Development of microbial populations in the anaerobic hydrolysis of grass silage for methane production

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Abstract

Six batch leach bed (LB) reactors, installed in parallel and connected to a common upflow anaerobic sludge blanket reactor, were fed with grass silage and operated at 35 (±1) °C. The development and distribution of microorganisms, which firmly and loosely attached to solid materials, and presented in the leachate in the LB reactors, were investigated by 16S rRNA gene-based terminal restriction fragment length polymorphism and clone library analyses. The phylotypes and their relative abundance changed in the respective bacterial community throughout the 49-day run and showed differences between the communities. Large numbers of phylotypes were detected from day 10 onwards. On day 17, the majority of phylotypes in the bacterial community firmly attached to solid residues affiliated to the classes Clostridia and Bacteroidetes. There were high numbers of the phylotypes in the leachate bacterial community. They were closely related to members of classes Clostridium, Bacteroidetes, Betaproteobacteria, Alphaproteobacteria, Gammaproteobacteria, and OP10. The Clostridium-like species clearly dominated the bacterial community. Archaea were only found in the solid residues on day 17 and in the leachate on days 10 and 17. The majority of the Archaea fell within the hydrogenotrophic genus Methanobacterium. The organism assigned to the acetoclastic genus Methanosarcina was only present in the solid residues.

Introduction

Anaerobic digestion of biomass as a technology for the production of methane-rich biogas has received considerable attention. Methane-rich biogas can be converted into heat and electricity or used as transportation fuel, and is thus economically viable. Cellulosic materials are the most abundant, economic, and sustainable renewable natural resource produced from photosynthesis (Lynd et al., 2005).

The anaerobic digestion of cellulosic material to methane is a multistep process consisting of hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which are mediated by hydrolytic, fermentative, and acetogenic bacteria as well as methanogenic Archaea (Boone et al., 1993; Chynoweth & Pullammanappalli, 1996). Hydrolysis is generally considered to be a rate-limiting step (Noike et al., 1985; Veeken & Hamelers, 1999; Mata-alvarez et al., 2000). Cellulose hydrolysis in bioreactors has been shown to be facilitated by surface-attached bacteria (O’Sullivan et al., 2005; Song et al., 2005). Therefore, knowledge of the microbial ecology during anaerobic hydrolysis is of importance for increasing the rate of hydrolysis and the overall efficiency of the anaerobic digestion process.

Anaerobic digestion of cellulosic materials to methane has been demonstrated as one-stage batch leach bed (LB) processes (Lehtomäki et al., 2008). In these systems, digesters are filled with fresh substrate and the leachate circulated over a bed of organic matter, in which it is then allowed to follow the various degradation steps. Overall degradation is stimulated by leachate circulation, as this induces more efficient dispersion of nutrients and degradation products (Lissens et al., 2001). In one-stage processes, the operational conditions are designed to favour methanogenic populations (Cirne et al., 2007). Two-stage processes are an alternative to one-stage processes. In a two-stage reactor, the biomass is located in the first stage, where hydrolysis–acidification occurs. The generated leachate is circulated through a second-stage methanogenic reactor and then...
reintroduced over the top of the biomass in the first stage. The operational conditions for both stages can be independently optimized and thus the conditions for hydrolytic and methanogenic microorganisms are well suited (Cirne et al., 2007). Although two-stage processes have been used in both laboratory (Cirne et al., 2007; Lehtomäki et al., 2007) and pilot trials (Svensson et al., 2005), the role of the microbial community in the rate-limiting hydrolytic stage of anaerobic digestion of energy crops to methane has only recently been assessed (Cirne et al., 2007). Nevertheless, the links between the microbial community and the operating conditions and digestion performance of the hydrolytic stage remain incomplete.

The aim of the present study was to investigate the development of the native microbial community in the hydrolytic stage of two-stage anaerobic digestion of grass silage, and to assess the distribution of microorganisms in hydrolytic reactors.

Materials and methods

Source of biomass

Grass silage was obtained from central Finland (Lehtomäki et al., 2008). It was prepared from grass (75% Timothy Phleum pretense, 25% meadow fescue Festuca pratensis) harvested at the early flowering stage, chopped after 24 h of prewilling, and ensiled in a bunker silo with the addition of lactic acid bacteria inoculant (AIV Bioprofit, Kemira Growhow Ltd). The composition of grass was analysed in accordance with the standard methods described previously (Lehtomäki et al., 2008). The characteristics of the grass silage are presented in Table 1.

The reactor setup has been described in detail by Lehtomäki et al. (2007). In brief, a two-stage process, consisting of six parallel 1000-mL hydrolytic LB reactors (LB1–6) and a methanogenic upflow anaerobic sludge blanket reactor (UASB), was operated at 35 °C.

Table 1. LB reactor performance data (Lehtomäki et al., 2007)*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Operational day</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>pH</td>
<td>4.1</td>
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<tr>
<td>Klasson lignin (% TS)</td>
<td>13.0</td>
</tr>
<tr>
<td>Carbohydrates (% TS)</td>
<td>45.0</td>
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<tr>
<td>Extractives (% TS)</td>
<td>8.4</td>
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<tr>
<td>Protein (% TS)</td>
<td>10.4</td>
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<tr>
<td>VS removal (%)</td>
<td>–</td>
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<tr>
<td>SCOD (g L⁻¹)</td>
<td>–</td>
</tr>
<tr>
<td>VFA (g L⁻¹)</td>
<td>–</td>
</tr>
<tr>
<td>CH₄ concentration (%)</td>
<td>–</td>
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</table>

*pH, SCOD, and VFA are for leachate, CH₄ content for produced biogas, and the rest are for the material in the LB reactors.
the manufacturer’s instructions. Genomic DNA from the solid residue was isolated using a DNeasy® Plant Mini kit (Qiagen Inc.). The extracted genomic DNA was purified with a Wizard® DNA Clean-Up System (Promega Inc.). The DNA extraction was performed in duplicate for each sample. The same terminal restriction fragment length polymorphism (T-RFLP) profile was obtained from the replicate DNA extractions of each sample.

**T-RFLP analysis**

The 16S rRNA genes for the T-RFLP analyses were amplified using the PCR primer sets 27f-6-carboxyfluorescein (FAM)/1492r (Sait et al., 2003) and Ar109f/Ar912r-FAM (Lueders & Friedrich, 2003) specific for the bacterial and archaeal 16S rRNA genes, respectively. PCR was performed in a 100-μL reaction mixture containing approximately 100 ng of DNA template, 1 × PCR buffer, 20 μmol of each deoxynucleoside triphosphate, 2 U of DyNaZyme™ II DNA polymerase (Finnzymes), and 0.05 μmol 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. Approximately 100 ng of bacterial PCR products were digested in a 10-μL reaction volume with 10 U of MspI for 3 h at 37 °C. The same amount of archaeal PCR products was digested with 10 U of TaqI for 3 h at 65 °C. Fluorescently labelled T-RFs were separated on an ABI Prism® 3100 automated sequencer (Applied Biosystems) using an internal size standard (GeneScan™ 500 LIZ®; Applied Biosystems). T-RFLP electropherograms were analysed with GENEMAPPER® software version 3.1 (Applied Biosystems). T-RF sizes between 50 and 500 bp with a peak area higher than 50 fluorescence units were used in the analysis. The analysis was performed twice for each DNA extraction. The replicate profile, with a total fluorescent peak area close to the average total fluorescent peak area of the T-RFLP profiles in the comparison dataset, was selected for the analysis. Profiles from different samples were standardized to the profile with the smallest total fluorescent peak area intensity. A 2% threshold was subsequently applied. The relative abundance of T-RF within a profile was calculated on the basis of the standardized peak area. It should be mentioned that distinct sequences that share a terminal restriction site, as well as the possible appearance of pseudo T-RFs, introduce biases and oversimplify the real diversity and abundance of phylotypes. To minimize this problem, clone libraries were constructed and analysed in parallel with the T-RFLP analysis.

The standardized relative area of each peak was subjected to principal component analyses (PCA) using R statistics software v2.7 (R Development Core Team, 2008).

**Cloning, sequencing, and phylogenetic analysis**

The highest number of T-RFs was detected in the samples taken on day 17. Moreover, there were significant differences in the bacterial T-RFs in the leachate fraction and the solid residues. Unique archaeal T-RF was found in the profiles from the firmly and loosely attached biomass. Therefore, the bacterial 16S rRNA gene clone libraries were generated with DNA extractions from the solid and leachate fractions. Archaeal clone libraries were generated from the firmly and loosely attached biomass on day 17. The PCR amplification was performed using primer sets 27f-1492r and Ar109f-Ar912r for the bacterial and archaeal 16S rRNA genes, respectively, as described above; the only exception was the final extension, carried out at 72 °C for 20 min. Amplified 16S rRNA gene fragments were cloned using the pGEM-T Easy vector system (Promega Inc.) and sequenced from both directions on an ABI Prism® 3100 sequencer as described previously (Wang et al., 2009). The obtained sequences with a size of 1381 and 794 bases for bacterial and archaeal 16S rRNA genes, respectively, were checked for chimeric artefacts as described in an early study (Wang et al., 2009). A homology search of the bacterial 16S rRNA gene sequences was performed using the PHLECT program in RDP II (Wang et al., 2009). A homology search of the archaeal sequences against the database (GenBank) was performed with BLASTN. The sequences were aligned with the CLUSTALW package. Finally, phylogenetic trees were constructed using the neighbour-joining method with a PHYLIP 3.65 software package, as described previously (Wang et al., 2010). Operational taxonomic units (OTUs) were defined for the completely similar sequences (100%).

**Nucleotide sequence accession numbers**

The nucleotide sequences reported in this paper were deposited in the NCBI nucleotide sequence databases under accession numbers EU887962–EU888017.

**Results**

The microbial community in the hydrolytic reactors was investigated on each termination day. Analyses of the microbial community firmly and loosely attached to solid residues on day 49 were hindered by the lack of solid residues. In addition, the leachate microbial communities on days 1 and 3 were not investigated due to the small amount of available materials.

**Bacterial community dynamics revealed by T-RFLP fingerprint**

The T-RFLP fingerprints of the bacterial 16S rRNA gene revealed a total of 40 T-RFs during the run (Fig. 1). The number of T-RFs detected in the solid fractions was rather
low on days 1, 3, and 6. From day 10, the number of T-RFs increased considerably. As shown in Fig. 1, a T-RF of 437 bp clearly dominated the profile on days 1 and 3, whereas a T-RF of 179 bp was the dominant component on day 6 (Fig. 1). However, the T-RFs of 437 and 179 bp were only identified in small proportions in the later profiles. Instead, a T-RF of 279 bp dominated the profile on day 10, and a T-RF of 90 bp on day 17. Altogether, more T-RFs appeared as the run proceeded, and the dominant T-RF in the respective profile also shifted.

Shifts also occurred in the T-RFs and their relative abundance in the leachate fractions during the run. The T-RF of 437 bp was also the major component (81.2%) in the leachate profile on day 6, but it was absent in the later profiles (Fig. 1). Another T-RF of 409 bp appeared to be dominant on day 10, but it was not found in either the later leachate profiles (days 17 and 49) or the profiles derived from the solid residues (Fig. 1). On day 17, T-RFs of sizes 394 and 146 bp appeared, respectively accounting for 25.5% and 23.9% of the total profile, respectively. However, on day 49, the T-RFs were evenly distributed in the profile and no individual T-RF could be singled out. The T-RFLP profiles also revealed that a number of T-RFs were only present in the profiles derived from the leachate fractions. The phylogenotypes and their relative abundance in the two fractions were different.

The T-RFs in the biomass loosely attached to solid residues mostly reflected those in the solid residues and the leachate fractions, with the exception that a T-RF of 403 bp, with a relative abundance of 32.4%, was only found in the loosely attached biomass on day 1 (Fig. 1). The T-RF of 437 bp was dominant in the profiles until day 17, whereas the T-RF of 90 bp was abundant on day 49. Notably, the T-RF of 179 bp prevalent in the solid residues was only present in a couple of loosely attached biomass samples as a minor component of the profiles. In contrast, some T-RFs, such as T-RFs of 285 and 472 bp, were found in greater abundance in the loosely attached biomass than in the solid residues.

The covariation of the bacterial T-RFLP profiles over time and between the leachate, loosely attached, and firmly attached samples was evaluated by PCA. The plotted data clearly show a distinct grouping between the samples before day 10 and those thereafter (Fig. 2a). Another important finding in the plotted data was the observation of a great difference in T-RFLP profiles before day 10. In addition, there is a clearly visible differentiation between the profiles derived from the leachate, loosely attached, and firmly attached samples. A significant difference between the

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**Fig. 1.** Relative abundance of the bacterial 16S rRNA gene fragments retrieved from the biomass attached and loosely attached to solid residues, and in the leachate throughout the run based on T-RFLP analysis. The length of T-RFs in base pairs (bp) is indicated.
profiles from the three fractions on day 6 was observed. This suggests that the bacterial community structure was dynamic and in a state of constant change. The T-RFLP profiles after day 10 were grouped together by the first two principal components (PC1 and PC2). However, the T-RFLP profiles of the firmly attached samples can be distinguished from those of the leachate samples according to the third principal component (PC3). This indicates that the bacterial community tended to be stable during these operational days, whereas the bacterial community in the leachate was distinct from that attached to the solid biomass.

Archaeal community dynamics revealed by T-RFLP fingerprint

Amplification of the archaeal 16S rRNA genes from the loosely attached biomass and leachate fractions was possible on days 10 and 17, and from the solid residues on day 17. The archaeal T-RFLP fingerprints revealed a total of seven T-RFs (Fig. 3).

A T-RF of 89 bp clearly dominated all the profiles, with the highest abundance of 94.4% in the leachate fraction on day 10 (Fig. 3). In addition, a T-RF of 184 bp appeared more abundant (32% of the profile) in the leachate profile on day 17 than on day 10. Moreover, the relatively abundant T-RF of 281 bp, accounting for 22.5% of the profile, was also observed on day 17. More T-RFs were observed in the loosely attached biomass than in the leachate fractions. The T-RFLP profiles from the loosely attached biomass were indistinguishable on days 10 and 17 (Fig. 3). The profiles derived from the loosely attached biomass mostly included the T-RFs found in the leachate fractions, with the exception of T-RF of 376 bp, which was found to be unique to this fraction. A T-RF of 84 bp, with a relative abundance of 12.5%, was only detected in the solid residues on day 17, whereas the T-RFs of 184, 376, and 389 bp, which were present in the other fractions, were not detected in this profile (Fig. 3). The fact that a unique T-RF was found in the solid residues profiles and the loosely attached biomass profiles indicated the differential distribution of methanogenic Archaea in the reactor.

The PCA of the archaeal T-RFLP profiles shows a clear difference among the profiles derived from the leachate,
loosely attached, and firmly attached samples on day 17 (Fig. 2b). The profiles from the leachate and loosely attached biomass on day 10 were grouped together with that from the loosely attached sample on day 17 by the first two principal components (PC1 and PC2). However, the T-RFLP profile from the loosely attached sample on day 10 can be distinguished from that from day 17 by the third principal component (PC3).

Bacterial clone libraries and phylogenetic analysis

Two clone libraries were constructed from the solid residues and the leachate fraction on day 17; these were referred to as the SB library (35 clones) and LB library (39 clones), respectively. In total, 20 OTUs were identified for the SB library and 29 for the LB library. The distribution of the OTUs within each clone library showed differences even at the class level. In the SB library, most OTUs were affiliated to the classes Clostridia and Bacteroidetes, whereas a single clone was found to be closely related to species within each of the phylum Proteobacteria, Actinobacteria, or the recently proposed candidate division OP10. In the LB library, the OTUs were affiliated to the classes Clostridia, Bacteroidetes, Betaproteobacteria, Alphaproteobacteria, Gammaproteobacteria, and OP10. In addition, one OTU was not affiliated to a specific class, but fell within the phylum Firmicutes. The LB library exhibited the greatest phylogenetic variety at the class level, which is consistent with the results from the T-RFLP profiles. The OTUs affiliated with the class Clostridia dominated in both the SB and LB libraries, accounting for 60.0% and 35.9% of the total clones, respectively. The OTUs closely related to the class Bacteroidetes were the next most prevalent in the SB library, representing 31.4% of the clones. However, the OTUs closely related to the phylum Proteobacteria were the next most prevalent in the LB (30.8% of the clones) and much more abundant than in the SB (2.9% of the clones) library.

The phylogenetic tree showed fewer similar and a large number of distinct phylotypes in the two bacterial communities (Fig. 4).

Class Clostridia

Within the class Clostridia, the phylotypes displayed more diversity in the LB than SB library. The majority of OTUs (92%) in the SB library clustered together and fell within the family Clostridiaceae, whereas the OTUs in the LB library were related to a number of families, such as the Clostridiaceae, Peptostreptococcaceae, Eubacteriaceae, Peptococcaceae, Acidaminococcaceae, and Syntrophomonadaceae. Only two OTUs, S14 (L30) and S26 (L39), were present in both libraries. S14 (L30) was closely related to Acetanaerobacterium elongatum (AY487928), which ferments several kinds of mono-, di-, and oligosaccharides and was isolated from the waste water sludge of a paper mill (Chen & Dong, 2004). The expected T-RF size of S14 (L30) was 279 bp, which matched the T-RF of 279 bp recognized in the T-RFLP profiles derived from the solid residue on days 10 and 17, and from the leachate on days 10 and 49 (Fig. 1). S26 (L39) was found to be close to Clostridium cellulosi- parum (X71856), a cellulolytic strain (Rainey & Stackebrandt, 1993). The supposed T-RF with size of 493 bp was very close to the T-RF of 491 bp resolved by the T-RFLP fingerprint; however, the T-RF of 491 bp was only found in the leachate profile on day 17 (Fig. 1).

The most abundant sequence in the SB library was S44 (Fig. 4), which accounted for 17.1% of the total clones. The supposed T-RF size of S44 was 179 bp, which corresponded to the prevalent T-RF of 179 bp identified in the solid residue T-RFLP profiles throughout the reactor operation but not in the leachate profiles. Along with the other seven OTUs, this OTU was closely related to Clostridium leptum (M59095).

In the LB library, the abundant sequence was L9, with the expected T-RF size of 158 bp (Fig. 4), which was closely related to Anaerovorax odorimutans (AJ251215), an obligately anaerobic bacteria producing butyrate and acetate (Matthies et al., 2000).

Class Bacteroidetes

Within the class Bacteroidetes, one OTU, S40 (L3), was commonly present in both clone libraries (Fig. 4). This was the dominant clone in the SB library, representing 22.9% of the clones. The expected T-RF size was 90 bp, which corresponded to the dominant T-RF of the solid residue profile identified by the T-RFLP fingerprint (Fig. 1). The T-RF was also detected in the leachate profiles on days 10 and 49 (Fig. 1). OTUs derived from the SB library formed separate clusters within the class Bacteroidetes. The OTUs retrieved from the LB library clustered on a branch of the phylogenetic tree. Among the OTUs within the class Bacteroidetes, L38 was the dominant sequence in the LB library, accounting for 10.2% of total clones. The expected T-RF size of L38 did not match any of the T-RF sizes detected from the T-RFLP fingerprint.

Family Proteobacteria

There were considerable differences within the family Proteobacteria between the two clone libraries (Fig. 4). In the LB library, three OTUs fell within the class Alphaproteobacteria, five OTUs were affiliated to the class Betaproteobacteria, and one was closely related to the class Gammaproteobacteria. In the SB library, only one OTU showed affinity to the class Gammaproteobacteria. The OTU L15, which was affiliated to the class Alphaproteobacteria, was the most abundant clone.
in the Proteobacteria cluster in the LB library, and represented 10.2% of all the clones. The supposed T-RF size of L15 was 168 bp, which was nearest to the T-RF of 164 bp detected only in the leachate fraction on day 17 by T-RFLP fingerprint (Fig. 1). OTUs within the Betaproteobacteria cluster were affiliated to hydrogen-oxidizing bacteria such as Alcaligenes and Hydrogenophaga, or the nitrate-reducing bacteria Achromobacter insolitus. Two OTUs, L4 and S29, were found to fall within the class Gammaproteobacteria, although they were at a considerable distance from each other on the phylogenetic tree. The closest cultured relative to OTU L4 was Stenotrophomonas acidaminiphila (AF273080), isolated from a lab-scale UASB reactor treating petrochemical wastewater (Assih et al., 2002). In the SB library, only one OTU, S29, was affiliated with the family Proteobacteria, and it displayed 99% sequence identity to Shigella flexneri (X96963).

**Archaeal clone libraries and phylogenetic analysis**

Two archaeal clone libraries referred to as the SA library (23 clones) and WA library (34 clones), respectively, were also constructed from the solid residues and the loosely attached biomass on day 17. A total of six OTUs were retrieved for the SA clone library and five for the WA library. Among the retrieved OTUs, four (SA1 through SA4) were affiliated with the class Thaumarchaeota, while one (WA1) was affiliated with the class Euryarchaeota. A phylogenetic tree was generated on Fig. 4, which shows the phylogenetic affiliation of the bacterial 16S rRNA gene fragment sequences from two clone libraries. Construction of the libraries from the solid residues and the leachate fraction referred to as S and L, respectively, was carried out on day 17. Bootstrap values from 100 replicates are shown for each node. The scale bar represents an estimated 10% difference in nucleotide sequence.
the basis of the clone sequences and their highly similar sequences in the database (Fig. 5). The most abundant clones (85.7%) were phylogenetically related to the genus *Methanobacterium*, whereas one OTU derived from the SA library was affiliated to the genus *Methanosarcina*.

**Methanobacterium**

Within the *Methanobacterium* cluster, SWA1 clearly dominated in both libraries, representing 47.8% of the clones in the SA library and 64.7% in the WA library. As shown in Fig. 5, the OTU was closely related to *Methanobacterium conglobense* (AF233686), a nonmotile, mesophilic, hydrogenotrophic, methanogenic archaeon isolated from an anaerobic digester used in the treatment of raw cassava-peel waste in the Democratic Republic of the Congo (Cuzin et al., 2001).

The other three OTUs, SWA2, SWA3, and SWA4, together accounted for 43.4% and 32.3% of the total clones in the SA and WA libraries, respectively. The remaining OTU, SA1, in the SA library was nearest to WA1, retrieved from the WA library in the phylogenetic tree (Fig. 5). The closest cultured relative of these two OTUs was *Methanobacterium beijingerense* (AY350742), isolated from the granular sludge of a mesophilic UASB reactor treating brewery wastewater in China (Ma et al., 2005).

**Methanosarcina**

Within the genus *Methanosarcina*, clone SA2, from the SA library, showed 98% sequence similarity to the archaeon *Methanosarcina siciliae* isolated from marine canyon sediments (Fig. 5), which is able to use acetate for growth and methanogenesis (Elberson & Sowers, 1997).

**Discussion**

Anaerobic cellulolytic capability is mainly distributed among bacteria of the genus *Clostridium*; however, only a few species within this genus are generally able to degrade cellulose (Lynd et al., 2002). This could explain the relatively low number of the bacterial phylotypes in the early operational stages of the present study. Analyses of the reactors’ performance indicated that 16% of the VS removal (Table 1) had been approached after the first day of operation. In addition, the VFA concentrations peaked on day 3 and decreased steadily thereafter (Lehtomäki et al., 2008). This result, together with those of the community-based studies, suggested that rapid growth of the native cellulolytic bacteria and a fast rate of hydrolysis occurred during the first 3 days in the reactors. A similar observation was made in a study of cellulose-hydrolysing populations during batch anaerobic digestion of crystalline cellulose (Song et al., 2005) and grass silage (Cirne et al., 2007), where a peak hydrolysis rate on a volumetric basis was observed on day 3 for crystalline cellulose, and high SCOD value and minimum pH were observed on day 2 for grass silage.

The number of bacterial phylotypes in the reactor was considerably higher during operational days 10, 17, and 49 than on days 1, 3, and 6. The changes in the bacterial community were related to substrate availability and to the prevailing environmental conditions. After 10 days of digestion, extractives were the most rapidly removed component; reaching 80% of total removal (Table 1) (Lehtomäki et al., 2008). The conversion of cellulosic materials into VFA, by the hydrolytic and fermentative group of microorganisms in turn stimulated the growth of microorganisms capable of utilizing these substrates in the reactors. Furthermore, the dominant component of the T-RFLP profiles on operational days 10, 17, and 49 was also different from that on days 1, 3, and 6. Hence, the bacterial population clearly changed as the structure of the food-chain in the reactors altered. A study of the microbial community in the hydrolytic stage of two-stage anaerobic digestion of energy crops (grass and clover) indicated that although the genus *Clostridium* was present in the early stage of operation, the population decreased when the rate of hydrolysis became a limiting factor in the overall degradation (Cirne et al., 2007).
The clone library analyses indicated different bacterial distribution in the reactor. The majority of the bacteria firmly attached to solid residues fell into class Clostridia and Bacteroidetes. Most phylotypes within the Clostridia were closely related to the fibrolytic, butyrate-producing anaerobic bacterium C. leptum (Duncan et al., 2002). The most abundant phylotype corresponded to the dominant T-RF of 179 bp, identified in the solid residue T-RFLP profiles throughout the reactor operation. Phylotypes within the class Bacteroidetes were related to Bacteroides nordii or Prevotella denticola, which are known for their anaerobic degradation of cellobiose, glucose, and mannose to acetate or succinate (Willems & Collins, 1995; Song et al., 2004). A recent study by Hernon et al. (2006) showed that the dominant fermentative bacteria belonged to the Bacteroidetes phylum and represented 31% of the clone library derived from a mesophilic anaerobic reactor degrading carbohydrate-rich waste. Bacteria belonging to the phylum Bacteroidetes were also found to be predominant in a bacterial community that was able to degrade long-chain fatty acids in a continuously stirred tank reactor, suggesting that they play important roles in long-chain fatty acid degradation (Shigematsu et al., 2006) as well as fermentation of complex carbohydrates (Hernon et al., 2006).

The phylotypes in the leachate fraction displayed more diversity than those firmly attached to the solid residues. The phylotypes affiliated with Clostridia or Bacteroidetes were also prevalent in the leachate fractions, but were closely related to members capable of using a broad range of substrates for acetogenesis. Furthermore, the phylotypes assigned to the Proteobacteria family were mainly enriched in the leachate and closely related to the aerobic members that were isolated from anaerobic environments, such as Shinella granulii (AY995149) from a UASB reactor (An et al., 2006) and Aquamicrobium defluvii (Y15403) from activated sewage sludge (Bambauer et al., 1998). The T-RFLP profile for the leachate fractions also demonstrated the highest numbers of unique T-RFs. A previous study (Cirne et al., 2007) also indicated that members of Alphaproteobacteria,Betaproteobacteria, and Gammaproteobacteria were commonly present in the leachate fraction, but not in other fractions.

No archaeal population was found until day 6. This is consistent with the methane concentration in the gas of the LB reactors, which remained below 1% until day 10 (Lehtomäki et al., 2008). No cellulolytic members of the domain Archaea have yet been identified (Lynd et al., 2002). Notably, archaeal species emerged from day 10 onwards, a finding in accordance with the performance of the reactors (Lehtomäki et al., 2008). The methane concentration in the gas from the LB reactors started to increase slowly, reaching 14% on day 41. The results obtained from the analyses of both the microbial community and the performance of the reactors suggest that methanogenesis occurred when appropriate substrates for the methanogenic Archaea, produced by other microorganisms, were present in the hydrolytic reactors in which hydrolysis and acidogenesis of cellulosic materials was expected to occur. This result is consistent with that of a previous study (Cirne et al., 2007), which indicated the appearance of Archaea in the hydrolytic stage around day 10. However, no archaeal population was detected on day 49 in the present study, indicating either low abundance or absence of Archaea in the microbial community.

Archaea were also detected in the biomass attached to the solid residues, in contrast to previous reports based on FISH (Cirne et al., 2007). Members of the Methanosarcinaceae were found to dominate in manure digesters containing high levels of ammonia and VFA (Karakashev et al., 2005). The complex structure of cellulosic materials might restrain the movement of acetate, VFA, H₂, and CO₂ produced by the hydrolytic and the fermentative bacteria adherence to solid particles in the soluble phase. The remaining intermediates might stimulate the growth of methanogens capable of converting these substrates to methane, including the hydrogenotrophic Methanobacterium and acetoclastic Methanosaarcina. A previous study has shown that cellulose hydrolysing and methanogenic populations exist wholly in cellulose-associated biofilms during batch anaerobic digestion of crystalline cellulose (Song et al., 2005). The authors further found that the methanogens were predominantly present in the ball-shaped colonies within the biofilm. A study conducted elsewhere found that the methanogenic activity in mature digesters was entirely associated with the solids rather than the supernatant (Nopharatana et al., 1998).

All of the Archaea in the leachate fractions fell within the hydrogenotrophic genus of Methanobacterium. They were closely related to organisms that can utilize hydrogen, carbon dioxide, and formate in the forming of methane, but not acetate. The reactor performance study indicated that the production of methane correlated with the production of CO₂ but not with the concentration of acetate in the UASB reactor (Lehtomäki et al., 2008). The leachate was circulated from the LB reactors to UASB until day 17; therefore, the microorganisms in the leachate in the LB reactors might also reflect those in the UASB reactor. The phylotypes closely related to members of Proteobacteria capable of utilizing acetate and reducing nitrate were mainly enriched in the leachate. It is likely that they might compete better for the same electron donors, acetate, of acetoclastic Archaea, inhibiting the growth of acetoclastic Archaea.

The 16S rRNA gene-based fingerprinting techniques have been used frequently to monitor the diversity, structure, and dynamics of microbial communities in environmental samples. T-RFLP profiles (Mladenovska et al., 2006) and a clone library (Klocke et al., 2007; Freeman et al., 2008) have several advantages over other fingerprinting methods.
T-RFLP profiling is highly reproducible and has high sample throughput and resolution, whereas the clone library enables the resolution of a complete target community. However, there are limitations to the molecular methods for understanding metabolic activities within habitats. Using the molecular methods in combination with microbial cultures should provide more consolidated information on community structure–activity relationships.

The present study showed the microbial population dynamics and differential distribution in the hydrolytic stage of two-stage anaerobic digestion of grass silage. The bacterial and archaeal groups involved and their shifts are identified here. The knowledge from this study could provide a theoretical basis for the improvement of rate-limiting hydrolysis and the development of strategies to increase methane production.

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References


Willems A & Collins MD (1995) 16S rRNA gene similarities indicate that *Hallela seregens* (Moore and Moore) and *Mitsuokella dentalis* (Haapsalo et al.) are genealogically highly related and are members of the genus *Prevotella*: emended description of the genus *Prevotella* (Shah and Collins) and description of *Prevotella dentalis* comb. nov. *Int J Syst Bacteriol* 45: 832–836.