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Biogas production from wheat straw: community structure of cellulose-degrading bacteria

Li Sun, Bettina Müller and Anna Schnürer*

Abstract

Background: Wheat straw is one of the most abundant crop residues produced in the world, making it highly interesting as a substrate for biogas production. However, due to the complex structure, its degradability and gas yield are low. The degradability can be improved by pre-treatment, making the material more accessible to microbial degradation.

Methods: To investigate the microbial response to straw as a feed stock for biogas production, this study examined the community structure of cellulose-degrading bacteria in lab-scale biogas digesters operating with manure, alone or in co-digestion with straw, with and without pre-treatment (steam-explosion) at different temperatures. The community was studied by targeting the functional gene encoding glycoside hydrolases of families 5 and 48 using T-RFLP, clone libraries and qPCR.

Results: In general, bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* dominated the cellulose-degrading bacteria community in all digesters. The degree of similarity to the characterised bacteria was often low, and some clones were more closely related to the uncultured bacteria. The addition of straw, pre-treatment of straw and increasing operating temperature all affected the cellulose-degrading community structure, with differing responses in the *cel48* and *cel5* communities. Both communities changed in response to temperature, while only the *cel5* community was affected by the addition of straw and *cel48* community by straw pre-treatment.

Conclusions: The addition of straw, pre-treatment of straw and operating temperature all affected the cellulose-degrading community in biogas digesters, but there were no major differences in the digester performance and gas yield.

Keywords: Biogas; Cellulose; Community structure; Temperature; Glycoside hydrolases; *Cel48*; *Cel5*

Background

Emission of greenhouse gases (e.g. the fossil fuel-derived CO_2) has become a global concern, but about 88% of global energy consumption still derives from fossil fuels [1]. To meet the environmental challenges, the EU member states have decided to increase the proportion of renewable energy up to 20% of total consumption by 2020 [2]. Anaerobic digestion (AD) is highly interesting in this context, as it can function in waste treatment, production of a renewable energy carrier (biogas) and production of an organic fertiliser [3]. Various kinds of organic materials can be used as substrates for biogas production, such as sewage sludge, manure, organic municipal waste and agricultural wastes [4]. Among the agricultural wastes, straw is an interesting feedstock for biogas production [3]. For example, wheat straw is the most abundant crop residue in Europe and second most abundant in the world [5]. However, the character and composition of straw, with cellulose fibres intertwined with hemicelluloses and lignin, make it difficult to degrade [6]. Moreover, the use of straw for biogas production is restricted by its high C/N ratio and low levels of trace elements, limiting microbial growth and activity. The biogas yield from straw can be increased by pre-treatment, making the material more accessible to microbial degradation [3,6,7]. Furthermore, by co-digestion with more nitrogen-rich materials, for example, animal manure or food waste, the nutrient limitation can be overcome [8-11].

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The microbial degradation of organic material to biogas consists of four steps: (1) hydrolysis of complex organic polymers to soluble compounds; (2) fermentation of the products of hydrolysis into intermediate compounds such as fatty acids and alcohols; (3) anaerobic oxidation of these intermediate products to produce acetate, H_2 and CO_2; and (4) methane production by methanogenic Archaea [12]. In the case of straw, it has been suggested that the crystalline structure of the lignocelluloses obstructs degradation in the initial step and thus the hydrolysis of these insoluble compounds becomes the rate-limiting step [13,14]. Numerous studies have examined anaerobic cellulose-degrading bacteria and their enzymatic capabilities in order to clarify the degradation mechanisms and identify ways to enhance degradation rates. However, most of these studies have been performed on samples from gut and soil ecosystems [3,13,15,16] and only a few have examined cellulose-degrading bacteria in biogas digesters [17-19].

In general, anaerobic degradation of cellulose can be performed by physiologically diverse taxa of microorganisms [3,13,20]. The degradation proceeds through the action of different cellulases, such as endoglucanases, exoglucanases and β-glucosidases, together with different enzymes attacking hemicellulose [3,13,21]. The enzymes can either be soluble or, as in the anaerobic bacteria, combined in cell-bound cellulosomes [3,13]. The glycoside hydrolases are grouped into 120 amino acid sequence-based families according to the international classification of carbohydrate-active enzymes (CAZy database). In anaerobic bacteria, the cellulose-degrading glycoside hydrolases are mainly allocated to the glycoside hydrolase families 5, 9 and 48 [22-24]. Recently, Pereyra et al. in 2010 [24] designed and validated consensus degenerated primers to amplify the glycoside hydrolase genes of families *cel5* and *cel48* with the aim of enabling the characterisation and quantification of cellulose-degrading bacteria in biogas digesters.

The aim of the present study was to provide further knowledge about cellulose-degrading bacteria in biogas processes. Specific objectives were to examine the response in the community of bacteria to the addition of straw, non-treated and pre-treated by steam explosion, and to investigate the effect on the community by a change in operating temperature from mesophilic to thermophilic. The microbial community structure and the abundance of anaerobic cellulose-degrading bacteria were studied by targeting the functional gene encoding the glycoside hydrolases of families 5 and 48 [24] and by performing terminal-restriction fragment length polymorphism (T-RFLP), clone libraries and quantitative polymerase chain reaction (qPCR).

Methods

Digester samples

The samples used for the community analysis originated from four laboratory-scale anaerobic reactors (RM, RS, $R^{Tc}SS$ and $R^{37}SS$), operating semi-continuously and fed 6 days per week for a total of 455 days [25]. All digesters were started at mesophilic temperature (37°C) and operated with an organic loading rate of 2.8 g VS L^{-1} day^{-1} and a hydraulic retention time (HRT) of 25 days. Digester RM processed cow manure as the only substrate during the whole period of operation, while digester RS operated with mechanical chopped (un-treated) straw and cow manure [25]. Digesters $R^{Tc}SS$ and $R^{37}SS$ were fed with the same substrate, processed steam-exploded straw [22% of total volatile solids (VS)] and cow manure (78% of total VS), and were initially run at 37°C. Digester $R^{Tc}SS$ was then operated under the same conditions for the whole experimental period, while digester $R^{37}SS$ was subjected to a temperature rise from day 90. At first the temperature was raised (by approximately 1°C/week) to reach an operating temperature of 44°C on day 140. The digester, now called $R^{44}SS$, was then operated with these conditions at approximately 3 HRT. On day 226 the temperature was raised again (by approximately 1°C/week) to reach 52°C in the digester, now called $R^{52}SS$, on day 299 [25]. Again, the digesters were operated at around 3 HRT to ensure wash-out of the non-active microbial population. The methane yields were similar in all digesters (0.13 to 0.17 N L CH_4/kg VS), with no increase in response to straw addition, steam-explosion treatment or temperature change [25].

DNA extraction

Samples (20 mL) were withdrawn from each digester after operation at approximately 3 HRT under different management conditions and stored at −20°C. For digesters $R^{37}SS$, $R^{44}SS$ and $R^{52}SS$, samples were taken on days 89, 224 and 402, corresponding to the operating temperatures of 37°C, 44°C and 52°C. Digester $R^{Tc}SS$, which operated at 37°C for the whole period, was sampled after the same period of operation, i.e. days 89, 224 and 402, and these samples were named $R^{Tc}SS$, S1 to S3. Total genomic DNA was extracted from triplicate samples using the method described by Westerholm et al. [26]. Aliquots of 200 μL digester sludge were used for the DNA extraction, and 60 μL water was used in the final elution of DNA.

PCR amplification, cloning and sequence analysis

Primers targeting the glycoside hydrolase families 5 (*cel5*_392F: 3′-GAG CAT GGG CTG GAA YHT NGG NAA-5′ and *cel5*_754R: 3′-CAT CAT AAT CTT TGA AGT GGT TTG CAA TYT GDK TCC A-5′) and 48 (*cel48*_490F: 3′-TNA TGG TTG AAG CTC CDG AYT

AYG G-5′ and *cel48*_920R: 3′-CCA AAN CCR TAC CAG TTR TCA ACR TC-5′) were used to study the cellulose-degrading bacterial community structure [24]. Polymerase chain reaction (PCR) amplification was performed with Maxima Hot Start PCR Master Mix (Fermentas, Thermo Fisher Scientific, Hudson, NH, USA) using the following programme: initial denaturation at 95°C for 5 min, 40 or 60 cycles of denaturation at 95°C for 45 s, annealing at 51°C (*cel5*, 60 cycles) or 56°C (*cel48*, 40 cycles) for 45 s and elongation at 72°C for 45 s, followed by a final extension step of 7 min at 72°C. Triplicate extractions of DNA were diluted 10, 20, 50 and 100-fold and subsequently used as templates to find the optimal dilution to reduce inhibition during the PCR. For the optimal dilution, triplicate PCR runs were conducted for each sample. The resulting products from nine PCR reactions were pooled and purified with QIAquick gel extraction kit (Qiagen, Hilden, Germany) to reduce the potential bias. Clone libraries were constructed for the *cel5* community with samples from $R^{44}SS$ and for the *cel48* community using samples from RM, $R^{37}SS$ and $R^{44}SS$ by ligation of the gel-purified PCR amplicon into pCR™4-TOPO® vector (Invitrogen Life Technologies, Grand Island, NY, USA), followed by transformation of the ligation product into TOP10 One Shot® chemically competent *Escherichia coli* (Invitrogen), according to the manufacturer's instructions. The sequences obtained were quality-checked and edited with the software package Geneious, version 5.6.5 (Biomatters Ltd., Auckland, New Zealand) and subsequently assigned to operational taxonomic units (OTU). The sequences were compared with sequences available in the NCBI GenBank. Alignment of cloned sequences and selected reference sequences, as well as sequences from uncultured bacteria, was conducted using the programme MUSCLE [27]. The phylogenetic trees were constructed with the MEGA programme version 5 using the maximum likelihood method and WAG model [28]. The confidence of the trees was tested by bootstrap resampling analysis for 1,000 replicates.

T-RFLP

Both *cel5* and *cel48* genes were amplified, using the PCR conditions described above. The 5′ ends of the primers *cel5*_754R and *cel48*_920R were labelled with 6-carboxyfluorescein (FAM). For each sample, nine PCR reactions were pooled together and purified with QIAquick gel extraction kit (Qiagen). The pooled amplicons of *cel5* and *cel48* were digested overnight at 37°C with restriction enzyme *MboI* (New England Biolabs, Wilbury Way Hitchin, Herts, UK) and *AluI* (Fermentas), respectively. Fluorescently labelled terminal restriction fragments (T-RFs) were separated and detected with ABI3730xl capillary sequencer (Applied Biosystems, Cheshire, UK). GS ROX 500 internal size standard (Applied Biosystems) was included in all assays. T-RFLP profiles were processed by Peak Scanner software (Applied Biosystems). The relative abundance of the individual T-RFs was calculated by dividing the peak area by the total area of all peaks. T-RFs constituting less than 1% of the total peak area were excluded as background.

qPCR

To quantify the abundance of *cel48*, qPCR was performed using CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The PCR programme used was 10 min at 95°C, followed by 55 cycles of 40 s at 95°C, 30 s at the annealing temperature of 56°C, and 30 s at 72°C. A standard curve was constructed with genomic DNA from *Clostridium cellulolyticum* (DSM 5812) for primer *cel48*, following the protocol described previously [26]. Each reaction contained 10 μL of Maxima qPCR master mix with SYBR Green (Fermentas), 3 μL of DNA template and 0.5 μM of each primer with a final volume of 20 μL. Melt curve analysis and the qPCR data processing were conducted as described previously [26].

Nucleotide accession numbers

All sequences were deposited in the NCBI GenBank database. Clones obtained by primer pair *cel*48 can be identified by the accession numbers KC789646-50 (OTU01), KC789651-58 (OTU02), KC789659-64 (OTU03), KC789665-66 (OTU04), KC789667-69 (OTU05), KC789670 (OTU06), KC789671-73 (OTU07), KC789674-83 (OTU08), and KC789684 (OTU09). Clones obtained by primer pair *cel*5 can be identified by the accession numbers: KC789685-86 (OTU01), KC789687 (OTU02), KC789688-92 (OTU03), KC789693-97 (OTU04), KC789698-700 (OTU05), KC789701-11 (OTU06), and KC789712 (OTU07), KF193414-17 (OTU08).

Results and discussion

T-RFLP

Cel48

The cellulose-degrading community structure in the different digesters, managed with different operating strategies and substrates, was examined by T-RFLP analysis combined with the construction of clone libraries. The T-RFLP profile obtained with primer *cel48* (Figure 1a) was similar for digesters RM (with manure only, 37°C) and RS (with manure and un-treated straw, 37°C). The communities identified in both digesters were mainly represented by the T-RFs of 249, 322 and 328 bp. The T-RF 328 bp dominated, with a relative abundance of 54.9% and 66.9% in RM and RS, respectively. The similar

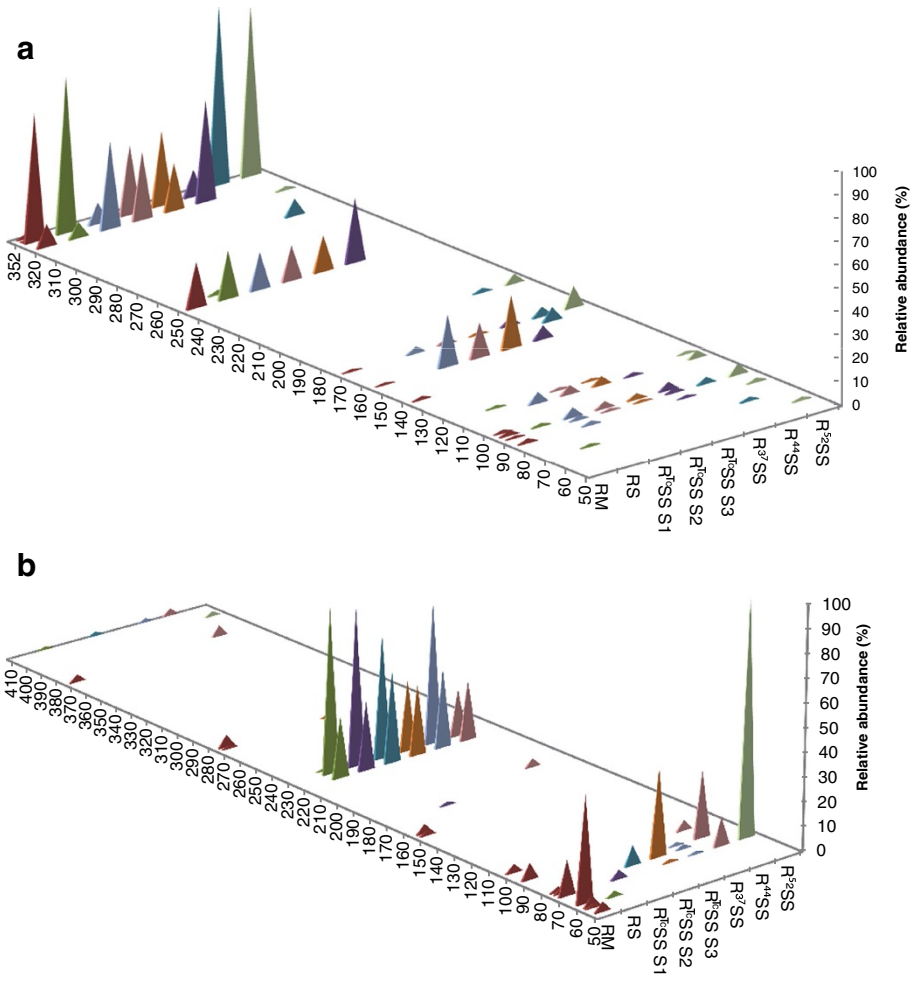

Figure 1 T-RFLP profile. T-RFLP profile representing the community of **(a)** glycoside hydrolases gene family 48 (*cel48*) and **(b)** glycoside hydrolases gene family 5 (*cel5*) in four lab-scale digesters processing: (1) manure (RM); (2) mechanically chopped (un-treated) straw and manure (RS); (3) steam-exploded straw and manure, operating temperatures 37°C, 44°C and 52°C ($R^{37}SS$, $R^{44}SS$ and $R^{52}SS$); and (4) steam-exploded straw and manure, operating constantly at 37°C ($R^{Tc}SS$ S1 to S3), where S1, S2 and S3 correspond to the same sampling point as $R^{37}SS$, $R^{44}SS$ and $R^{52}SS$.

T-RFLP profile observed in the digesters RM and RS suggests that straw did not have an impact on the community relative to manure alone. However, the community in digester $R^{Tc}SS$ (manure with steam-explored straw, 37°C) was slightly different to that in digesters RM and RS, suggesting that pre-treatment of the straw enabled new organisms to be active in degradation (Figure 1a). The T-RF at 322 bp had a higher abundance in $R^{Tc}SS$ (20.3% to 37.3%) than in RM (9.8%) and RS (6.7%). Moreover, the community in $R^{Tc}SS$ was also complemented with a new T-RF of 157 bp (14.8% to 22.7%). Analysis of samples from digester $R^{Tc}SS$, at three different time points (days 89, 224 and 402) showed a similar pattern, illustrating the stability over time and strengthening the hypothesis that the observed difference between this digester and RM and RS was caused by pre-treatment of the straw. Digester $R^{37}SS$ started with the same operating parameters and substrate as digester $R^{Tc}SS$, but was subjected to a temperature change. On day 89, both $R^{37}SS$ and $R^{Tc}SS$ had the same operating conditions and here the T-RFLP profiles had the same patterns. However, after changing the temperature in $R^{44}SS$, there was an apparent shift in the community and a new fragment of 355 bp became most dominant, with a relative abundance of 76.2% (Figure 1a). In addition, two new T-RFs of 294 bp (abundance 7.2%) and 168 bp (5.9%) appeared in the $R^{44}SS$ profile. None of these three T-RFs were detected in the reactors operating at 37°C. On day 540, a similar T-RFLP profile was observed in digester $R^{52}SS$ as in $R^{44}SS$, but the T-RFs at 168 and 294 bp were absent.

Cel5

In contrast to the results obtained using primer pair *cel48*, the T-RFLP profiles for *cel5* were different in digesters RM and RS (Figure 1b). In RM the community was dominated by two T-RFs of 64 bp (abundance 44.1%) and 74 bp (14.5%). These T-RFs were not present in RS, and instead the community was dominated by T-RFs 222 bp (26.0%) and 228 bp (69.1%). The two digesters were run in similar conditions, with the chopped, non-steam-exploded wheat straw in digester RS being the only difference. Thus, the results suggest that the presence of additional straw affected the *cel5* community structure. When comparing the T-RFLP profiles of RS, R^{Tc}SS S1 and R^{37}SS, a similar pattern was observed, suggesting that the steam explosion of the straw had no effect on the *cel5* community. This result is also in contradiction to the results obtained when analysing the *cel48* community, which changed in response to pretreatment of the straw. However, the *cel5* profile of R^{Tc}SS changed over time with an enrichment of T-RFs 81 bp (8.2% abundance at S2 and 36.3% at S3) during operation. It is unclear if this change was caused by the steam-exploded straw or just time. This change in community did not have an effect on the digester performance (e.g. the gas yield) [25]. However, analysis of the methane potential in batch tests using inocula from the digesters, taken at the same time points as the samples for community analysis, illustrated different cellulose-degrading efficiency at the different time points, possibly explained by the community shift [25]. R^{Tc}SS and R^{37}SS shared similar profiles at the first sampling point on day 89. However, on day 224, at the second sampling point at 44°C (R^{44}SS), the T-RFs 85, 95, 183 and 380 bp emerged in the profile with a relative abundance of 27.5%, 4.2%, 3.5% and 4.7%, respectively. None of these T-RFs was present in R^{Tc}SS, which ran at 37°C over the whole study period. T-RF 74 bp also increased in response to the increase in temperature, with abundance of 11.8%, compared to 3.8% and 1.5% at sampling points R^{Tc}SS S1 and S3, respectively. At the third sampling point (R^{52}SS), the T-RFLP was dominated by this T-RF of 74 bp, representing 98% of the relative abundance. In line with the T-RFLP of the *cel48* community, this illustrates the impact of temperature on the cellulolytic community. Furthermore, it suggests a relatively low complexity of the *cel5* community at thermophilic conditions, i.e. dominated by only one T-RF (Figure 1b).

Clone libraries and phylogenetic analysis

To identify the T-RFs in the T-RFLP analysis, clone libraries were constructed for digester R^{44}SS with primer *cel5* and for digesters RM, R^{Tc}SS and R^{44}SS with primer *cel48*. The sequence analysis recovered most of the T-RFs in the T-RFLP profiles from *cel48* community (Table 1). For *cel5* one major peak (T-RFs 64 bp) was not identified in the RM sample. The phylogenetic relationship of the sequences obtained was investigated by comparing them to the database, followed by maximum likelihood tree construction (Table 1, Figures 2 and 3). In general, the degree of similarity to the characterised bacteria was low, and some clones were more closely related to uncultured bacteria. For *cel48*, all of the sequenced clones belonged to the class of *Clostridia*, within the *Firmicutes*; mainly two families, *Clostridiaceae* and *Ruminococcaceae*. Previous studies have reported the importance of the phylum *Firmicutes* for the hydrolysis of cellulosic materials in biogas digesters, particularly the class *Clostridia* [29-31]. The genus *Ruminococcus* commonly present in the rumen was also identified in the clone sequences obtained with primer *cel48*. This genus has previously also been found in anaerobic digesters [32]. For *cel5*, the clones obtained all belonged to the phyla *Bacteroidetes* and *Firmicutes*, which is in agreement with other studies on biogas reactors. By targeting 16S rRNA genes, high abundance of representatives of both phyla have been found in biogas digesters operating with plant-based materials and/or manure, including a CSTR biogas digester fed

Table 1 Summarised clone sequences of *cel5* and *cel48*

Clone	T-RFs	Most closely related microorganism	Identity (%)	Accession number
Cel48				
OTU01	249	*Acetivibrio cellulolyticus*	69.5	ZP_09463651
OTU02[a]	328	*Clostridium josui*	72.9	BAA32430
OTU03	322	*Clostridium straminisolvens*	79.0	ACV92097
OTU04	168	*Ruminococcus champanellensis*	61.8	CBL17316
OTU05	352	*Ruminococcus albus*	46.1	YP_004105715
OTU06	157	*Ruminococcus flavefaciens*	68.7	ZP_06145360
OTU07	294	*Acetivibrio cellulolyticus*	74.3	ZP_09463651
OTU08	355	*Clostridium straminisolvens*	80.0	ACV92097
OTU09	328	*Clostridium cellulolyticum*	100	YP_002505088
Cel5				
OTU01	74	*Eubacterium siraeum*	49.5	CBK96866
OTU02	173	*Flavobacterium johnsoniae*	65.7	YP_001193127
OTU03[b]	222	*Eubacterium cellulosolvens*	52.5	ZP_10167476
OTU04[c]	228	*Marinilabilia salmonicolor*	63.8	ZP_11227339
OTU05	380	*Flavobacterium johnsoniae*	62.9	YP_001193127
OTU06[d]	85	*Mahella australiensis*	60.4	YP_004463133
OTU07	380	*Flavobacterium johnsoniae*	62.9	YP_001193127
OTU08	81	*Echinicola vietnamensis*	57.4	YP_007223444

[a]More closely related to uncultured bacterium from sulphate-reducing reactors (identity 81.3%, ACV50351); [b]More closely related to uncultured bacterium from buffalo rumen (identity 54.3%, ACA61144) [c]More closely related to uncultured bacterium from human gut (identity 68.3%, ADD61911); [d]More closely related to uncultured bacterium from biogas digester (identity 100%, AEV59735).

with fodder beet silage and cattle manure [30], a biogas digester processing pig manure [33] and a biogas reactor operating with maize silage, bruised grain, cattle manure and pig manure [34]. In the present study, representatives of the class *Clostridia* comprised 26.0% and 31.1% to 38.2% of the total *cel5* community in digester RS and $R^{Tc}SS$, respectively. Furthermore, the proportion increased in the digester subjected to a temperature rise, from 36% at 37°C ($R^{37}SS$) to 64.8% at 44°C ($R^{44}SS$) and 98.0% at 52°C ($R^{52}SS$). An increase in the abundance of *Clostridia* in response to temperature has been reported in previous studies on biogas digesters degrading a variety of different substrates [19,35,36].

Cel48

The T-RF 249 bp represented by OTU01 was present in all samples from digesters running at 37°C, comprising between 15.0% and 27.6% of the population. T-RF 294 bp (represented by OTU07) was present in digester $R^{44}SS$ (44°C) with a relative abundance of 7.2%. These OTUs were related to *Acetivibrio cellulolyticus* (identities 69.5% and 74.3%, respectively) isolated from municipal sewage sludge. This cellulolytic bacterium is able to grow on cellulose, cellobiose and salicin, but cannot metabolise simple sugars such as glucose, fructose or xylose. Acetic acid, hydrogen and carbon dioxide are formed as major fermentation products from cellulose or cellobiose. The optimal growth temperature range is 20°C to 40°C, and no growth occurs at 43°C [37]. The presence of a species grouping with *A. cellulolyticus* in RM, RS, $R^{Tc}SS$ and $R^{37}SS$ might suggest its importance during the degradation of cellulolytic material at mesophilic temperature (37°C to 44°C). The T-RF 294 bp was only detected in $R^{44}SS$, indicating the possible existence of a thermo-tolerant mesophilic strain of this species. However, in line with the growth pattern of the characterised species, neither of the two T-RFs was detected in digester $R^{52}SS$ (52°C). Species of the genus *Acetivibrio* have previously been identified in biogas digesters, including a CSTR (35°C) fed with fodder beet silage and cattle manure [30], a production-scale biogas plant (41°C) fed with maize silage, green rye and chicken manure [38], and a biogas digester processing pig manure [33]. In the phylogenetic tree in this study

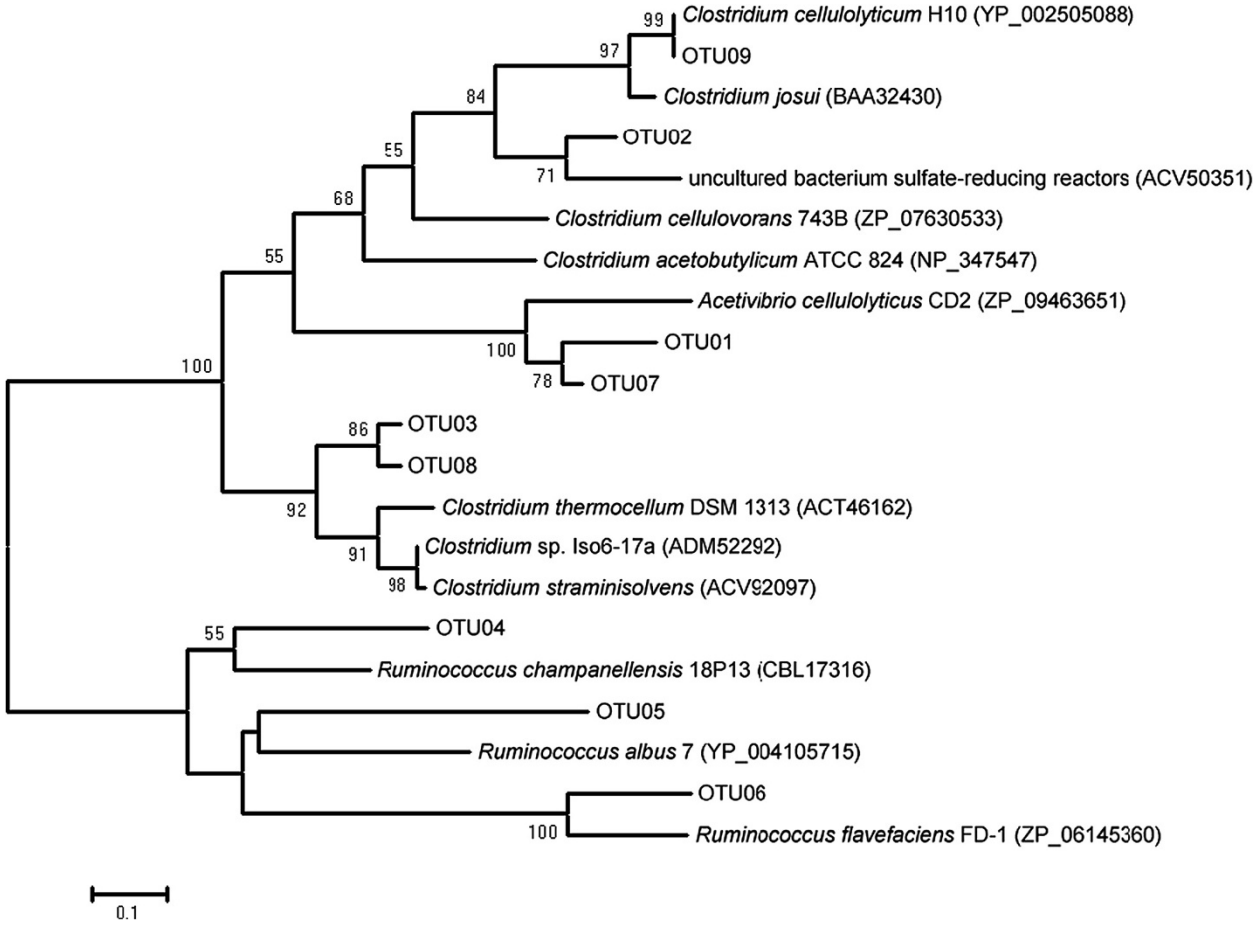

Figure 2 Phylogenetic tree of sequences from glycoside hydrolases gene family 48 identified in lab-scale digesters.

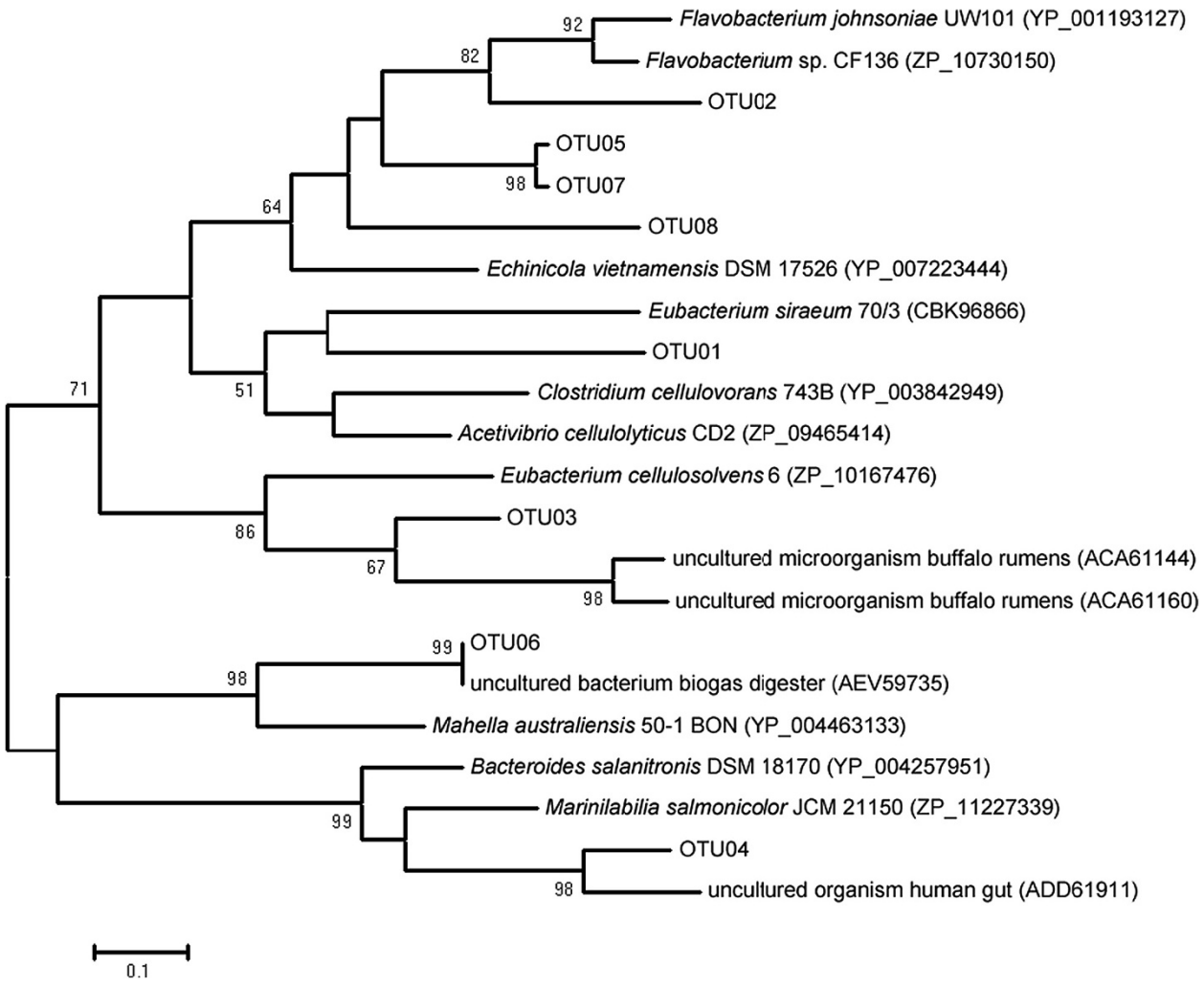

Figure 3 Phylogenetic tree of sequences from glycoside hydrolases gene family 5 identified in lab-scale digesters.

(Figure 2), OTU06, OTU04 and OTU05 grouped together with representatives of the genus *Ruminococcus*. T-RF 157 bp (OTU06) was detected in all the mesophilic digesters operating with steam-exploded straw ($R^{Tc}SS$ and $R^{37}SS$) and was grouped together with *Ruminococcus flavefaciens* (68.7% identity). This bacterium was isolated from rumen fluid and can utilise both cellulose and cellobiose as substrates for growth [39]. The presence of such a species might suggest competitiveness specifically for the degradation of the steam-exploded wheat straw. T-RF 352 bp (OTU05), closely related to *Ruminococcus albus* (46.1% identity), was only detected at 1% in digester RM. This bacterium has been isolated from rumen fluid and can utilise cellulose and cellobiose. *R. flavefaciens* is different from *R. albus* by producing succinate as the major fermentation product [40]. The T-RF 168 bp (OTU04) was only observed in the digester operating at 44°C ($R^{44}SS$). This clone was most closely related to *Ruminococcus champanellensis* (61.8% identity), a cellulolytic bacterium isolated from human faeces that ferments cellulose, cellobiose and xylan, produces acetate and succinate as the main fermentation products and grows optimally from 33°C to 39°C (optimum 39°C) [41]. In a previous study, the abundance of *Ruminococcus* in a biogas digester was shown to increase after pre-treatment of sludge from a wastewater plant [32]. A *Ruminococcus* sp.-like clone was also recovered in another biogas process operating with synthetic wastes under thermophilic conditions [42]. Clones with T-RFs 322 and 355 bp (OTU03 and 08) were closely related to *Clostridium straminisolvens*, with an identity of 79.0% and 80.0% respectively. The former was observed in all the mesophilic digesters, while the latter was only found in $R^{44}SS$ and $R^{52}SS$. The type strain $CSK1^T$ of *C. straminisolvens* was isolated from an enrichment originating from an artificial compost process [43]. When using cellulose as the sole carbon and energy source, the optimum growth temperature has been shown to be 50°C to 55°C, with no growth below 45°C, and acetate, lactate, ethanol, hydrogen and carbon

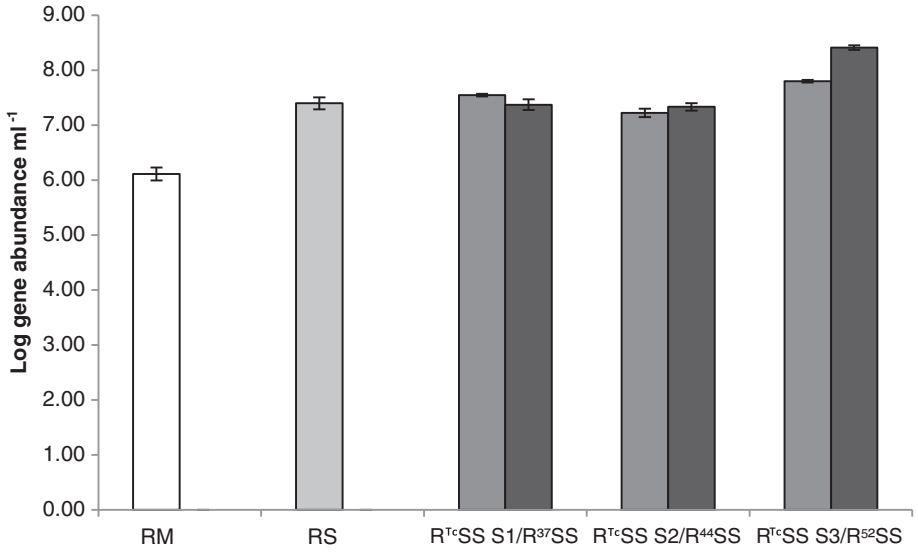

Figure 4 Real-time PCR analysis of the abundance of glycoside hydrolases gene of family 48 (*cel48*). Real-time PCR analysis of the abundance of glycoside hydrolases gene of family 48 (*cel48*) in four laboratory-scale digesters processing: (1) manure (RM); (2) mechanically chopped (un-treated) straw and manure (RS); (3) steam-exploded straw and manure, operating temperature 37°C, 44°C and 52°C (R^{37}SS, R^{44}SS and R^{52}SS); and (4) steam-exploded straw and manure, operating constantly at 37°C (R^{Tc}SS S1 to S3), where S1, S2 and S3 correspond to the same sampling point as R^{37}SS, R^{44}SS and R^{52}SS.

dioxide as the main fermentation products [43]. The common presence of bacteria related to *C. straminisolvens* in the digesters investigated here suggests both an important role in degradation and the possible existence of uncultured mesophilic strains. The T-RF 328 bp, representing OTU02 and OTU09, was detected in all mesophilic digesters (RM, RS, R^{Tc}SS and R^{37}SS), with a higher abundance in digesters RM (54.9%) and RS (66.9%). OTU02 was most closely related to an uncultured clone sequence (identity 81.3%) from mine drainage treatment systems with a high content of lignocellulosic carbon source [24]. The most closely related characterised bacterium is *Clostridium josui* isolated from compost and capable of fermenting various substrates such as cellulose, cellobiose, arabinose, glucose, maltose, ribose, xylose and xylan at temperatures between 25°C and 60°C [44]. OTU09 is identical to a characterised bacterium *C. cellulolyticum*, isolated from decayed grass, which can grow on cellulose, arabinose, cellobiose, fructose, glucose and xylose within the temperature range 25°C to 45°C.

Cel5

By analysing the cloned sequences generated from primer set *cel5*, two T-RFs of 222 and 228 bp (OTU03 and 04) were abundant in digesters receiving straw at 37°C (RS, R^{Tc}SS and R^{37}SS) and 44°C (R^{44}SS). OTU03 grouped together with an uncultured microorganism from rumen (Figure 3) with an identity of 54.3% [45]. The most closely related characterised strain is *Eubacterium cellulosolvens* [46] isolated from rumen, which is capable of fermenting cellulose and cellobiose as well as several monomeric sugars. OTU04 is similar (identity 68.3%) to a cellulolytic uncultured microorganism from the human gut [47]. The most closely related characterised bacterium is *Marinilabilia salmonicolor*, which is capable of fermenting various substrates such as cellulose, cellobiose, different monomeric sugars, inulin and starch [48,49]. T-RF 85 bp (OTU06) is closely related to an uncultured bacterium (identity 100%, AEV59735) detected in a biogas digester, but, unfortunately, information on this digester has not yet been published. The closest known relative is *Mahella australiensis* (identity 60.4%) isolated from an oil reservoir, which can ferment arabinose, cellobiose, fructose, galactose, glucose, sucrose, xylose and pyruvate [50]. T-RF 173 bp (OTU02) is similar to *Flavobacterium johnsoniae* (identity 65.7%) commonly present in soil and freshwater [51]. Recently, genome analysis predicted numerous glycoside hydrolases and esterases, suggesting the capability for hemicellulose utilisation [52]. Clones with a T-RF of 380 bp (representing OTU05 and 07) were rather distantly related to *F. johnsoniae*. A clone with T-RF 74 bp was close to *Eubacterium siraeum* (49.5% identity) isolated from human faeces [53]. The T-RF 81 bp, enriched over time in the R^{Tc}SS, was most closely related to *Echinicola vietnamensis* (57.4% identity) isolated from seawater [54] but has also high similarity to *Cellvibrio japonicus* isolated from soil [55]. The former is a gliding bacterium degrading starch, but not cellulose, cellobiose or glucose and with optimal growth temperature ranges from 30°C to 32°C. The latter is

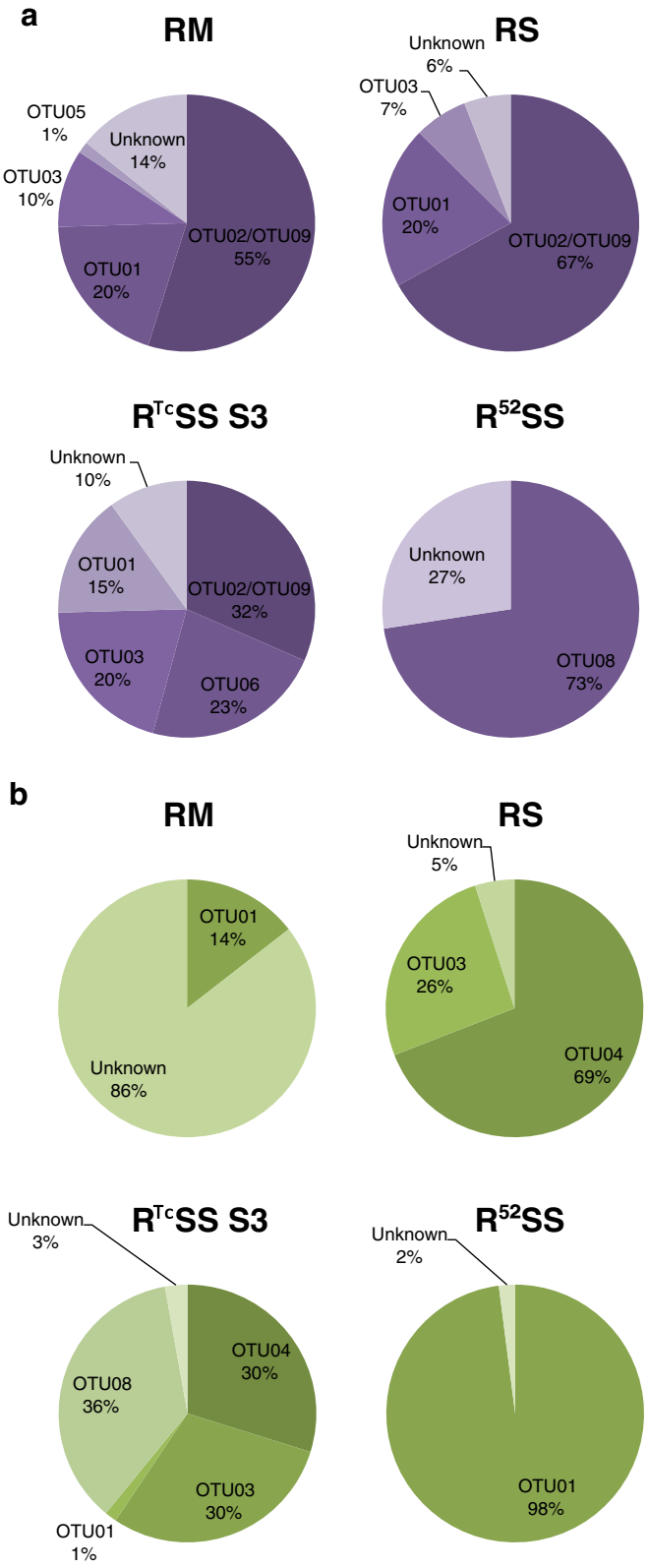

Figure 5 Summarised relative abundance of each OTU based on the T-RFLP profiles. (a) *cel48* and **(b)** *cel5* in four laboratory-scale digesters processing: (1) manure (RM); (2) mechanically chopped (un-treated) straw and manure (RS); (3) steam exploded straw and manure, operating at temperature 37°C (R^{37}SS S3); and (4) steam-exploded straw and manure, operating at 52°C (R^{52}SS).

considered as a specialist for plant degradation harbouring a lot of different kinds of glycoside hydrolases [54,55]. This T-RF had a relative abundance of 8.2% and 36.3% at the last two sampling occasions (Figure 1b).

It is worth noting that the clone library of *cel5* revealed that this primer set picked up a large proportion of a non-specific sequence of 483 bp (29 out of 91 clones sequenced). Thus this primer pair was not used for qPCR assay and the T-RF of 436 bp derived from the amplicon 483 bp was excluded from the T-RFLP results.

qPCR

The glycoside hydrolase family 48 gene (*cel48*) was quantified in all lab-scale digesters using qPCR (Figure 4), with a reaction efficiency varying between 97.2% and 98.3%, while the linear correlation coefficient r^2 was between 0.977 and 0.992. The abundance of *cel48* in digester RM was $1.3 \pm 0.3 \times 10^6$ copies/mL. The digesters with straw (treated and un-treated) had a tenfold increase in copy number; RS ($2.5 \pm 0.6 \times 10^7$ copies/mL), $R^{Tc}SS$ ($1.7 \pm 0.3 \times 10^7$ to $6.3 \pm 0.3 \times 10^7$ copies/mL) and $R^{37,\ 44,\ 52}SS$ ($2.2 \pm 0.3 \times 10^7$ to $2.6 \pm 0.3 \times 10^8$ copies/mL). The abundances of *cel48* were apparently relatively stable at all three sampling points in both $R^{Tc}SS$ and $R^{37,\ 44,\ 52}SS$, and thus the temperature did not have any significant effect on *cel48* abundance.

Conclusions

In summary, the quantitative information on the abundance of *cel48* obtained by qPCR assay revealed that straw in the substrate stimulated a tenfold increase in the numbers of this gene copy. Community analysis by T-RFLP combined with construction of clone libraries showed that complementing manure with non-treated straw for co-digestion clearly changed the *cel5* community (Figure 5b), while the *cel48* community remained approximately the same, irrespective of straw addition. On the other hand, pre-treatment of the straw by steam explosion initially had an impact on the *cel48* community only (Figure 5a). However, the profile in the *cel5* community in the digester operating with steam-exploded straw changed over time and became separated from the digester operating with un-treated straw (Figure 5b). Both communities responded to the increase in operating temperature, e.g. new sequence types appeared and the complexity of the population decreased, resulting in one or two dominant sequence types (Figure 5). Furthermore, many sequenced clones were more similar to sequences from uncultured organisms, particularly in the *cel5* community, reflecting the demand of isolation and characterization of new species. In spite of the changes in the cellulose-degrading community structure, there was no major difference in the biogas yield of the digesters studied.

Abbreviations

AD: anaerobic digestion; *Cel5*: glycoside hydrolase families 5; *Cel48*: glycoside hydrolase families 48; HRT: hydraulic retention time; OTU: operational taxonomic unit; PCR: polymerase chain reaction; T-RF: terminal restriction fragment; T-RFLP: terminal restriction fragment length polymorphism; VS: Volatile solids.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LS carried out the laboratory work, participated in the planning of the study as well as analysis of the obtained data and was main responsible for writing the manuscript. BM provided analytical assistance, participating in planning of the study and in writing the manuscript. AS designed, conceived and coordinated the study and participated in analysis of data and in writing the manuscript. All authors read and approved the final manuscript.

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