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ORIGINAL PAPER

Microbial community structure in anaerobic co-digestion of grass silage and cow manure in a laboratory continuously stirred tank reactor

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Abstract The impacts of feeding ratio and loading rate on the microbial community during co-digestion of grass silage with cow manure in an anaerobic laboratory continuously stirred tank reactor were investigated by 16S rRNA gene-based fingerprints. The microbial community remained stable when the reactor was fed with cow manure alone and with up to 20% of grass silage in feedstock at an organic loading rate (OLR) of 2 kg VS m^{-3} day^{-1}. Large changes in the bacterial community were observed when the loading ratio of grass was increased to 40%, while there was little change in the archaeal community. During the increase in OLR from 2 to 4 kg VS m^{-3} day^{-1} the bacterial community structure showed few differences, whereas *Archaea* was undetectable. Sequencing of the major DGGE bands indicated that the phylum *Bacteriodetes* predominated in the bacterial community. Two unclassified bacteria with high abundance survived throughout the operation of the reactor.

Keywords Microbial community · Anaerobic digestion · Terminal restriction fragment length polymorphism (T-RFLP) · Denaturing gradient gel electrophoresis (DGGE) · Continuously stirred tank reactor (CSTR)

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Introduction

Anaerobic co-digestion of animal manure and organic wastes or crops to methane-rich biogas is increasingly used, as methane can be converted to heat, electricity or replace traffic biofuel. Besides providing energy, greenhouse gas emissions are also expected to decrease from waste and manure management (Amon et al. 2006; Clemens et al. 2006). Co-digestion of crops with animal manure has been demonstrated in continuously stirred tank reactors (CSTRs) in the laboratory (Fischer et al. 1983; Fujita et al. 1980; Hashimoto 1983; Hills 1980; Somayaji and Khanna 1994; Weiland 2003) and on the farm scale (Kaparaju et al. 2002). A variety of start-up strategies, feeding compositions, organic loading rates (OLRs) and hydraulic retention times (HRTs) have been evaluated, but the microbial community structure has only recently been

linked to operating conditions and digestion performance. Stable performance of anaerobic digestion systems, as measured by methane yield, can be greatly related to steady microbial community structure (McMahon et al. 2001). However, a flexible community structure can maintain a stable ecosystem function (Fernandez et al. 1999, 2000). Thus, the link between the performance and microbial community structure remained uncertain. A community-based approach to anaerobic digestion should therefore produce more knowledge about the relations between microbial community structure and operating and functional parameters.

The anaerobic digestion of organic material to methane is a multi-step process mediated by Bacteria and methanogenic Archaea (Chynoweth and Pullammanappallil 1996). The polymers are hydrolysed into soluble compounds under fermentative conditions. Acidogens and acetogenic bacteria convert these intermediates into acetate and one-carbon compounds. These compounds in turn can be converted directly by methanogenic Archaea into methane and carbon dioxide. Generally, hydrolysis is considered to be the rate-limiting step in the anaerobic digestion of particulate organic material (Noike et al. 1985; Veeken and Hamelers 1999). Therefore, information on the microbial ecology and mechanisms of hydrolysis during anaerobic digestion is of importance in seeking to increase the rate of hydrolysis and the overall efficiency of the anaerobic digestion process. A number of bacteria well known for their cellulolytic capabilities, and mostly belonging to the order *Clostridiales*, have been studied (Burrell et al. 2004; Chachkhiani et al. 2004; Lynd et al. 2002; O'Sullivan et al. 2005). However, the investigation of microbial community structure taking part in the anaerobic co-digestion of crops and animal manure continue to be lacking.

Comparative analyses of anaerobic microbial communities by culture-based methods have limitations because of syntrophic interactions, low growth rates, unknown growth requirements and obligate anaerobiosis. The advent of small-subunit rRNA-based molecular fingerprinting techniques has made it possible to study complex anaerobic microbial communities without the cultivation of microorganisms (Hugenholtz et al. 1998). Among these fingerprinting methods, terminal restriction fragment length polymorphism (T-RFLP) (Dunbar et al. 2001; Kitts 2001) and denaturing gradient gel electrophoresis (DGGE) (Muyzer 1999) are widely used for the differentiation of communities and for the comparison of the relative phylotype richness in environmental samples. They were also used for identifying specific organisms in a community in conjunction with gene sequence information. Both methods have several advantages over other fingerprinting methods, since they have high sample throughput, and enable the resolution of a complete target community. Very recently, DGGE and T-RFLP profiling have been used in exploring microbial community structure in the anaerobic digestion of industrial wastes (Connaughton et al. 2006) and manure (Mladenovska et al. 2006), and co-digestion of food waste and biosolids (McMahon et al. 2001). It is anticipated that knowledge on both the structure of microbial communities and the relations between microbial community structure and digester performance during anaerobic co-digestion of crops and manure would also be obtained by DGGE and T-RFLP profiling.

The aim of the present study was to investigate the microbial community structure involved in the co-digestion of grass silage with cow manure, and to assess the impact of the feed component ratio and OLR on the dynamics of the microbial community in a laboratory CSTR.

Materials and methods

Source of biomass

Biomass source have been described in detail by Lehtomäki et al. (2007). In brief, grass silage was initially prepared from grass comprising 75% of timothy *Phleum pretense* and 25% of meadow fescue *Festuca pratensis* grown on a local farm in Central Finland (Kalmari farm, Laukaa). The grasses were harvested at the early flowering stage and chopped after 24 h of pre-wilting, and were further ensiled in a bunker silo with the addition of a commercial silage additive (lactic acid bacteria inoculant AIV Bioprofit, containing 60% *Lactobacillus rhamnosus* and 40% *Propionibacterium freudenreichii* spp. *shermanii*, Kemira Growhow Ltd.). The grass silage was further chopped to a particle size of approximately 3 cm in the laboratory and then stored at −20°C. Before feeding to the reactor, the grass silage was allowed to

thaw overnight at 4°C and was then mixed with manure. Cow manure was obtained from a storage tank with a retention time of up to 6 months on several occasions during the study and stored at 4°C. The composition of cow manure batches was also analyzed at each time when they were obtained and showed very little variation. Inoculum was taken from the farm's mesophilic digester, which treated dairy manure and industrial confectionary by-products.

Reactor set-up and operation

Two 5 l CSTR reactors were operated at 35 ± 1°C and with a continuous stirring rate of 300 rpm. First the reactors were inoculated with 4 l of inoculum. Thereafter, the reactors were fed semi-continuously with cow manure at OLR of 2 kg VS m^{-3} day^{-1} once a day for 5 days $week^{-1}$ with a HRT of 20 days for 27 days. Before feeding, an equal amount of digestate was withdrawn. The two reactors showed nearly identical specific methane yields and VS removals (Lehtomäki et al. 2007). Subsequently one reactor was continued similarly for another 28 days before stopped, while in the other reactor feeding of grass silage along with manure was then initiated by replacing 10% of the feedstock volatile solids (VS) with grass silage while maintaining constant OLR and HRT. The proportion of grass silage in the feedstock was then stepwise increased to 40% of the feedstock VS. The OLR was then increased first to 3 and then 4 kg VS m^{-3} day^{-1} while the HRT was decreased to 18 and 16 days, respectively. Increase in OLR was obtained as feed VS was increased from 4.0 to 5.4 and to 6.4 kg VS/m^{-3} day^{-1} whereas decreased HRT was obtained by slightly increasing the volume of added feed. In total, the 318 days trial was divided into seven different operational periods (P1–P7) characterized by a change either in the feedstock (grass silage proportion) or in the OLR (Table 1).

DNA extraction and PCR-amplification of 16S rRNA genes

For the microbial community analyses, 5 ml of sample from the reactor was collected on each sampling day, as shown in Table 1, and stored immediately at −80°C. Genomic DNA was extracted from the samples after a physical disruption of the biomass using a FastPrep® Instrument and a Fast DNA® SPIN Kit for Soil (Qbiogene, Inc., CA, USA) according to the manufacturer's instructions.

The 16S rRNA genes for T-RFLP fingerprinting were amplified using the PCR primer sets 27f-6-carboxyfluorescein (FAM)/1492r (Sait et al. 2003) and Ar109f/Ar912rt-FAM (Lueders and Friedrich 2003) specific for the bacterial and archaeal 16S rRNA genes, respectively. PCR was performed in 100-µl reaction mixture containing approximately 100 ng of DNA template, 1× PCR buffer, 200 µM of each deoxynucleoside triphosphate, 2U of DyNAzyme™ II DNA polymerase (Finnzymes, Espoo,

Table 1 Operational conditions and performance of the reactor

Operational periods	P1	P2	P3	P4	P5	P6	P7
Run days (day)	0–27	28–55	56–84	85–141	142–203	204–266	267–318
Sampling day (day)	15; 27	36; 50	64; 77	92; 106; 134	148; 162; 190	218; 225; 253	281; 295; 309
Grass silage in the feed (% VS)	0	10	20	30	40	40	40
OLR (kg VS m^{-3} day^{-1})	2	2	2	2	2	3	4
HRT (day)	20	20	20	20	20	18	16
Specific methane yield (m^3 kg^{-1} VS)[a]	0.15 ± 0.05	0.14 ± 0.02	0.18 ± 0.01	0.27 ± 0.03	0.25 ± 0.02	0.23 ± 0.01	0.19 ± 0.02
VS removal (%)[b]	26	41	42	43	46	53	52

pH remained between 7.3 and 7.6 and VFA (acetic and propionic acids) concentrations were lower than 300 mg l^{-1} in the digestates throughout the run

OLR Organic loading rate, *HRT* Hydraulic retention time

[a] Calculations as average ± SD of the measurements during the last 2 weeks of each operational period

[b] Calculated on basis of average values

Finland), and 0.5 μM of each primer. The PCR conditions consisted of initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 90 s and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min.

The 16S rRNA genes for DGGE analysis were amplified by nested PCRs. The primer sets 27f/1492r and Ar109f/Ar912rt were used for the initial PCR amplification. Cycle conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 10 min. The PCR products were purified with a GFX™ PCR DNA and Gel Band Purification kit (Amersham, NJ, USA) and further used as templates for the second PCR amplification. The primers used for the secondary PCR were universal forward primer 533f and reverse primer 907r^{GC} with a 40-base pair GC-clamp attached to the 5′terminus. The secondary PCR amplification was performed using a touchdown program consisting of initial denaturation at 94°C for 3 min followed by denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s. The annealing temperature was reduced by 0.5°C every cycle until it reached 50°C. A further 10 cycles was carried out under these conditions prior to the final extension cycle, which was performed at 72°C for 10 min. All PCR products (5 μl) were visually verified using 1 or 1.5% agarose gel electrophoresis and ethidium bromide staining (BioRad, CA, USA).

T-RFLP analysis

Prior to digestion, amplicons were purified with a GenElute™ PCR clean-up kit (Sigma) according to the manufacturer's instructions and were quantified by using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA). Approximately 100 ng of purified DNA was digested in a 10-μl reaction volume with 10 U of MspI and TaqI for Bacteria and Archaea, respectively. The digestions were carried out for 3 h at 37°C for MspI and 65°C for TaqI. Fluorescently labeled terminal restriction fragments (T-RFs) were separated on an ABI Prism® 3100 automated sequencer (Applied Biosystems, CA, USA) using an internal size standard (GeneScan™ 1200 LIZ®; Applied Biosystems, CA, USA). T-RFLP electropherograms were analyzed with GeneMapper® software version 3.1 (Applied Biosystems, CA, USA). T-RF sizes between 50 and 1,200 bp with a peak area of ≥50 fluorescence units were used in the analysis. The analysis was performed in triplicate for each DNA extraction. The replicate profile with a total DNA quantity close to the average DNA quantity of the T-RFLP profiles in the comparison data set was selected for the analysis. Profiles from different samples were manually aligned by inspection of the size of peaks in bases according to Sait et al. (2003). T-RF profiles were then standardized to the profile with the smallest total fluorescent units of peak areas following the procedure suggested by Dunbar et al. (2001). After standardization, a percentage threshold was applied to remove all T-RFs that contributed <2% to the total area of all the T-RFs in a T-RFLP profile. The relative abundance of a detected T-RF within a given profile was then calculated on the basis of the standardized peak area according to Schwarz et al. (2007).

For comparison of the bacterial T-RFLP profiles, a binary vector representing the presence (1) or absence (0) of T-RFs in the profile was constructed for each sample. A Sorensen–Dice similarity coefficient was used as a measure of the similarity of binary vectors, and a matrix of pairwise comparisons was generated (Jackon et al. 1989). Agglomerative hierarchical clustering was performed using the similarity matrix of the Sorensen–Dice coefficients and the Nearest Neighbor method and was displayed as dendrograms. The analyses were performed by using the SPSS 14.0 software for windows (SPSS Inc. USA).

DGGE

DGGE was performed as described by Bodelier et al. (2005) using the INGENYphorU-2 × 2 system (Ingeny, The Netherlands). Aliquots of 25 μl of each of the GC-clamped amplicons were loaded into the 6% polyacrylamide gels with denaturing gradient ranging from 30 to 60% where 100% denaturant contained 7 M urea and 40% formamide. The gels were run at 100 V and 60°C for 20 h. Following this, the gels were stained for half an hour in Sybr-Gold™ (Invitrogen, USA) nucleic acid stain (1:10000 dilution in 1× TAE buffer) and photographed with a Kodak 1D

v.3.5.4 system (Kodak, USA) equipped with a UV illuminator.

Sequencing of 16S rRNA gene fragments and phylogenetic reconstruction

DGGE bands for sequencing were selected according to the emergence or disappearance of particular bands and the predominant bands for all the samples. Selected bands were excised and suspended in 20 µl of milli-Q sterilized water for 24 h at 4°C. The purity of selected bands was confirmed by a second DGGE. The elution from pure bands was used as templates in the PCR reactions, which were conducted with the primer set 533f/907r. PCR products were purified for sequencing with Econuclease I-SAP enzyme (Fermentas) according to the manufacturer's instructions. Sequencing was performed on an ABI Prism® 3100 sequencer (Applied Biosystems, CA, USA) using an ABI BigDye® terminator v3.1 cycle sequence kit (Applied Biosystems, CA, USA) as specified by the manufacturer. The nucleotide sequence of each band was confirmed by sequencing two or three parallel bands.

Raw sequence data were assembled and checked with the DNAMAN software package (Lynnon Biosoft, USA). An equal portion (about 372 bp) of sequences were aligned and uploaded to the *MyRDP* space maintained at the Ribosomal Databases Project II (Cole et al. 2007). A homology search of aligned sequences against the sequences available in databases RDP II (release 9.46 January 2007) was performed with the SeqMatch program at the RDP II. Finally, a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) with the PHYLIP 3.65 software package and the Jukes-Cantor distance model (Felsenstein 1985). Bootstrap re-sampling analysis for 100 replicates was performed to estimate the confidence of the tree topology (Felsenstein 1985).

Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper were deposited in the NCBI nucleotide sequence databases under accession numbers EF597504 through EF59 7510.

Results

The microbial community structure in the reactor was investigated during the seven different periods (P1–P7) from samples taken soon after the feeding or OLR change (early E, day 9–15), after ca 1 HRT operation (middle M, day 22–29) and after ca 2 HRTs operation (late L, day 49–50) (Table 1).

Bacterial community dynamics

The T-RFLP fingerprints of bacterial 16S rRNA gene fragments revealed a total of 32 terminal restriction fragments (T-RFs) during the run (Fig. 1). In the beginning of the run (P1M) 12 T-RFs were detected with relatively abundant T-RFs of sizes 65, 85, 157, 536 and 540 bp, which together accounted for 73.7% of the profile. The inclusion and increase of grass silage in the feedstock led to the reduction or emanation of some of these T-RFs and the appearance of some new T-RFs. For instance, T-RFs with sizes of 176, 186, 296, 359 and 536 bp disappeared as the proportion of grass in the feedstock increased. On the contrary, there were two T-RFs of 87 and144 bp appeared. Several minor T-RFs of 128, 133, 134 and 144 bp were detected only during the reactor was fed with 30% grass silage in the feedstock. The most richness community (15 T-RFs) was detected during this operational period (P4M).

Increase of the OLR also caused the disappearance of the T-RF of 85 bp and the appearance of a T-RF of 422 bp. In addition, T-RFs of 83 and 162 bp were undetectable when increasing the OLR (from P5L onwards) whereas a certain amount of T-RF with the size of 180 bp was present.

The relatively abundant T-RFs of 157 and 540 bp occurred throughout the run. The T-RF of 65 bp was also present in most of the samples, showing at its highest abundance up to 36.9%, but it was undetected in several case samples.

The similarity of the bacterial T-RFLP profiles within the data set was expressed as a Sorensen–Dice pairwise similarity coefficient. These values were then used to generate distances, and a dendrogram was constructed from these values to depict the similarities between the different profiles (Fig. 2). The dendrogram clearly shows a distinct grouping between the samples before P5L, with a continually increasing proportion of grass in feedstock while

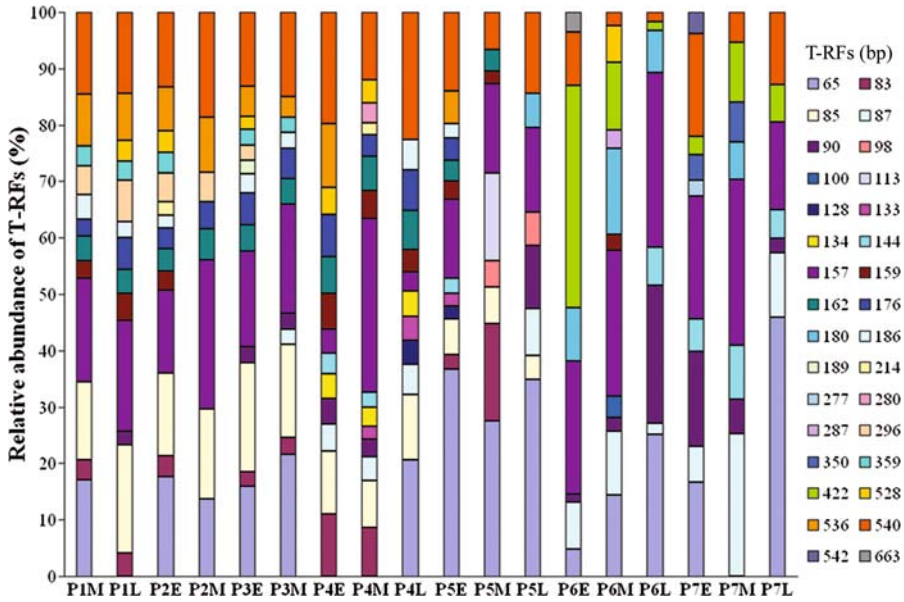

Fig. 1 Relative abundance of bacterial 16S rRNA gene fragments retrieved from the reactor samples throughout the operational periods P1 to P7 based on T-RFLP analysis. The operational periods were characterized by a change in either the applied loading ratio of grass silage to manure or the organic loading rate. Each operational period was divided into an early (*E*) (day 9–15), middle (*M*) (day 22–29) and late (*L*) (day 49–50) stage. The length of *T-RFs* in base pairs (bp) was indicated

maintaining a constant OLR, and those thereafter. T-RFLP profiles before P5L were clustered in a group in the dendrogram. T-RFLP profiles during the increase of the OLR while maintaining the constant feeding ratio were clustered in another group, as shown in Fig. 2. Another important finding that can be concluded from the dendrogram is that the T-RFLP profiles during increasing the proportion of grass in the feedstock were more closely related to the previous sampling event but more distinct from the period when the reactor was fed with cow manure alone (P1). In addition, a great difference in T-RFLP profiles was observed after the proportion of grass was increased to 40%. This suggests that the bacterial community structure was dynamic and in a state of constant change along with the increase in the proportion of grass in the feedstock. Moreover, increasing the grass up to 40% led to a significant change in the bacterial community structure in the reactor. In contrast, there was less difference in the T-RFLP profiles during the increase in the OLR than during the change in the feed ratio, as shown in Fig. 2.

The bacterial DGGE band patterns revealed distinct bands during the runs as shown in Fig. 3. The largest number of bands was recorded when 40% of the feedstock was grass and an OLR of 2 kg VS m^{-3} day^{-1} was applied (P5L), represented the greatest level of bacterial diversity. Nine bands

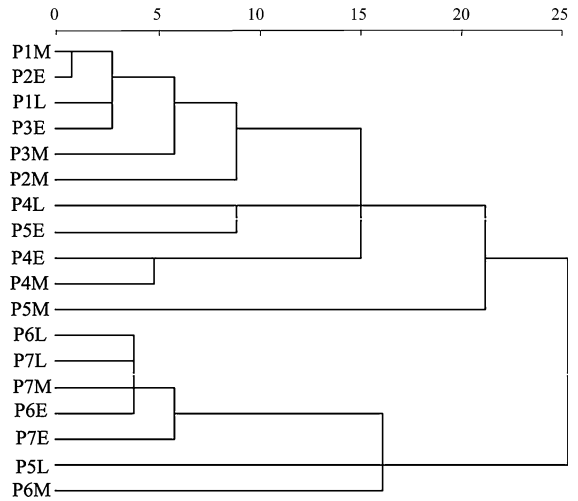

Fig. 2 Clustering of the bacterial 16S rRNA gene-based T-RFLP profiles based on Sorensen–Dice similarity coefficient. P1 to P7 refer to different operational periods, and *E*, *M* and *L* refer to the different stages in each period. The dendrogram was constructed by using the Nearest Neighbor method on the basis of the similarity matrix of Sorensen–Dice coefficients

were visualized when the reactor was fed with cow manure alone (P1). The DGGE bands also revealed three clearly different band patterns among the analyzed samples. The bacterial community during the reactor was fed with cow manure alone and with up to 20% grass silage in feedstock (P1 to P3M) displayed a similar DGGE band pattern, whereas samples taken during feeding with 30–40% grass

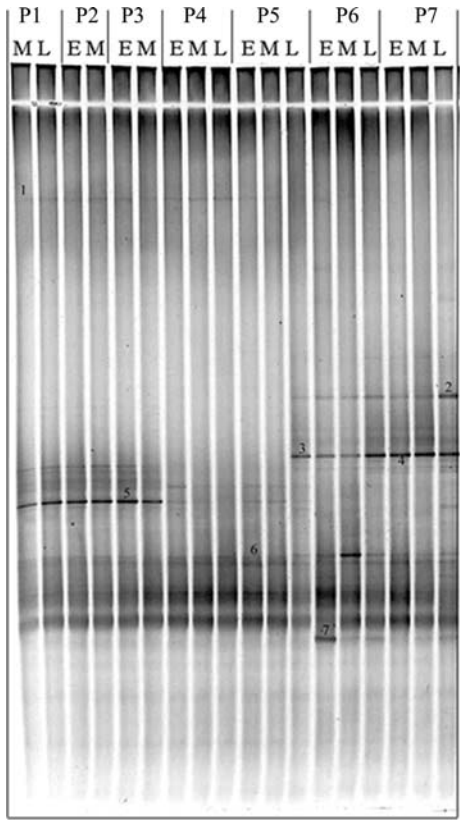

Fig. 3 DGGE band patterns of bacterial 16S rRNA gene fragments obtained from the reactor samples throughout the operation. The sequences of the indicated *bands* (B1 to B7) were identified. Each *lane* represents the bacterial community structure from an individual sampling event. *P1* to *P7* refer to different operational periods, and *E*, *M* and *L* refer to the different stages in each period

silage in feedstock (P4, P5E and P5M) formed another band pattern. The bacterial community after P5L showed a similar band pattern, which exhibited great dissimilarity with the previous band patterns. This was consistent with the results obtained from the T-RFLP profiles. The bacterial community shift between P5M and P5L might imply that the bacterial structures at P5E and P5M were under transit state while the bacterial structure at P5L was under steady state to feeding condition with 40% grass silage. The DGGE band patterns further indicated during the run the emergence of new bands. For example, eight new bands were detected after P5L, and one of them (B3) appeared as a dominant band. Conversely, evidence of the reduction or elimination of some phylotypes during the run was also observed. Specifically, five bands were absent from the samples collected during the increase of the OLR, one of which (B5) appeared as a dominant band in the samples from P1 to 3. In addition, DGGE revealed that two phylotypes were abundant in the bacterial community from P1 to P3, whereas one phylotype was clearly dominant in the bacterial community after P5L. However, from P4 to P5M, it seemed the bacteria were evenly distributed in the community and no individual phylotype could be singled out. Change also occurred in the bacterial community structure in the reactor during the increase in the loading rate, although the changes in community composition were not as significant as those observed in the previous samples taken during the changes in the feed ratio. The observations obtained from the DGGE band patterns were comparable to those derived from the T-RFLP profiles.

Archaeal community structure

The archaeal 16S rRNA gene could be amplified only from the samples during the increase of grass proportion in the feedstock (before P5L). The archaeal T-RFLP fingerprints indicated a total of four T-RFs as shown in Fig. 4, implying lower diversity of the archaeal community than the bacterial population in this reactor. A T-RF of 185 bp clearly predominated in the reactor. In addition, the T-RFLP fingerprints were undistinguishable until the middle operational period of 30% grass in feedstock (P4M), suggesting the archaeal community remained stable during these operational periods. A T-RF of 281 bp

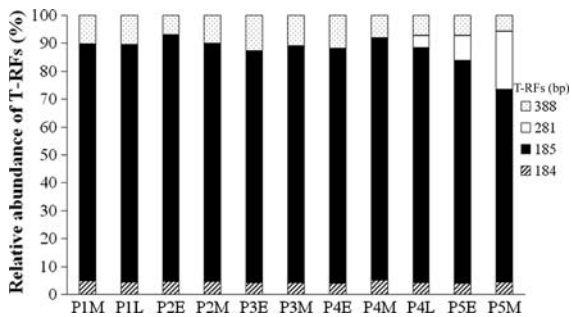

Fig. 4 Relative abundance of archaeal 16S rRNA gene fragments retrieved from the reactor samples throughout the operational periods *P1* to *P5* based on T-RFLP analysis. The operational periods were characterized by a change in the applied loading ratio of grass silage to manure. Each operational period was divided into an early (*E*) (day 9–15), middle (*M*) (day 22–29) and late (*L*) (day 49–50) stage. The length of *T-RFs* in base pairs (bp) was indicated

with increasing in the relative abundance was detected when increasing the grass proportion from 30 to 40%.

The DGGE analysis of archaeal 16S rRNA gene displayed a similar band pattern during increasing the feed ratio of grass in feedstock (data not shown), indicating there was no change in the archaeal community during these operational periods of the reactor.

Sequence analysis of the abundant bacterial groups

A total of seven distinct sequences were identified. Comparative analyses with nucleotide databases and phylogenetic reconstruction revealed that four sequences (B2, B3, B4 and B5) were affiliated with the phylum *Bacteroidetes* while one (B7) belonged to the family *Clostridiaceae* lineages. In two sequences (B1 and B6) phylogenetic identification failed. The phylum *Bacteroidetes* apparently predominated in the population, indicating that their important role in the co-degradation of cow manure and grass silage within the reactor. The emergence of a *Clostridiaceae* -like organism during increasing the OLR typified by the sequence illustrated in Fig. 5, was noted. In particular, this organism was observed in high abundance at the beginning of increasing the OLR (Fig. 3). In addition, the emergence and even the predominance of two *Bacteroidetes*-like organisms (B2 and 3) were observed after the late operational period at 40% grass in feedstock and the OLR of 2 kg VS m^{-3} day^{-1} (Figs. 3, 5). In parallel, there was apparent reduction in the abundance and even the disappearance of *Bacteroidetes*-like organism B5. Two unclassified bacterial organisms (B1 and B6) more related to the *Actinobacteria* were detected in all the reactor samples throughout the operation (Figs. 3, 5).

Discussion

The 16S rRNA gene-based fingerprinting techniques have been frequently used to monitor the diversity, structure and dynamics of microbial population in environmental samples. T-RFLP profiles (Mladenovska et al. 2006) and DGGE band patterns (Bodelier et al. 2005; Connaughton et al. 2006) were found to reflect the change in the microbial population. In the present study, the microbial community dynamics in a CSTR during anaerobic co-digestion of cow manure and grass silage were investigated by 16S rRNA gene-based T-RFLP and DGGE analyses. All the samples were identically prepared and were analysed simultaneously, thus the potential bias should have affected the results equally. Furthermore, two molecular fingerprinting techniques were employed to investigate the change in the bacterial community structure during reactor operation, which

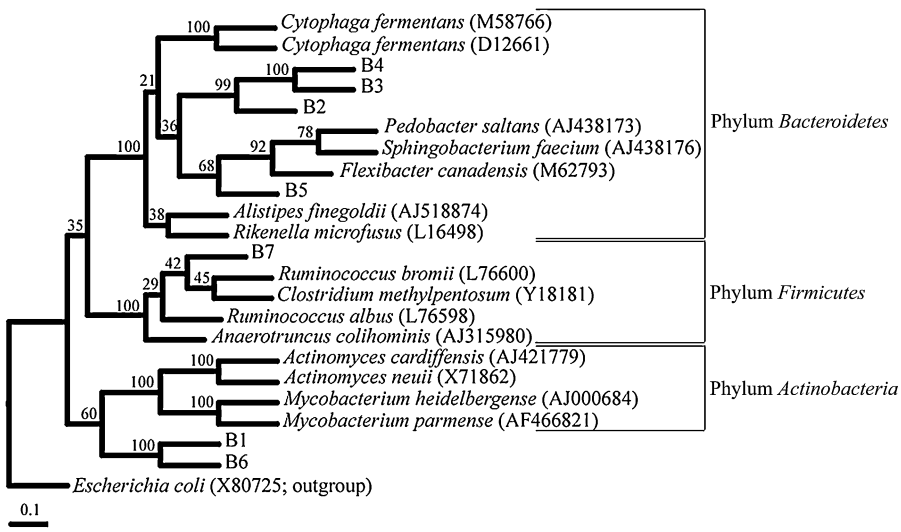

Fig. 5 Phylogram showing the phylogenetic affiliation of the bacterial partial 16S rRNA gene sequence from re-amplified excised DGGE bands. *Bootstrap* values from 100 replicates are shown for each *node*. The *scale bar* represents an estimated 10% difference in nucleotide sequence. The *tree* is rooted using *Escherichia coli* as outgroup

eliminated the pitfall of a single technique and supported the interpretation of results derived from one technique.

The microbial community structures revealed by the T-RFLP profiles were in good agreement with those resolved by DGGE band patterns. However, the number of band detected by the DGGE pattern is relatively low compared to that of T-RFs revealed by the T-RFLP profile. A similar observation was made by Moesender et al. (1999), where the T-RFLP approach was more sensitive than DGGE, as indicated by the higher number of OTU detected by T-RFLP when investigating marine bacterial communities by both methods. Better resolution of complex microbial communities in lake sediment was indicated by T-RFLP than DGGE analysis when the Shannon–Wiener diversity indices obtained by using these two fingerprinting techniques were compared (Koizumi et al. 2003; Schwarz et al. 2007).

The present 16S rRNA gene-based microbial community study revealed that both the bacterial and the archaeal community structure remained stable during operational phases P1 to P3M when the reactor was fed with cow manure alone and with up to 20% grass silage in feedstock. A bacterial phylotype (B5) within the phylum *Bacteriodetes* was clearly dominant, and mostly reflected community composition in the inoculum and cow manure. The phylotype, B5, had 94% sequence identity with an uncultured clone, AHU24, which was obtained from a CSTR fed with a synthetic wastewater containing acetate as the sole carbon source (Shigematsu et al. 2003). The *Bacteroidetes* form a phylogenetically highly diverse group and are known as hydrolytic fermentative degraders of polymers in mainly anaerobic habitats. Members of the class *Bacteroidetes* are also abundant in the intestinal tracts and feces of warm-blooded animals, and they are facultative anaerobes. A recent study by Hernon et al. (2006) showed that the most dominant fermentative bacteria belonged to the *Bacteroidetes* phylum, representing 31% of the cloned library, in a mesophilic anaerobic reactor degrading carbohydrate-rich waste. The members of the phylum *Bacteroidetes* capable of fermenting a range of carbohydrates were further specified by the authors. Bacteria belonging to the phylum *Bacteroidetes* were also found to be predominant in the bacterial community that was able to degrade long-chain fatty acids in a CSTR, suggesting that they play important roles in long-chain fatty acid degradation (Shigematsu et al. 2006). The inoculum in the present study was collected from a mesophilic farm digester treating dairy manure and industrial confectionery (sweets and chocolates) by-products. A microbial community capable of efficient anaerobic digestion of carbohydrate-rich or fat-rich substrates was therefore present in the inoculum. Feeding with cow manure alone and with a low ratio of grass (10–20%) in the feedstock might have stimulated the growth of microbes able to convert the easily degradable substrates to methane, mainly the VFA present in the manure. The bacteria responsible for cellulose hydrolysis might not have been abundant.

The bacterial population clearly changed when the ratio of grass in the feedstock was increased to 40%. Contrary to the bacterial community structure, there was little change in the archaeal population with only a new phylotype appeared at the operational phases P4L, P5E and P5M. The more diverse bacterial population was observed when the reactor was fed with 30% grass in the feedstock (P4E to P5E). The study of the reactor performance by Lehtomäki et al. (2007) showed that the highest specific methane yield was obtained during this operational period. This result suggests that the versatile substrate resource produced oscillations in the structure of the bacterial community and allowed the coexistence of more species (Saikaly and Oerther 2004). However, a large change in bacteria population occurred when the grass ratio was further increased to 40% (P5). Compared to the P4, during P5 the bacterial population became less diverse while some individual bacteria became more abundant. Increasing the proportion of grass further to 40% decreased the specific methane yield by 6% as shown by Lehtomäki et al. (2007). Sequence analyses of excised DGGE bands indicated the emergence of two new phylotypes (B2 and B3), which belonged to the phylum *Bacteroidetes*, whereas the dominant phylotype (B5), affiliated also to *Bacteroidetes*, during P1 to P3 declined. The phylotype (B3) predominant in the bacterial community after P5L showed 95% sequence identity with the uncultured bacterium clone p-2534-18B5 found in the intestinal tracts of Danish pigs (Leser et al. 2002). The clone was affiliated with the *Rikenella microfusus* subgroup (RDP req. no. 2.15.1.2.1). Another abundant phylotype, B2, had 94% sequence identity with the uncultured clone

HsW01-040 from gut wall of termites (Nakajima et al. 2006). Termites harbor a dense microbial community in their guts, and gut microbes are responsible for the efficient decomposition of plant litter. The closest relatives of these phylotypes to the uncultured clones from plant digestive gut microbial communities suggest that these bacteria might play an important role in grass degradation.

A further change in the bacterial community structure was recorded when a number of loading rate increases were applied to the reactor during the final two operational phases P6 and P7, although the differences were not as significant as those observed when the grass ratio in the feedstock was increased. The archaeal population could not be detected during the operational phases P5L, P6 and P7, which indicated low abundance of the archaeal population in the microbial community. Increasing the loading rate from 2 to 4 kg VS m^{-3} day^{-1} caused a reduction in HRT from 20 to 16 days. Reduction in HRT may have resulted in enrichment of the microbial species outcompeting for the essential resources in the reactor, whereas species with less competitive advantage are removed from the bioreactor because of washout. The relatively low bacteria diversity and high abundance of individual bacteria were observed, with only seven T-RFs being detected at the conclusion of P7L. This has also been observed by Dearman et al. (2006), who showed that bacterial diversity during the treatment of organic wastes for hydrogen production increased as HRT was extended. The analyses of the performance of the reactor indicated that the decrease in HRT led to the inefficient hydrolysis of solid material, mainly cellulose, the decrease of the specific methane yield by 26%, and the increase of ammonia concentration in the digestates (Lehtomäki et al. 2007). Ammonia was considered inhibitory for aceticlastic methanogens and hydrogenotrophic methanogens under mesophilic conditions (Angelidaki and Ahring 1993). A phylotype, B7, which fell into the family *Clostridiaceae* was detectable during increasing the OLR (P6 and P7). Members of the *Clostridiaceae* found in a methanogenic landfill leachate reactor (Burrell et al. 2004; O'Sullivan et al. 2005) and in a thermophilic methanogenic bioreactor digesting wastepaper (Shiratori et al. 2006) are capable of anaerobic cellulose hydrolysis. The *Bacteroidetes*-like bacteria B3 was prevalent in the reactor with the exceptions of P6E, where a *Clostridiaceae*-like bacterium B7 was predominant, and of P7L, where a *Bacteroidetes*-like bacterium B2 was also abundant in the reactor. The evidence indicated that the essential resource and environmental conditions in reactor during these operational phases favoured the proliferation of these bacteria. We assume that the phylotype B2, B3 and B7 might be important for the degradation of grass silage in the reactor; hence increasing the grass proportion in the feedstock may lead to the enrichment of these bacteria.

Significant amounts of the phylogenetic group B1 and B6 survived throughout the whole operation, and thus were adaptable to a wide range of conditions. This suggests that the natural population found in the inoculum is at least adequate for the degradation of some components in the feedstock. Unfortunately, conclusions as to the possible functions of these two phylotypes could not be drawn since they failed to fall into any of the known phylogenetic groups, although they were closely related to the phylum *Actinobacteria*. The *Actinobacteria* are a group of Gram-positive bacteria, which play an important role in the decomposition of organic materials, such as cellulose and chitin. A few members, such as *Actinomyces israelii*, can grow under anaerobic conditions (Stackebrandt et al. 1997).

Conclusions

The microbial community structure remained stable when the reactor was fed with cow manure alone and with up to 20% of grass silage in feedstock at an OLR of 2 kg VS m^{-3} day^{-1}, although the number of the archaeal phylotypes was much smaller than that of the bacterial phylotypes in the reactor. Changes in the bacterial community towards a more diverse bacterial population were observed when the loading ratio of grass was increased to 30%. Large changes in bacterial community structure with less observed phylotypes occurred when the grass ratio was further increased to 40%. Contrary to the bacterial population, there was little change in the archaeal population. The structure of the bacterial community showed fewer differences during the increase in OLR from 2 to 4 kg VS m^{-3} day^{-1} than during the changes in the feed ratio. Archaea was undetectable during the increase in OLR, which indicated low

abundance of Archaea in the microbial community. The present community-based studies together with the previous analyses of reactors performance suggested a microbial community with most diverse in bacterial population to be related to the highest specific methane. Phylotypes which belonged to the phylum *Bacteroidetes* predominated in the bacterial community. A *Clostridiaceae*-like bacterium appeared after the OLR was increased. Two unclassified bacteria with high abundance survived throughout the operation and thus showed adaptability to a wide range of substrate conditions.

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