected the strains able to

Table 1
Axenic cellulolytic strains used in this study.

Strain	DSM number	DSMZ cultivation media number
Caldicellulosiruptor saccharolyticus	DSM 8903	640
Thermoanaerobacterium thermosaccharolyticum	DSM 571	61
Thermoanaerobacter pseudethanolicus	DSM 2355	144
Caldanaerobacter subterraneus subsp. *subterraneus*	DSM 13054	899
Clostridium cellulolyticum	DSM 5812	520

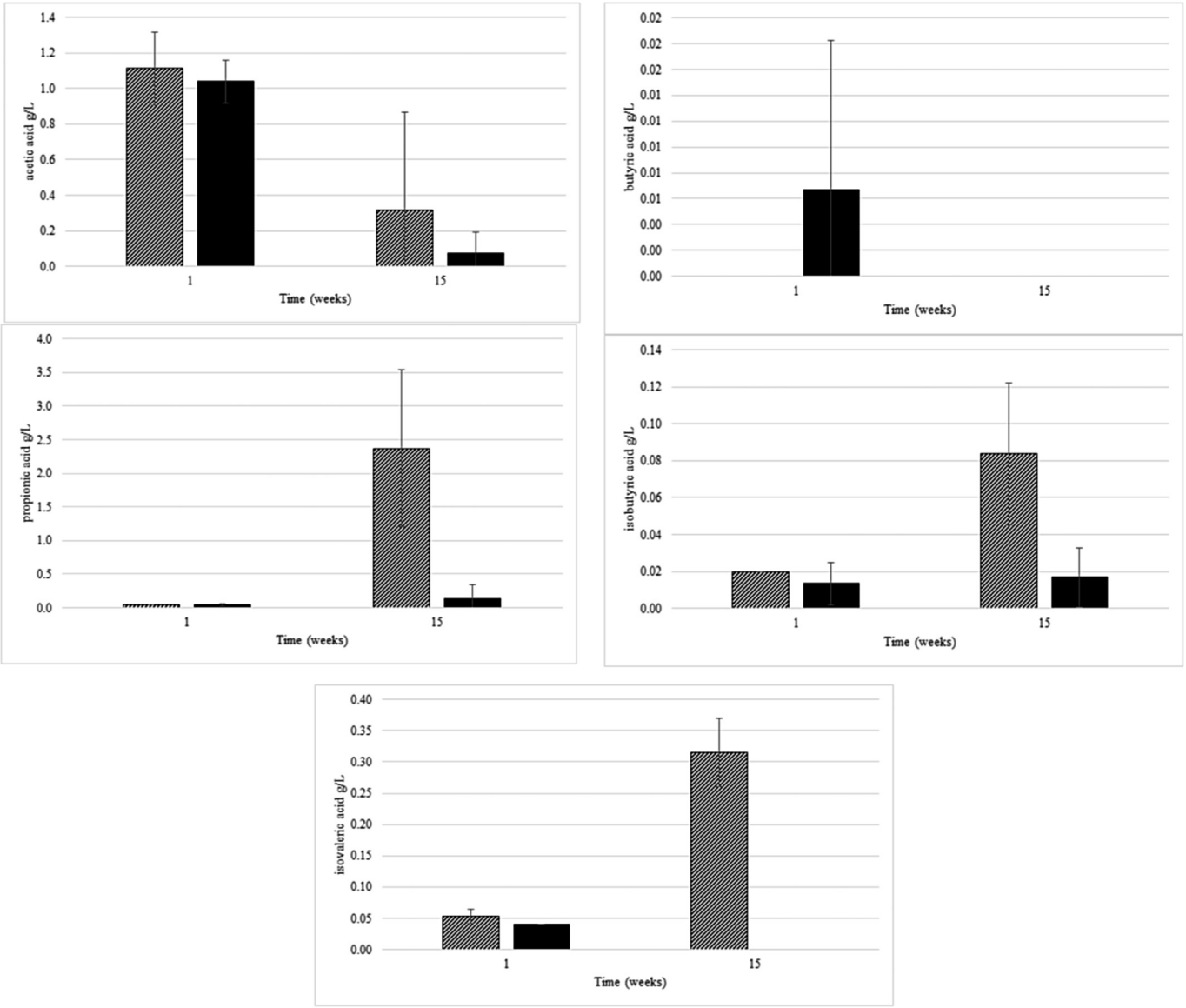

Fig. 1. VFA contents at the beginning and at the end of the first enrichment phase (16 weeks) using glucose (striped columns) or α-cellulose (black columns) substrates. Values are the mean of three parallel samples and the error bars indicate the standard deviation.

grow at 55 °C. The surviving microbial community showed the same biological activity at both mesophilic and thermophilic temperatures as the non-acclimated controls (data not shown). In some bioaugmentation experiments 54 mL sludge and 6 mL (10 v/v%) of AD1 or AD2 consortia (4.5×10^8 cells/mL) were used essentially with the same results. In these experiments corn stover was used as substrate in a dosage of 8 g oDM/L. Corn stover was collected from a local farm and dried at 55 °C. The dried corn stover was pretreated mechanically by shredding and sieving to <2 mm particles (Kakuk et al., personal communication) with a Retsch cutting mill SM 100, was stored at room temperature and sealed to keep from humidity. Mechanical pretreatment reduces particle size and crystallinity of cellulose and increases the specific surface to bacterial access [25]. The mechanical pretreatment of ensiled meadow grass enhanced the methane yield by 8–25% [38]. Composition of the corn stover was 32 ± 2% cellulose, 23 ± 2% hemicellulose and 14 ± 1% lignin. C/N ratio of the used substrate was 47.1.

2.3. Analytical methods

Volatile fatty acids (VFA) were determined by high performance liquid chromatography (Hitachi Elite, equipped with ICSep ICE-COREGEL 64H column and refractive index detector L2490) using the following parameters: solvent of 0.1 N H_2SO_4, flow rate of 0.8 mL/min, column temperature of 50 °C, and detector temperature of 41 °C.

The composition of the evolved biogas was measured by taking 100-μL aliquots from the headspace and injecting into a gas chromatograph (6890N Network GC System, Agilent Technologies) equipped with a 5 Å molecular sieve column (length 30 m, I.D. 0.53 megabore, film 25 μm) and a thermal conductivity detector. Nitrogen was used as carrier gas.

The dry matter content was quantified by drying the biomass at 105 °C overnight and weighing the residue. Further heating of this residue at 550 °C until its weight did not change yielded the organic dry matter content.

An Elementar Analyzer Vario MAX CN was used to determine C/N. The instrument works on the principle of catalytic tube combustion under an O_2 supply at high temperatures (combustion temperature: 900 °C, postcombustion temperature: 900 °C, reduction temperature: 830 °C, column temperature: 250 °C). The desired components were separated from each other with the use of specific adsorption columns (containing Sicapent, in CN mode) and determined in succession with a thermal conductivity detector. Helium served as flushing and carrier gas.

The rate of gas formation was measured by the water displacement technique, using the ideal gas law, the measured volume was converted to a value at standard temperature and pressure [39].

pH was determined with a Radelkis OP-211 pH meter.

2.4. Enzyme assay

β-glucosidase activity was assayed using 4-nitrophenyl-β-D-glucopyranoside (pNPG) (Sigma) as substrate. The enzymatic reaction mixtures contained the following components: 2 mL sample from the reactor was centrifuged and mixed with 750 μL of 0.1 M sodium acetate buffer (pH 5.0) and 250 μL of pNPG (20 mM). The samples were incubated at 55 °C for 30 min. The amount of *p*-nitrophenol released was measured at 400 nm after addition of 200 μL of 1M Na_2CO_3 to the reaction mixtures.

2.5. DNA extraction

The whole genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB)-based method from 10 mL cellulose degrading consortia as described previously [31]. The concentration of the purified DNA was measured spectrophotometrically (NanoDrop ND-1000 Technologies, Washington, DC, USA), and its integrity was determined by agarose gel-electrophoresis.

2.6. Next-generation DNA sequencing

Sequencing was performed using Ion Torrent PGM 316 chip (Thermo Fisher Scientific). The reads were analyzed and quality values were determined for each nucleotide. From the enriched consortium AD1 578,372 reads containing more than 119 million bp were identified, in the case of AD2 these values were 515,436 and 111 million, respectively. The average read lengths were 231and 246 bp. The individual sequences were further analyzed by using the MG-RAST software package [40]. The MG-RAST server computes results against several reference datasets (protein and ribosomal databases) [41] as previously described [42].

2.7. Statistical analysis

Statistical analysis was done by using Student's t-distribution (two tailed, two-sample unequal variance). Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Enrichment of cellulose degrading thermophilic consortia

When the enrichment culture was fed with 10 g/L glucose, the VFA levels, particularly that of propionic acid, elevated to the alarming level of 2 g/L. Although the VFA content was above the inhibitory threshold, apparently it had no effect on the pH and the biogas production indicating a good buffering capacity of the system. Siegert et al. [43] found that the fermentation of glucose was slightly inhibited at VFA concentrations above 4 g/L and was more than halved above 8 g/L which indicated that the fermentation of glucose was less sensitive to inhibition caused by VFA. To monitor proper operation, the VFA contents (Fig. 1) and pH (Fig. 2) were measured weekly along with the β-glucosidase enzyme activity (Fig. 3), which reflects the cellulose hydrolysis.

New fermentations were started using the enriched and the non-adapted sludge in order to examine if the first enrichment step was successful and further enrich the two isolated consortia. *Caldicellulosiruptor saccharolyticus,* a thermophilic bacterium possessing cellulase activity, was used as positive control. This bacterium proved to be a suitable strain for biogas bioaugmentation earlier [29]. *C. saccharolyticus* enhanced the biogas yield by 12% in the non-adapted reactors but in the reactors containing the enriched first consortia the effect was negligible. Without the contribution of *C. saccharolyticus* the enriched consortia yielded 14% more biogas than the non-adapted one (Fig. 4). Biogas yield from 1 g oDM was 463.4 ± 4.4, 482.0 ± 10.8, 411.6 ± 25.2 and 456.1 ± 20.5 mL in the case of enrichment, enrichment supplemented with *C. saccharolyticus*, non-adapted and non-adapted supplemented with *C. saccharolyticus*, respectively. These values are 65.3–79.8% of the observed values of 604 and 630 mL/g VS by Richards et al. [44]. The results corroborate that the adaptation of the thermophilic microbial community to cellulose was successful and our enrichment cultures brought about a similar bioaugmentation effect as *C. saccharolyticus* did. The cellulose degrading microbes were cultivated and subcultured 8 times after the second phase of enrichment experiments. The consortia apparently remained stable in its biological activity. The two mixed cultures were marked as AD1 and AD2 and used in subsequent experiments.

3.2. Bioaugmentation effects of AD1 and AD2

In the subsequent experiments corn stover was used as substrate. Corn stover is a lignocellulosic agricultural residue produced in vast amounts annually. It is a cheap and potentially suitable substrate for biogas production except for its high lignocellulose content. The biogas yield and methane content in the batch tests were monitored in every 1–2 days. The added cultures had positive effects. The possible contribution of the growth medium to the enhanced biogas yield has been tested in separate experiments and was excluded from the calculations. Therefore, the elevated methane yields were solely due to the addition of AD1 and AD2 to the fermenters. Fig. 5 presents a typical experiment. Although the two enriched consortia were treated and handled separately, they showed a very similar bioaugmentation behavior enhancing the

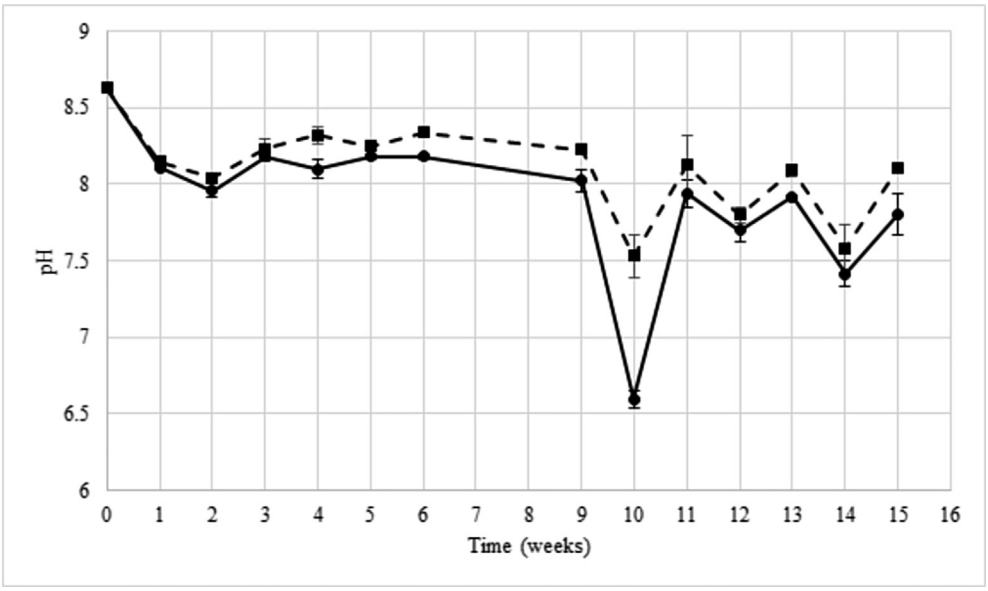

Fig. 2. pH changes during the enrichment on glucose (dashed line) or cellulose (solid line). Weekly substrate supply was gradually increased to 10 g/L in 10 weeks (see Fig. 3), then set back to 6 g/L/week.

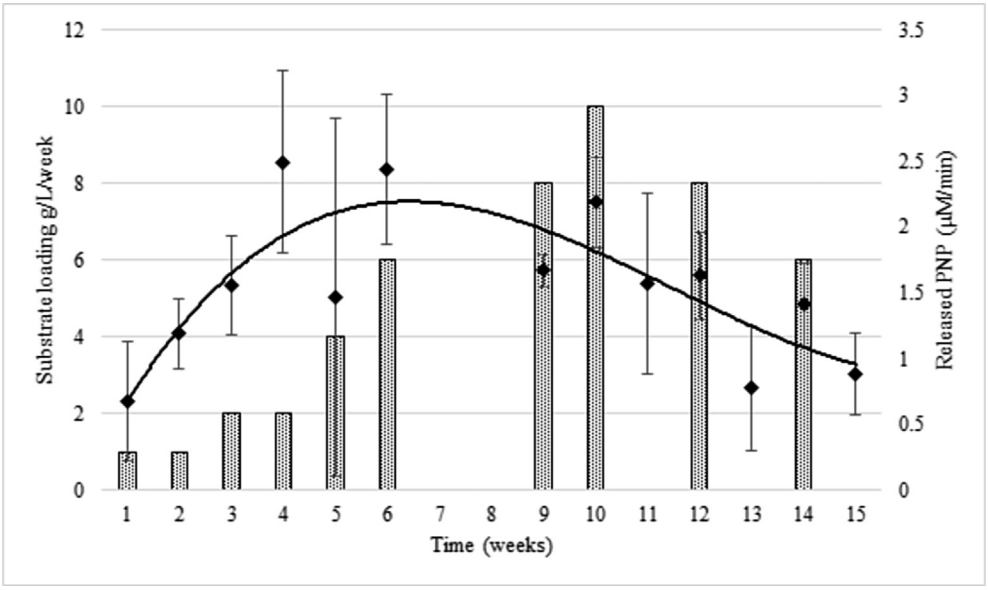

Fig. 3. β-glucosidase activity changes during cellulose adaptation (◆). Increasing activity can be observed along with the higher substrate uptake and decreasing with lower or absent substrate load. The dotted columns indicate the substrate load.

methane yield by 22–24%. Methane yields were 167.1 ± 0.1, 163.7 ± 1.3 and 134.2 ± 0.3 in case of bioaugmentation with AD1, AD2 and control, respectively. It should be noted that the methane yields from the control corn stover was significantly lower in our experiments than those obtained using corn stover of similar particle size in the same laboratory (Kakuk et al., personal communication). The somewhat distinct properties of corn stover samples used (C/N = 47.1 vs. 52.3) and the different inocula may explain the differences and alerts in general for the possible substantial differences in methane potential determinations due to slightly different conditions employed in the tests.

In previous studies [34,37,39,45–47] several attempts have been made to facilitate the decomposition of cellulose-rich substrates and increase biogas/biomethane yields by bioaugmentation. The most relevant results, i.e. thermophilic anaerobic mixed cultures enhancing biogas yield and/or kinetics, and conditions are summarized in Table 2. The published results are difficult to rigorously compare with those reported here. From the diverse sets of data and in spite of the varying experimental designs the biogas yield data and the degree of bioaugmentation relative to the non-augmented controls may be informative. It is clear and not surprising from the data in Table 2 that the biogas yields strongly differ depending on the substrate used and the percent increase upon bioaugmentation fluctuates similarly in these studies. The 165 mL CH_4/g oDM yield obtained for corn stover, a recalcitrant and lignin-rich lignocellulosic biogas substrate and the biomethane yield increase (22–24%) achieved by AD1 and AD2 seem to be in the outstanding range. The taxonomic groups involved in the enriched

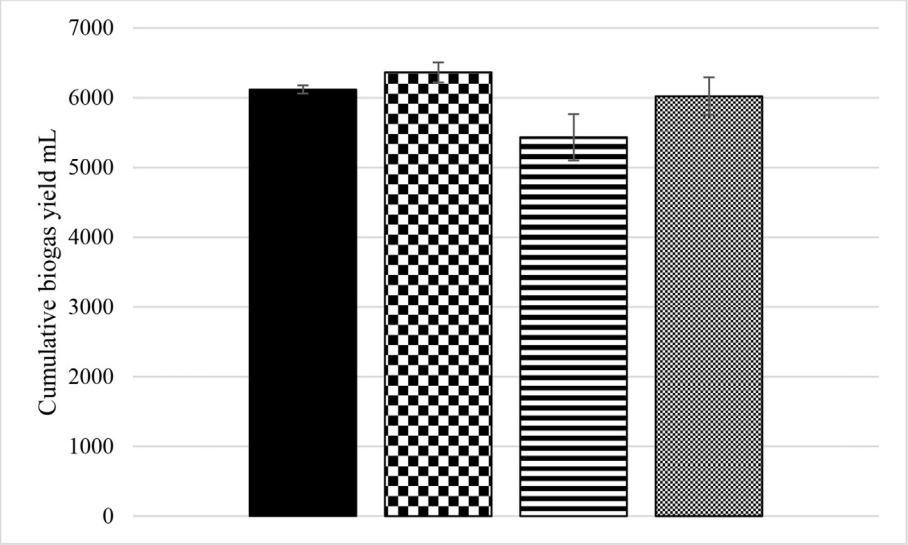

Fig. 4. Cumulative biogas yields in the second enrichment phase (18 weeks, from 13.2 g α-cellulose) and the effect of *C. saccharolyticus* addition. Enriched culture from the first phase without *C. saccharolyticus* (black) and with *C. saccharolyticus* (checked) compared to non-adapted sludge alone (striped) and supplemented with *C. saccharolyticus* (dotted). ($p \leq 0.2$).

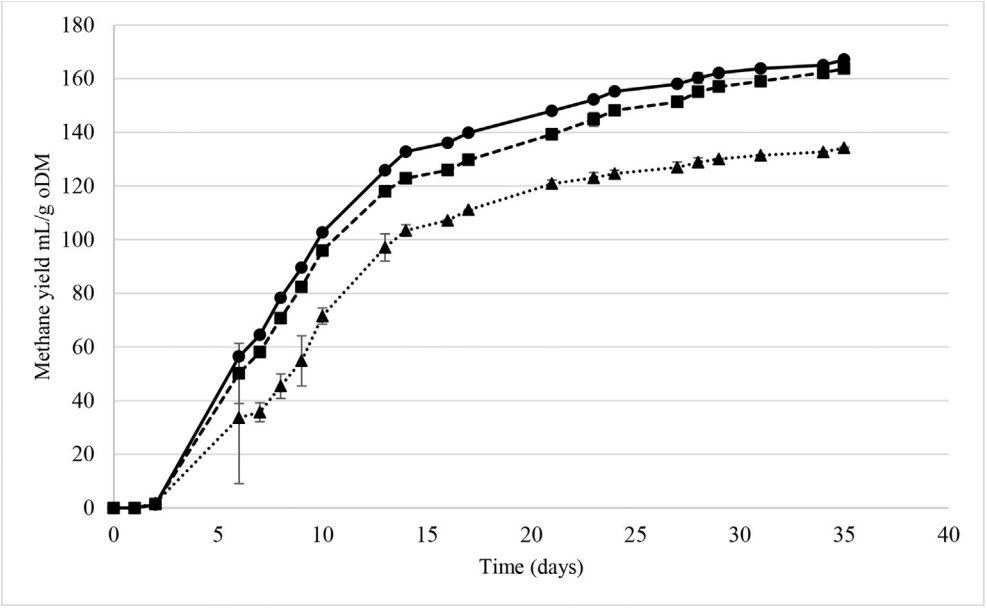

Fig. 5. The augmentation effect of AD1 (solid line) and AD2 (dashed line) on the biogas fermentation from corn stover compared to the control (dotted line). The samples contained 2 g corn stover, 50 mL of acclimatized inoculum and 10 mL of AD1 (●) or AD2 (■). Controls (▲) received no bioaugmentation consortia. In several cases the error bars (standard deviation) are smaller than the symbols (mean value of three parallel samples). ($p < 0.05$).

thermophilic bioaugmentation consortia were poorly identified in most previous cases. Nevertheless, it is noteworthy that representatives of the orders *Thermoanaerobacterales* and *Clostridiales* were frequently found as key components of the enrichment cultures.

3.3. Metagenome analysis

The good quality sequence data allowed the identification of the most abundant strains at species level. *Thermoanaerobacterium thermosaccharolyticum* (99.81 and 99.80%), *Caldanaerobacter subterraneus* (100 and 100%), *Thermoanaerobacter pseudethanolicus* (99.92 and 99.99%) and *Clostridium cellulolyticum* (100 and 100%) were identified as the best match of the most predominant strains. The numbers in brackets indicate homology to the strains estimated by MG-RAST (Fig. 6). Both AD1 and AD2 displayed essentially the same composition albeit the separate isolation and enrichment from the same original biogas reactor effluent. The relative representation of these four strains were also very similar (Table 3).

T. thermosaccharolyticum frequently described in thermophilic biohydrogen production from cellulose [48,49] and other substrates in consortia [48,50–55] or in pure culture [56–59]. The *C. subterraneus* type strain, i.e. *Caldanaerobacter subterraneus* subsp. *subterraneus*, was first isolated from an oilfield reservoir in France [60]. *C. subterraneus* was also identified in thermophilic hydrogen producing consortia, sometimes together with *T. thermosaccharolyticum*, [52,55,61,62].

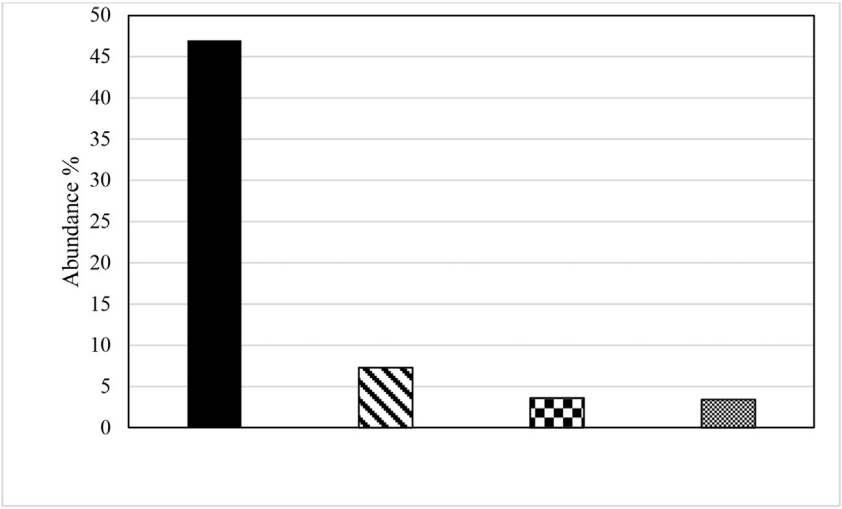

Fig. 6. Predominant strains and the DNA sequence abundance % identified in AD1: *Thermoanaerobacterium thermosaccharolyticum* (black), *Caldanaerobacter subterraneus* (striped), *Thermoanaerobacter pseudethanolicus* (checked) and *Clostridium cellulolyticum* (dotted).

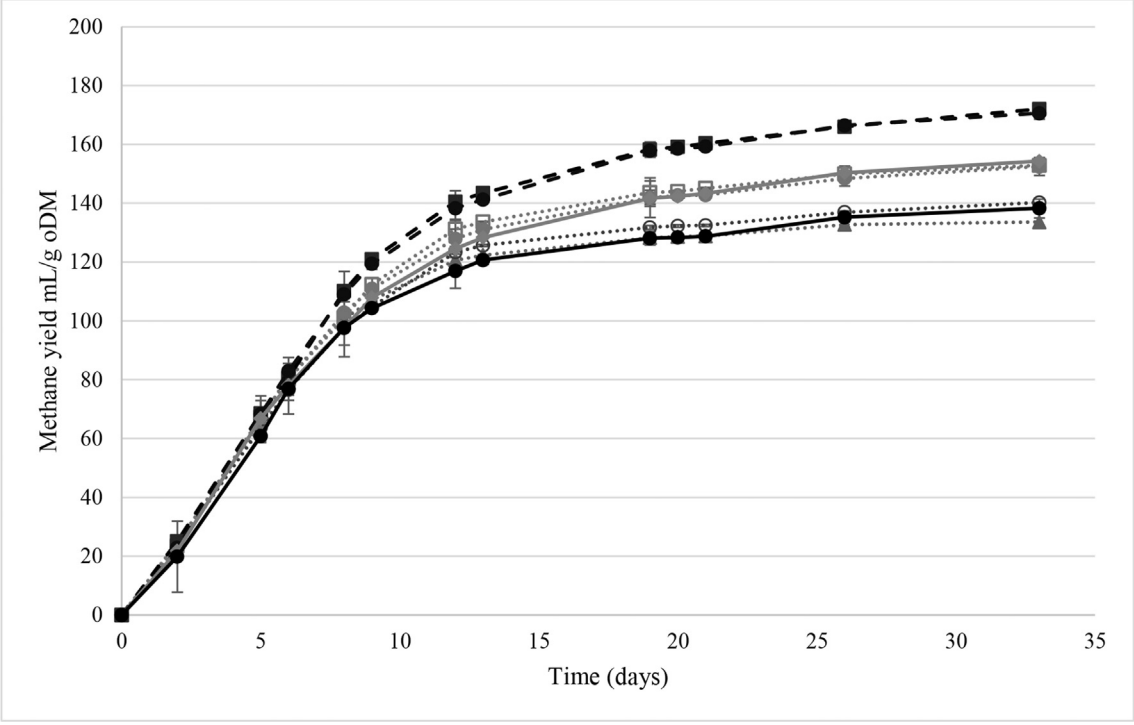

Fig. 7. Effect of AD1 (black square, dashed line), AD2 (black circle, dashed line), Thermoanaerobacterium thermosaccharolyticum (grey triangle, dotted line), Caldanaerobacter subterraneus (grey empty square, dotted line), Thermoanaerobacter pseudethanolicus (grey circle, dotted line), *Clostridium cellulolyticum* (grey empty circle, dotted line) and their mixture (grey diamond, solid line) compared to the controls (black circle, solid line). Symbols represent the mean value of three parallel samples and error bars indicate standard deviation; $p < 0.05$ except in case of C. cellulolyticum.

T. pseudethanolycus was isolated from Octopus Spring in Yellowstone Park [63] as a good candidate for consolidated bioprocessing [64] to produce bioethanol in co-culture with *C. thermocellum* [65,66]. The mixed culture A7, isolated from a Mexican oil field, comprised the strain *T. pseudethanolicus* [67].

C. cellulolyticum was described as a mesophilic cellulose degrading bacterium, which produces hydrogen from cellulose [68–70]. In the cellulolytic biofilm of a thermophilic two-phase leach-bed biogas reactor *C. cellulolyticum* was one of the prevalent species [71]. In co-culture with *Clostridium acetobutylicum*, *C. cellulolyticum* was used for consolidated bioprocessing [72]. Peng et al. reported 13% methane yield enhancement from wheat straw upon employing *C. cellulolyticum* for bioaugmentation [33].

Next generation sequencing of the whole DNA samples identified the predominating orders being *Thermoanaerobacterales* at 70% and 73% and *Clostridiales* at 10% and 11% abundances for AD1 and AD2, respectively. The same orders characterized the thermophilic consortia enriched under completely different conditions from various sources at diverse locations (Table 2). This suggests that similar enrichment communities may develop from completely

Table 2
Thermophilic lignocellulose degrading mixed cultures and their bioaugmentation potential reported.

Origin	Taxonomic category	Conditions	Substrate	Maximal bioaugmentation at thermophilic conditions	Reference
from MC3F	genera *Clostridium, Thermoanaerobacterium*, family *Rhodocyclaceae*	mesophilic (37 °C) and thermophilic (50 °C), facultative anaerobic	swine manure	145 mL CH_4/g VS yield; 10% increase	[37]
compost	not fully characterized, contains *Clostridium straminisolvens* CSK1, *Clostridium* sp. FG4b, *Pseudoxanthomonas* sp. strain M1-3, *Brevibacillus* sp. M1-5, and *Bordetella* sp.M1-6	thermophilic (50 °C), anaerobic	lignocellulose of municipal solid waste cotton stalk rotten maize silage	221 mL CH_4/g VS yield; 125.5% increase 118 mL CH_4/g VS yield; 136.4% increase 304 mL biogas/g VS; 74.7% increase	[39,45,46]
compost	*Clostridia* class	thermophilic (55 °C), anaerobic	lignocellulosic substrate	101 mL CH_4/g VS yield; 12% increase	[34]
soil	*Thermoanaerobacterium, Thermanaerovibrio, Clostridium* and many unidentified, uncultured	thermophilic (55 °C)	cassava residue	259.5 mL CH_4/g VS yield; 96.63% increase	[47]

Table 3
Similarities and differences between the most abundant community members of AD1 and AD2. Strains occurring in one consortium but not in the other are marked with grey background.

Closest match	AD1 Abundance %	AD2 Abundance %
Thermoanaerobacterium thermosaccharolyticum	46.99	48.61
Caldanaerobacter subterraneus	7.29	7.98
Thermoanaerobacter pseudethanolicus	3.62	4.68
Clostridium cellulolyticum	3.42	4.35
Thermosinus carboxydivorans	–	4.09
Clostridium thermoamylolyticum	2.98	2.31
Clostridium thermocellum	2.98	3.96
Bacillus pseudofirmus	2.64	3.56
Thermoanaerobacter sp. X514	2.15	2.31
Clostridium sardiniense	1.96	0.53
Eubacterium limosum	1.76	2.18
uncultured bacterium	1.76	1.25
Ralstonia solanacearum	1.71	–
Cupriavidus pinatubonensis	1.61	–
Thermoanaerobacterium saccharolyticum	0.98	0.66
Carnobacterium sp. AT7	0.93	1.06
Lactobacillus hilgardii	0.54	0.99

different sources provided that the community is selected under similar environmental selection pressure, i.e. lignocellulosic substrate under thermophilic conditions. Further studies are needed to corroborate this assumption. The methods used for the characterization of the stable lignocellulose decomposing enrichment cultures in the previous studies (Table 2) did not allow the identification of consortium members at species level or their relative richness in the community. Our results therefore will help the development of a rationally designed and optimized stable microbial preparation for the reproducible facilitation of lignocellulose degradation.

This is, however, not a simple task as the four strains enriched in high abundance were accompanied by strains of low abundance in the enrichment community. The members of AD1 and AD2, which are present in higher than about 1% relative abundance are listed in Table 3.

The intriguing question is: to what degree these strains, and perhaps those present in richness lower than the 1% threshold, contribute to the bioaugmentation effect if at all? To test this, the type strains of the four most abundant bacteria were purchased from the German Collection of Microorganisms and Cell Cultures (Table 1) and sterile pure cultures were grown in their respective media recommended by the supplier. After removing the growth medium by centrifugation 1–3 x 10^8 cells/mL cell suspensions were used under bioaugmentation conditions using corn stover as substrate. The results, in case of *T. thermsaccharolyticum*, control, *C. cellulolyticum*, *T. pseudethanolicus*, *C. subterraneus*, Mixture, AD2 and AD1 are 133.6 ± 1.3, 138.3 ± 1.5, 140.2 ± 1.1, 152.5 ± 3.1, 152.9 ± 2.2, 154.3 ± 1.1, 170.6 ± 2 and 172 ± 0.7 mL, respectively, presented in Fig. 7, indicate that addition of the four bacterial strains and their mixture (in the ratio matching the relative abundances shown in Fig. 6) to the biogas reactors enhanced the methane yield in some cases. Surprisingly, *T. thermosaccharolyticum,* the most abundant member of the enriched consortium and *C. cellulolyticum* alone did not display significant augmentation of biomethane production. The other two abundant strains performed better and so did the mixture of the four strains. It is important to note that neither the individual pure cultures nor their mixture were as efficient as the original communities, AD1 and AD2. This is a strong indication of the active contribution of bacteria present in low numbers. The four most abundant strains comprised 64.3 (AD1) and 69.6% (AD2) of the total microbial biomass, respectively.

4. Conclusions

Two stable and sustainable thermophilic cellulose degrading enrichment cultures have been established independently from the same thermophilic biogas effluent. The two consortia, marked as AD1 and AD2, enhanced the methane yield from pure α-cellulose

and from corn stover by 22–24%. Next generation whole genome DNA sequencing revealed the main orders and most abundant species in AD1 and AD2. In line with earlier findings, members of the orders *Thermoanaerobacterales* and *Clostridiales* play important role in thermophilic lignocellulose decomposition. The composition and biological activities of the most abundant members in the two enriched communities were very similar. Several additional members, occurring in significantly lower numbers have also been identified. At this level a few differences between AD1 and AD2 were identified. Community members below 1% relative abundance were disregarded.

Bioaugmentation of biogas production from mechanically pretreated corn stover using the pure cultures of the most abundant four strains was successful. Nevertheless, the mixture of the most abundant strains, containing the bacteria in the ratio corresponding to the enrichment community, did not achieve the same augmentation as AD1 and AD2 did, indicating additional contribution by the minor constituents of the enriched microbial community. Therefore, the contribution of the microbial strains to the overall performance and biological activity of the community may differ from their relative abundance.

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