Community Structure of Methanogens in the Hydrolytic Reactors of
Two-Stage Anaerobic Biogas Reactor

Jyväskylän yliopisto

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Master's Thesis

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PREFACE

This study was done at the University of Jyväskylä, Department of Biological and Environmental Science. The experimental part was performed in the summer and autumn of 2007. Writing was executed in the spring of 2008.

I would like to express my greatest compliments to PhD Hong Wang for advice and supervision of this study. I would also like to thank Professor Jukka Rintala for giving me the opportunity to work in his research project. I kindly thank everybody in the laboratory for all the help. I warmly thank my girlfriend and daughter who offered me the opportunity to finish this work. Special thanks to my good friend Kari for his accurate revision of the work. Thanks also to Ari for some general comments. Finally, I would like to express my gratitude to Sirkka who offered her help for babysitting.

University of Jyväskylä   **Abstract of Master's Thesis**
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Abstract:

Methane-rich biogas produced by anaerobic degradation of cellulosic materials can be used as renewable source of energy. Anaerobic degradation of cellulosic materials to methane is carried out by a complex microbial consortium including hydrolytic and fermentative bacteria and methanogenic archaea, which metabolize the terminal step, methanogenesis. Hence, knowledge on the archaeal community during methanogenic fermentation is of importance in seeking to increase the efficiency of methanogenesis.

The understanding of the archaeal community structure in methanogenic fermentation is incomplete. For efficient production of biogas in anaerobic biogas reactors, the archaeal community structure and dynamics in the hydrolytic stage of two-stage batch anaerobic digestion of grass silage was studied. The development of archaeal community in solid, loosely attached and leachate fractions were investigated by means of 16S rRNA gene-based terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses throughout the bioreactor operation.

In this study, the amplification of archaeal 16S rRNA gene fragments was successful only from the genomic DNA extractions retrieved from the loosely attached biomass and the leachate samples on operational days 10 and 17, and from the solid sample on day 17. However, no results were obtained on days 1, 3, 6, and 49. The presence of archaeal DNA correlated with methane production of the reactor, indicating that methanogens were enriched in the reactor. The T-RFLP profiles revealed totally 8 different terminal restriction fragments (T-RFs). The T-RF of 89 bp clearly dominated in the archaeal community. T-RFLP profiles of fractions were different indicating a difference in archaeal communities between fractions. Furthermore, 7 clone groups were retrieved from the solid and loosely attached fractions on the operating day 17. Closest cultured relatives of the sequences were *Methanobacterium curvum* (SEQ1), *Methanobacterium subterraneum* (SEQ2), *Methanobacterium formicum* (SEQ3, SEQ4), *Methanobacterium beijingense* (SEQ5, SEQ7) and *Methanosarcina barkeri* (SEQ6). The majority of archaea fell within the genus *Methanobacterium* capable of reducing CO_2 to methane using hydrogen as an electron donor.

This study provides more information on the community structure of methanogenic archaea in the hydrolytic stage of two-stage anaerobic digestion of grass silage.

Keywords: methanogenesis, T-RFLP, archaea, *Methanobacterium*, *Methanosarcina*

Jyväskylän yliopisto   **Pro gradu -tutkielman tiivistelmä**
Matemaattis-luonnontieteellinen tiedekunta

Selluloosapitoisen orgaanisen aineen anaerobisessa hajotuksessa syntyvää metaanirikasta biokaasua voidaan käyttää uusiutuvana energian lähteenä. Monimutkainen mikrobiyhteisö vastaa anaerobisesta hajoamisprosessista. Siihen osallistuu hydrolyyttisiä ja fermentatiivisiä bakteereja sekä metanogeenisiä arkkeja. Koska arkit vastaavat prosessin viimeisimmästä vaiheesta, metanogeneesistä, on anaerobisessa hajotuksessa toimivaa arkkiyhteisöä koskeva tieto tärkeää metaanin tuotannon tehostamiseksi.

Käsitys arkkiyhteisön rakenteesta metanogeenisessä fermentaatioprosessissa on puutteellinen. Tässä tutkimuksessa tarkasteltiin arkkiyhteisön rakennetta ja dynamiikkaa säiliörehua hajottavan kaksivaiheisen anaerobisen suotopetireaktorin hydrolyyttisissä reaktoreissa. Arkkiyhteisöä tutkittiin 16S rRNA geenien terminaalisten restriktiofragmenttien pituuspolymorfian (T-RFLP) sekä kloonikirjaston analyysilla. Analyysit suoritettiin kiinteälle fraktiolle sekä uutos- ja liuosfraktioille, jotka otettiin reaktorin eri operaatiopäivinä.

Arkkien 16S rRNA geenifragmenttien amplifikaatio, näytteistä erotetusta genomisesta DNA:sta, onnistui liuos- ja uuttofraktioista operaatiopäivinä 10 ja 17 sekä kiinteästä fraktiosta päivänä 17. Amplifikaatio ei onnistunut päivinä 1, 3, 6 ja 49. Arkki-DNA:n läsnäolo korreloi reaktorin metaanin tuotannon kanssa, mikä viittasi metanogeenien toimintaan. T-RFLP-profiileista löytyi kahdeksan eri terminaalista restriktiofragmenttia (T-RF). Arkkiyhteisö oli T-RFLP-profiilien mukaan erilainen eri fraktioissa. 89 emäsparin T-RF oli selkeästi runsain kaikissa profiileissa. Lisäksi seitsemän klooniryhmää eristettiin kiinteästä- ja liuosfraktiosta 17. operaatiopäivänä. Sekvenssien lähimmät karakterisoidut sukulaiset olivat *Methanobacterium curvum* (SEQ1), *Methanobacterium subterraneum* (SEQ2), *Methanobacterium formicum* (SEQ3, SEQ4), *Methanobacterium beijingense* (SEQ5, SEQ7) ja *Methanosarcina barkeri* (SEQ6). Suurin osa arkeista kuului *Methanobacterium*-sukuun, jonka jäsenet pystyvät pelkistämään CO_2:a metaaniksi käytämällä vetyä elektronin lähteenä.

Tämä tutkimus tuotti uutta tietoa kaksivaiheisen anaerobisen biokaasureaktorin hydrolyyttisissä reaktoreissa toimivan arkkiyhteisön rakenteesta.

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Abbreviations

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Abbreviations

16S rRNA	Prokaryotic small-subunit ribosomal RNA
18S rRNA	Eukaryotic small-subunit ribosomal RNA
AGE	Agarose Gel Electrophoresis
Ar109F	Forward primer for archaeal 16S rRNA gene
Ar912rt	Feverse primer for archaeal 16S rRNA gene
ARDRA	Amplified rDNA Restriction Analysis
DGGE	Denaturing Gradient Gel Electrophoresis
dNTP	dideoxy Nucleotide Triphosphate
Exo I	Exonuclease I
FAM	Carboxyfluorescein
FISH	Fluorescence *In Situ* Hybridization
FRB	Ferric iron-Reducing Bacteria
IPTG	Isopropyl β-D-1-thiogalactopyranoside
L	Leachate fraction
LB	Leach bed reactor
LB-ampicillin	Luria Broth ampicillin
PCR	Polymerase Chain Reaction
PBS	Phosphate Bufferd Saline
R1	Common reservoir 1 of the reactor
R2	Common reservoir 2 of the reactor
rcf	Relative centrifugal force
RDP	Ribosomal Database Project
rpm	revolutions per minute
rRNA	Ribosomal RNA
S	Solid fraction
SAP	Shrimp Alkaline Phosphatase
SEQ	Sequence
SP6	Reverse primer for amplification of 16S rRNA gene from pGEM-T plasmid
SRB	Sulphate Reducing Bacteria
SSCP	Single Strand Conformation Polymorphism

SSU	Small-Subunit
T7	Forward primer for amplification of 16S rRNA gene from pGEM-T plasmid
T-RFLP	Terminal Restriction Fragment Length Polymorphism
T-RF	Terminal Restriction Fragment
TGGE	Temperature Gradient Gel Electrophoresis
UASB	Upflow Anaerobic Sludge Blanket reactor
W	Loosely attached to solid substrate fraction
WU-BLAST	Washington University Basic Local Alignment Search Tool
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

1. INTRODUCTION

1.1 Methane as bioenergy

The global climate change has created an increasing awareness towards renewable energy sources. Conventional energy sources, mainly fossil fuels, are producing large amounts of CO_2 and therefore development of the renewable energy sources is an interest to the governments and consumers. In March 2007, European Council set targets for the European Union's climate and energy policy; a reduction of at least 20% in greenhouse gas emissions and a increase up to 20% share of renewable energies in energy consumption by year 2020 in each member state. A minimum target of 10% for the share of sustainable biofuels for EU transport was also endorsed (European Commission, 2008).

Biogas provides a clean carrier of renewable energy and it can be used to produce heat and electricity (Murray et al., 1999) or as a vehicle fuel. Methane-rich biogas produced from energy crops is shown to be resource efficient and environmentally friendly compared to the other biomass-based vehicle fuels available so far (Börjesson and Mattiasson, 2008).

The biogas production based on the anaerobic digestion has not been investigated sufficiently. Performance of anaerobic digestion systems has mostly been described by means of volatile fatty acids, pH value and ammonia concentration. However, there are other factors than these chemical parameters affecting the whole process. It is widely accepted that the composition of microbial community participating the degradation of plant biomass to methane is related to the methane yield (Dearman et al., 2006).

1.2 Overview of the methane fermentation

In methane fermentation, there are three main stages: hydrolysis and acidogenesis, acetogenesis and dehydrogenation, and methanogenesis as shown in Fig.1. In the process, polymeric materials are converted to methane and carbon dioxide under anaerobic

conditions. Each stage of the methane fermentation is mediated by functionally different microorganisms.
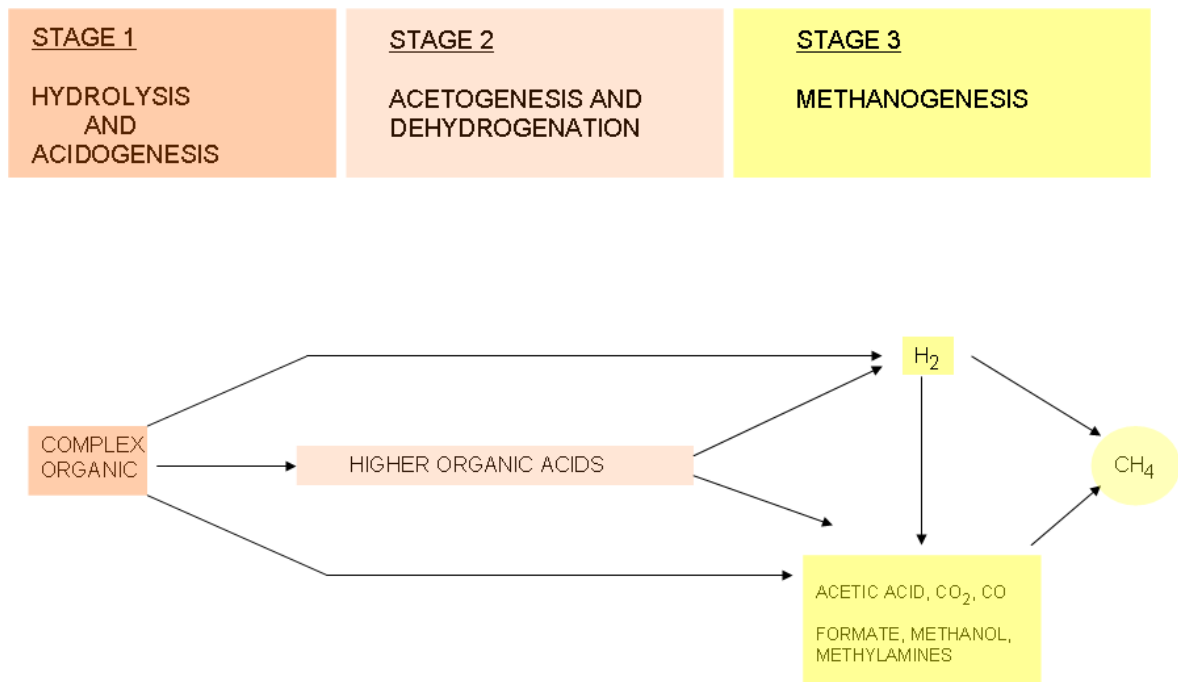
Figure 1. Different stages of methane fermentation. Adapted from Miyamoto, 1997.

In the first stage (hydrolysis and acidogenesis), polymeric materials such as cellulose, proteins and lipids are hydrolyzed to their monomers, for example to glucose, to amino acids or to fatty acids. Then the monomers are converted to higher volatile fatty acids, H_2 and acetic acid (Boone and Bryant, 1980; for review see Miyamoto, 1997).

In the second stage (acetogenesis and dehydrogenation), volatile fatty acids, H_2 and acetic acid formed previously are further metabolized. Acetogenic bacteria convert the formed higher volatile fatty acids *e.g.,* propionic and butyric acids to H_2, CO_2, and acetic acid. This second group of microorganisms contains species that are involved in β-oxidation of fatty acids to acetate and H_2 and in case of fatty acids with odd-number of carbons, also to propionate. In addition, there are species in this group that are able to decarboxylate propionate to acetate, CO_2, and H_2 (Boone and Bryant, 1980; Miyamoto, 1997).

In the third stage (methanogenesis), the acetate produced by the primary and secondary groups, is converted to methane by aceticlastic methanogenic archaea. CO_2 is reduced to

methane by hydrogenotrophic methanogenic archaea using H_2 as an electron donor (Boone D.R., and M.P. Bryant 1980). In addition, formate, methanol, methylamines, and CO are converted to methane by different methanogens (for review see Jones et al., 1987; Miyamoto, 1997).

Methane fermentation is a complex process, which is carried out by consortia reaction within a group of functionally different microorganisms. This process is thought to be the least energy producing mode of degradation. For instance, it releases only 15% of the chemical energy of hexose (for review see Schink, 1997). Aerobic or alternative anaerobic respirations produce considerable more energy. Because of the methanogenic degradation is so energy-poor process, the microorganisms involved are forced to cooperate efficiently. The interactions between metabolic groups during the methanogenesis are strong and syntrophic. The syntrophic interactions are thought to be a core of the methanogenic degradation (Schink, 1997). Syntrophic associations between methanogens and acetogens are demonstarated by a classical example of *Methanobacillus omelianskii* culture (Barker, 1940). *Methanobacillus omelianskii* was thought to be a pure culture of one organism, while now it is known to be in a syntrophic association between a fermenting bacterium (called S organism) and a methanogen. The bacterium produce acetate and H_2 from ethanol and the methanogen utilize produced H_2. The two prokaryotes cooperate in the conversion of ethanol to acetate and methane by interspecies hydrogen transfer. The fermenting bacterium is not able to grow in ethanol containing medium, in the absence of the hydrogen-scavenging partner methanogen (Bryant et al., 1967; Reddy et al., 1972).

1.3 Methanogenic Archaea

Methanogenic Archaea are the only organisms, which are able to couple their energy production to methanogenesis (Jones et al., 1987). Although there are few anaerobic bacteria able to produce methane as a by-product of their metabolism, they are not able to couple the production of methane to the production of energy. Methanogens live in very different environments such as in fresh water and marine sediments, digestive and intestinal tracks of animals, and in anaerobic waste digesters (Jones et al., 1987). All the

methanogens are obligatively anaerobes. Apparently, methanogens are present in every place where biological anaerobic degradation of organic compounds is occurring (Jones et al., 1987). Methanogens are an important part of the food chain in anaerobic conditions. All together, there are 28 genera of methanogens characterised (Ma et al., 2005), which are classified into three main nutritional categories based on their substrates for methane production (for review see Garcia et al., 2000). Hydrogenotrophs oxidize H_2 and reduce CO_2 to form methane, while methylotrophs reduce methyl compounds such as methanol, methylamines, or dimethylsulfide. Substrate to Aceticlastic (or acetotrophic) methanogens is acetate. Some species of methanogens cannot be placed in one single nutritional category. For example some species are hydrogeno-methylotrophs, using H_2 to reduce methanol to methane. Carbon monoxide can also be converted to methane, but it is not considered as an important methanogenic substrate.

1.4 Methane production decreasing factors

Three factors decrease a rate of methane fermentation. First, the hydrolysis of lingocellulose is known to be the bottleneck in degradation of cellulose-rich material. Second, substrate competition among other bacteria can reduce the amount of available substrates for methane production. Third, methane consuming bacteria, methanotrophs, can reduce methane yield.

The main reason for the difficulties in the degradation of cellulosic material is lignin (Hatfield et al., 1999; Grabber, 2005). Lignin plays a vital role in plant growth and development. It improves water conduction through xylem tracheary elements, enhancing the strength of fibrous tissues and limits the spread of pathogens in plant tissues. It also provides mechanical support of aerial parts of plants and assists resisting gravitational compressive forces (Iiyama et al., 1994). Because of the accumulation and progressive lignification of plant cell walls, the enzymatic degradability of cell walls declines during maturation (Grabber, 2005). In the plant cell wall lignin is cross-linked to other structural polysaccharides restricting their degradation by hydrolytic enzymes (Grabber, 2005). Cellulose typically comprises in the range of approximately 35-50% and lignin 5-30% of plant dry weight (Lynd et al., 1999; for review see Lynd et al., 2002). Lignocellulose is a

major organic component of different kinds of energy crops and the rate-limiting step during anaerobic digestion is hydrolysis of lignocellulose (for review see Mata-Alvarez et al., 2000; Noike et al., 1985). The microbes in the first stage of methane fermentation (Fig. 1) are responsible for the hydrolysis of the lignocellulose. Lignocellulose utilizing microbes are not well known, but in a group of Bacteria there are micro-organisms with cellulolytic capabilities in the aerobic order *Actinomycetales* and in the anaerobic order *Clostridiales* (Lynd et al., 2002).

Substrate competition between methanogens and other microbes may reduce methanogenesis. Energetically more favorable electron acceptor will be utilized first under substrate limiting conditions (Yagi et al., 1997). Hence, methanogenesis by archaea is suppressed if there are other electron acceptors than CO_2 for the anaerobic respriration such as Fe^{3+}, SO_4^{2-} or NO_3^{-} (Achtnich et al., 1995; Lueders and Friedrich, 2002). Sulfate reducing bacteria (SRB) are able to outcompete methanogens if sulphate concentrations are not limited (Dar et al., 2008; Robinson and Tiedje, 1984). Ferric iron-reducing bacteria (FRB) can also outcompete methanogens when ferric iron is present (Lueders and Friedrich, 2002).

The effect of methanotrophs to the methane production by methanogens is negative. Methanotrophs are unique in their ability to utilize methane as a sole carbon and energy source. Both aerobic and anaerobic species are known. Over 130 methanotrophs are characterized so far (for review see Hanson and Hanson, 1996). In natural anaerobic environments methane escapes to the atmosphere if it is not oxidized by methanotrophs.

1.5 Molecular analysis of microbial community

Classical way to study microbial populations from environmental sample is to prepare pure culture of microbes. However, analyzing microbial communities by cultivation does not give reliable picture about the numerically dominant or functionally important species in the analyzed sample. Only so called "weeds" of microbial world, which are able to grow easily on artificial growth medium are represented in the cultures. These weeds are estimated to constitute less than 1% of total microbial diversity and this distorted view of

microbial world caused by culture-dependent method has been called the "great plate-count anomaly" (Staley and Konopka, 1985).

Carl Woese and his colleagues were pioneers who realized that small-subunit (SSU) of ribosomal rRNAs (16S for prokaryotes and 18S for eukaryotes) can be used for phylogenetic classification of all organisms (Woese and Fox, 1977). In other words, SSU rRNA gene sequence can be used as a genetic fingerprint to differentiate species and the comparison of these fingerprints produces information about the evolutionary relationships between these species. In mid-1970s, the comparison of SSU rRNA gene sequences revealed that organisms can be divided into three main lineages or domains (Woese and Fox, 1977; for review see Woese, 1987). Two of these domains, Bacteria and Archaea, are prokaryotic and the third, Eucarya, is eukaryotic.

The sequence-based approach to phylogenetic classification of organisms created the framework for culture-independent methods to analyze microbial communities in the mid-1980s (for reviews see following articles: Forney et al., 2004; Hugenholtz, 2002; Robertson et al., 2005; Tiedje et al., 1999; Xu, 2006). These new methods, based on sequence data, have revolutionized our view of microbial ecology. For instance, sequences isolated from different environments have proved that Archaea, which were earlier found only from the extreme environments, are actually a cosmopolitan group.

In the molecular methods as shown in Fig. 2, the previously essential cultivation step of a microorganism to sequence its SSU rRNA gene, is bypassed. The total DNA of the environmental sample is extracted and the microbial DNA is analyzed. The SSU rRNA genes from the sample can be amplified by PCR using specific primers. The amplified SSU rRNA genes can be used to construct gene library and the genes can be sequenced from the clones of the library. Once a SSU rRNA gene has been sequenced, a comparative analysis against sequence databases may be achieved.

PCR amplified SSU rRNA genes can also be analyzed by other methods such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), amplified rDNA restriction analysis (ARDRA) and terminal restriction fragment length

polymorphism analysis (T-RFLP). Each method is based on differences in 16S rRNA gene sequences. They also can provide information about the relative abundance of different microbial groups in a sample. Because the sequencing is laborious, other approaches have gained more popularity.
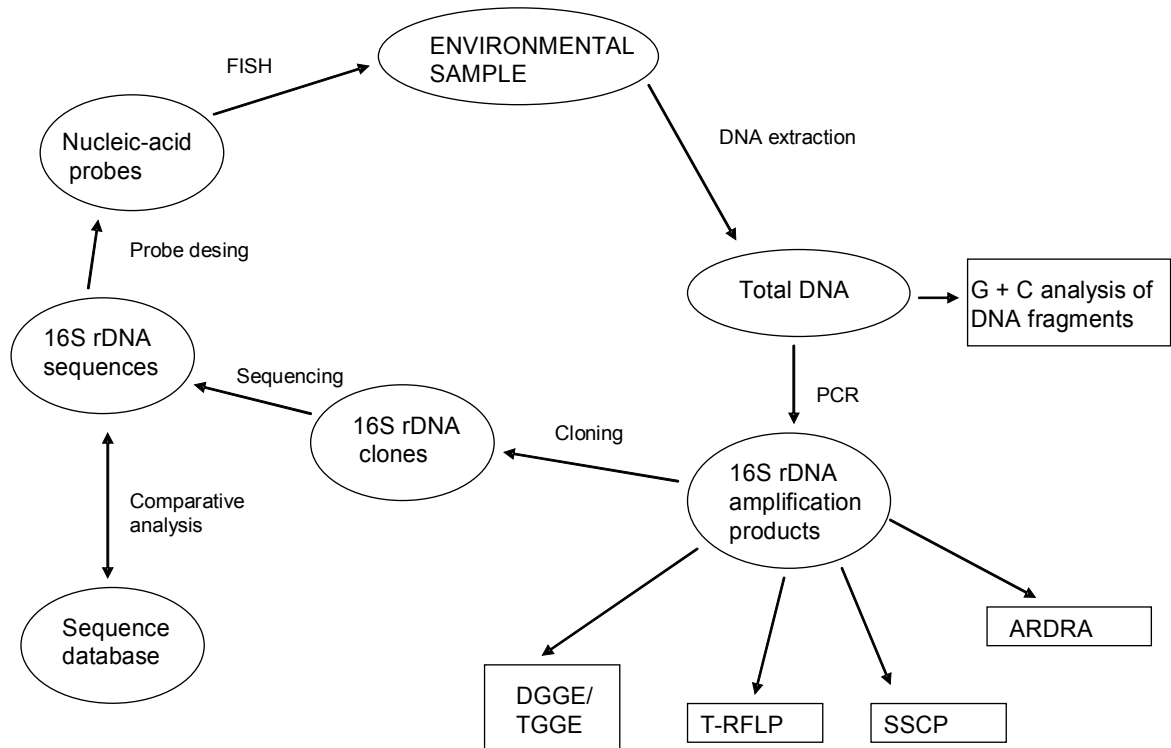
Figure 2. Schematic presentation of the methods for analyzing microbial communities by culture-independent genomic methods.

In the DGGE/TGGE, the analysis of PCR products that differ in sequence is based on the different helix stabilities in a denaturant or thermal gradient polyacrylamide gel. DNA fragments having the same length, but different sequences are possible to separate in a gel. Thus, sequence variants or, in the case of 16S rRNA gene fragments, ribotypes stop migrating at different positions in the gel. In theory, each band on the gel corresponds to one phylotype. This technique is sensitive and used to detect single base differences (Muyzer et al., 1993; for review see Muyzer and Smalla, 1998).

The separation of the PCR products by SSCP is based on conformational difference of the single-stranded products (Lee et al., 1996). The electrophoretic mobility of the DNA in

polyacrylamide gel is affected by its shape. The conformational differences are consequence of the primary sequences. Thus, different sequences can be separated into different bands in gel.

ARDRA is based on length polymorphism of the restriction fragments of PCR products (Smit et al., 1997). The length of different fragments can be determined in agarose or non-denaturing acrylamide gel electrophoresis, which gives distinguishable fingerprints for each microbial species in the sample.

In addition to the 16S rRNA gene analysis, the guanine plus cytosine (G + C) content of the DNA fragments in an environmental sample can be determined. This method provides coarse level resolution of the composition and the structure of the community (Holben and Harris, 1995; Tiedje et al., 1999). It is based on the fact that different prokaryotic groups have different G + C content. For example, prokaryotes with relative high G + C content (60-75%) are usually obligate aerobes and prokaryotes with relative low G +C content are mostly restricted to fermentative metabolism (Santo Domingo et al., 1998).

Specific 16S rRNA-targeted nucleic-acid probes for the organisms of interest are used to quantitatively visualize the target group. Techniques that use nucleic acid probes are whole-cell *in situ* hybridization (FISH) and membrane hybridization (for review see Amann et al., 1995).

Among these fingerprinting methods, T-RFLP (Dunbar et al., 2001; Kitts 2001) is widely used for the comparison of the relative phylotype richness and structure of communities in large environmental samples. In addition, it is used to identify specific organisms in a community in conjunction with gene sequence information.

1.6 T-RFLP analysis

In order to measure the size polymorphism of terminal restriction fragments, a PCR amplified marker is used in T-RFLP analysis. Three procedures in T-RFLP are:

comparative genomics, PCR and nucleic acid electrophoresis. The primers for amplifying 16S rRNA gene as well as other genes are designed by comparative genomics and the amplification of the target genes is done by PCR. The amplicons produced are digested by carefully selected restriction endonucleases and the gained restriction fragments are differentiated by size on high resolution (± 1 base) sequencing gel. The electrophoresis is performed on automated systems such as the ABI gel or capillary electrophoresis systems that provide digital output (for review see Marsh, 1999).

The reverse primer is fluorescently labeled at the 5′ terminus. Thus, only the fluorescent labeled terminal fragment of the restriction digest (T-RF) is detected by automated system. As a result the sequencer machine gives the peak (fragment) height, area and size in graphical and tabulated forms. The sequencer can discriminate between different fluorescent tags in a single gel lane and this enables the double and triple loading of the gel by differently tagged primers in the PCR amplification (Tiedje et al., 1999).

Sequencing technology allows the determination of the restriction fragment lengths down to ± 1.5 bases (Tiedje et al., 1999). With this technique any genetic marker with conserved sequence domains appropriate for primer design could be used. Nevertheless, 16S rRNA sequence database is relatively large, which makes it an ideal candidate (Cole et al., 2007; Marsh, 1999).

Often the members of the closely related phylogenetic groups have a same terminal restriction fragment size. The combined effect of 2-4 restriction enzymes in the experiment gives increasing confidence of any phylogenetic presumptions made (Tiedje et al., 1999). This method gives opportunity to discriminate ribotypes from different environmental samples, to align certain physiological abilities with taxonomic units and to gain greater understanding concerning interactions of the populations within a community (Tiedje et al., 1999).

Pseudo-T-RFs are one of the disadvantages of this technique. Pseudo-T-RFs are formed during PCR of 16S rRNA genes and this causes bias to the T-RFLP profile. Some of the amplicons formed during PCR of 16S rRNA genes from DNA extracts are at least partly

single-stranded and terminal restriction sites cannot be cleaved by the restriction endonucleases. Hence, "pseudo"-terminal restriction sites downstream from the expected primary restriction site can be detected by T-RFLP analysis (Egert and Friedrich, 2003).

1.7. The species problem

It is reasonable to remind that the general biological species concept is not applicable for asexual organism such as prokaryotes. According to biological species concept, only individuals, which are capable of interbreeding with opposite sex to produce fertile offspring, belong to the same species (Xu, 2006). For prokaryotes, different species concept has been applied. According to prokaryotic species concept two strains belong to the same species if their purified genomic DNA shows 70% or higher hybridization (Forney et al., 2004). This level of hybridization correspond 94% of average nucleotide identity (Konstantinidis and Tiedje, 2005). However, prokaryotic species concept is not applicable for animals and plants. For instance, the primates (humans, gibbons, orangutans etc.) would belong to the same species according to the prokaryotic species concept (Sibley et al. 1990). Thus, prokaryotic species concept and biological species concept are valid only their own scope.

2. Aim of the Study

Methanogenic archaea mediate the terminal step in anaerobic methanogenic fermentation process, which is also a key process within anaerobic digestion. However, the knowledge of archaeal community structure and relatively abundant species taking part in the anaerobic digestion of lignocellulosic materials in nature is still scarce. The aim of the present study was to investigate the dynamics of archaeal community in hydrolytic stage of two-stage processes during the anaerobic digestion of grass silage, and to assess the distribution of archaea in the reactor.

The specific aims of this study are as follows:

1. To elucidate the dynamics of the archaeal community in the hydrolytic reactors of two-stage anaerobic digestion of grass silage by T-RFLP analysis.
2. To reveal the distribution of archaea in the three fractions: solid residue, loosely attached biomass, and leachate in the hydrolytic reactors.
3. To phylogenetically recognize the archaeal population in the hydrolytic reactors and evaluate their potential functions.

3. Materials and methods

3.1 Substrates for the Biogas Reactor

The composition of the grass silage used as a substrate was 75% timothy (*Pheleum pretense*) and 25% meadow fescue (*Festuca pratensis*), which were harvested at early flowering state. The grass silage was obtained from a farm in central Finland (Kalmari farm, Laukaa). The grasses were chopped with an agricultural precision chopper after 24 h pre-wilting. Before ensiling the grass silage in a bunker silo, addition of a commercial lactic acid bacteria inoculant (AIV Bioprofit, Kemira Growhow Ltd.) was performed. The inoculant contained 60% *Lactobacillus rhamsonus* and 40% *Propionibacterium freudencreichii* spp. *Shermanii*. The inoculant had totally 5.8×10^{11} colony-forming units g^{-1}, diluted to 0.7 g 1^{-1} in tap water. The diluted inoculant was applied to the plant material in a ratio of 0.5% volume/weight. In the laboratory, the grass silage was chopped to a particle size approximately 3 cm with a garden chopper (Wolf Garden SD 180E) and then stored at -20 °C. Before feeding the grass silage to the bioreactor, it was allowed to thaw overnight at 4 °C (Lehtomäki et al., 2008).
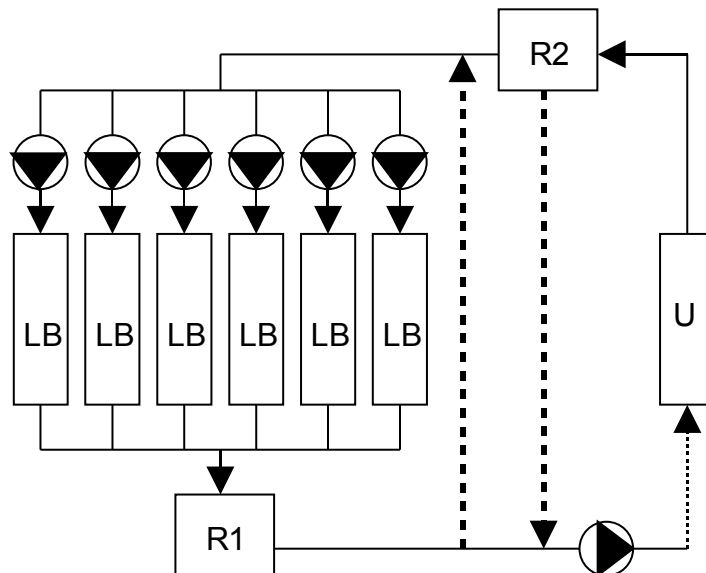
3.2 The Bioreactor

The samples for the archaeal community structure analysis were taken from a two-stage anaerobic digestion reactor (Fig. 3). The reactor consisted of two separate reactor units: six parallel hydrolytic leach bed reactors (LB1-LB6) and one upflow anaerobic sludge blanket reactor (UASB). The LBs were plastic columns and the UASB was a glass column both having liquid volume of 1000 ml. Leachate from the LBs was collected in a common reservoir (R1) and further circulated to the UASB. The effluent from the UASB was collected in a reservoir (R2) and recirculated to the top of the LBs. The recirculation was set to the same flow rate for each LB (Lehtomäki et al., 2008).

Before starting the run of the reactor, the UASB had been inoculated with granular sludge obtained from an internal circulation reactor treating wastewater from sugarbeet and

vegetable processing (Säkylä, Finland). Then the UASB was operated for two months with artificial wastewater by internal circulation. LBs were filled with 208 g wet weight of grass silage, which contained 50 g volatile solids at start and 250 ml tap water was added per reactor (1500 ml in total). All the LBs and UASB were operated at 35 (± 1) °C (Lehtomäki et al., 2008).

Before circulation to the UASB, LBs were initially operated with internal recirculation for 24h. Circulation to the UASB continued 17 days. LBs were terminated sequentially (LB1, LB2, LB3, LB4, LB5 and LB6) during the run on days 1, 3, 6, 10, 17 and day 49 (Lehtomäki et al., 2006).

Figure 3. The bioreactor architecture for the study.
Six LBs (leach bed reactors) were conjucted with a common UASB (upflow anaerobic sludge blanket reactor), marked as U. Dashed lines represent the flow of process liquid during internal recirculation. Leachate from LBs was collected in R1 (liquid reservoir) and UASB effluent was collected to R2 (Lehtomäki et al., 2008).

3.3 The samples

There were three types of samples or fractions taken to be analyzed. Leachate (L) fractions (15 ml) were taken from the R1. The solid substrate (S) and loosely attached to solid substrate (W) fractions were taken from the LBs. The solid residues were mixed thoroughly and two grams of sample was washed for four times with one volume of PBS to remove the loosely attached biomass. After each wash, the solid material was spinned down gently at 500 rpm for 5 min (Heraeus Biofuge Pico) and the supernatant was removed. The loosely attached solid substrate comprised all the collected supernatant fractions. The leachate and loosely attached fractions were concentrated by centrifugation at 5000 rpm for 5 min (Heraeus Biofuge Pico) and the pellets were collected. The taken samples were stored at -20 °C before DNA extraction.

There were totally 16 samples taken for the archael community structure analysis as described in Table 1.

Table 1. The samples taken during the reactor operation of 49 days[a].

Sample	Reactor	Operating day
S1; W1	LB1	1
S3; W3	LB2	3
S6; W6	LB3	6
S10; W10	LB4	10
S17; W17	LB5	17
L1	R1	1
L3	R1	3
L6	R1	6
L10	R1	10
L17	R1	17
L49	R1	49

[a] Solid residues (S) and loosely attached biomass (W) were taken from the LBs. Leachate (L) samples were taken from the R1.

3.4 Extraction of total DNA from the samples

For solid samples (S1, S3, S6, S10 and S17) QIAGEN DNeasy Plant Mini Kit were used, which is designed for DNA isolation from plant tissues. The extraction was done according to the instruction manual. Centrifugation steps were done by using Heraeus Biofuge Pico. In the steps 4 and 5 of the protocol, the centrifugal field was 16060 x g and in the steps 8, 10 and 12 the field was 6082 x g.

For leachate samples (L1, L3, L6, L10 and L17) Power Soil™ DNA Isolation Kit (Mo Bio Laboratories, inc.) were used. The extraction was done according to the user protocol of the instruction manual with the following exceptions: 50 µl of solution C6 was added to the tubes instead of 100 µl in the step 20 and all centrifugation steps were done by using Heraeus Biofuge Pico at 9503 x g instead of 10 000 x g.

Previously extracted and purified genomic DNA from the samples L49, W1, W3, W6, W10 and W17 were donated by Anna –Leena Keränen. She used FastDNA® SPIN Kit for Soil (Qbiogene, Inc.) for the exctraction of the DNA. Extraction was performed according to the instruction manual except the following modifications: 1. Cell lysis was performed by processing Lysing Matrix E Tubes in FastPrep®Instrument for 45 seconds at speed 5.5, then Lysing Matrix E Tubes were centrifuged at 14,000 x g for 10 minutes with Harrier 18/80 centrifuge (MSE, UK); 2. The binding matrixes were washed three times with 1 ml of 5.5 M guanidine thiocyanate to remove humic acid before loading on SPIN™ Filters; 3. DNA was eluted from SPIN™ Filters in 100 µl of DES (DNase/Pyrogen Free Water).

The extracted DNA samples were purified by Wizard®DNA Clean-Up System (Promega). The purification was done according to the vacuum manifold protocol of the instruction manual with the following exceptions: 30 µl TE buffer was used in step 7 instead of 50 µl. Centrifuging steps were done by using Heraeus Biofuge Pico at 16060 x g instead of 10 000 x g. After the purification the DNA concentrations of the samples were determined by ND-1000 Spectrophotometer.

3.5 T-RFLP analysis

The archaeal 16S rRNA gene in the total extracted genomic DNA was amplified for the T-RFLP analysis by PCR. Reverse primer was labelled by 6-carboxyfluorescein (FAM).

Archaeal 16S rRNA genes were amplified using Ar109f (5´-ACK GCT CAG TAA CAC GT-3´) as a forward primer and FAM-Ar912rt (5'-Fam-GTG CTC CCC CGC CAA TTC CTT TA-3') as a reverse primer. PCR was performed in a 100-µl reaction mixture containing 3 µl template DNA (64-335 ng of DNA depending on the sample), 10 µl of 10 x PCR buffer, 0.2 mM dNTPs, 1U of DyNAzyme™ II DNA polymerase (Finnzymes), 1M Betain and 0.5 µM of each primer. PCR was performed by MBS PCR system (Thermo Fisher Scientific). PCR reaction conditions were following: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 min. The final extension was carried at 72°C for 10 min.

The PCR products were verified by AGE (1 % agarose gel) and ethidium bromide staining. The gel was visualised by the Gel Doc 2000 gel documentation system (BioRad, UK). The PCR products were purified by GenElute™ PCR clean-up kit (Sigma) according the instructions of the manual with minor modification: The DNA was eluted in 30 µl of elution solution instead of 50 µl. All centrifuging steps were done by using Heraeus Biofuge Pico at 16060 x g. The DNA concentrations of the purified PCR products were determined by ND-1000 Spectrophotometer.

Approximately 100 ng of DNA in 10 µl volume was digested with 10 U of Tag I for 3 hours at 72°C. The digestion mixtures were separated to three parts: undiluted digestion mixture, diluted digestion mixture by 1:1 in water and diluted digestion mixture by 1:1.5 in water. 1 µl of sample from each part was mixed with 9.0 µl mixture of Hi-Di™ Formamide and size standard GeneScan™ LIZ 500 (1:128), and loaded onto 96-well plate. The mixture was heated at 95 °C for 3 minutes and chilled on ice for few minutes. Fluorescently labelled terminal restriction fragments (T-RFs) were separated on an ABI Prism® 3130 automated sequencer (Applied Biosystems).

T-RFLP profiles were standardized according to Dunbar et al., (2001). The sum of peak area in each profile was calculated to get the total DNA quantity of each profile (total fluorescent units of peak areas). Then T-RFLP profiles were standardized to the profile with the smallest total fluorescent units of peak areas. After standardization, 2% threshold was used to remove T-RFs that contributed less than 2% to the total area in the T-RFLP profile. The relative abundance of T-RFs in the profiles was calculated according to the standardized peak areas (Schwarz et al., 2007).

3.6 Clone library analysis

For clone library analysis, the samples S17 and W17 were chosen. The analysis consisted five different steps. First, the 16S rRNA genes were amplified by PCR and the PCR products were purified. Second, the purified PCR products were ligated to the pGEM-T plasmid. Third, the recombinant plasmids were transformed to *E. coli* and transformed *E. coli* cells were cultivated on selective LB-ampicillin agar plates supplied with X-Gal and IPTG. Fourth, positive colonies of transformed *E. coli* were chosen as templates for PCR. Finally, the PCR products were purified and sequenced using ABI Prism® 3130xl.

The Archaeal 16S rRNA gene was first amplified by PCR using Ar109f as a forward primer and Ar912rt as a reverse primer. PCR was performed in a 50-μl reaction mixture containing 2 μl of template DNA (119-432 ng of DNA depending on the sample), 5 μl of 10 x PCR buffer, 0.2 mM dNTPs, 1U of DyNAzyme™ II DNA polymerase (Finnzymes), 1M Betain, and 0.5 μM of each primer. PCR was performed by MBS PCR system (Thermo Fisher Scientific). PCR reaction conditions were following: initial denaturation at 94°C for 3 min. The initial denaturation was followed by 29 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s. The final extension was carried out at 72°C for 20 min. The PCR products were checked by AGE (1% agarose gel) and ethidium bromide staining. The gel was visualised by the Gel Doc 2000 gel documentation system (BioRad, UK).

Before the ligation the PCR products were cleaned by using GenElute™ PCR clean-up kit (Sigma). The purification was performed according to the instructions of the manual with

no deviation except all centrifuging steps were done by using Heraeus Biofuge Pico at 16060 x g. The samples were stored at 4 °C over night before ligation.

The amplified 16S rRNA gene fragments were ligated to pGEM-T plasmid by using Promegas pGEM®-T Easy Vector System according to the manual instructions. The ligation was performed for 1 hour at room temperature.

After the ligation reaction, the recombinant plasmids were transformed to electro competent JM109 *E. coli* by electroporation. 2 µl of ligation mixture was added to 60 µl *E. coli*, and they were mixed lightly by pipette and left for incubation for 10 minutes on ice. The mixture was transferred to BTX cuvette of 2mm gap (BTX®, Harvard Apparatus) for electroporation. The cuvettes were cooled beforehand on ice. The electroporation was done by BTX Electro Cell Manipulator ® 600 (BTX®, Harvard Apparatus) and the settings of electroporation are shown in the table 2. After electroporation 1 ml of SOC was added immediately into the mixture and incubated in the Excella™ Shaker E25 (New Brunswick Scientific) at 37 °C for 1 hour.

Table 2. Electroporation settings.

Resistance	129 ohm
Charging Voltage	2.45 kV
Field strength	~12.25 kV/cm
Pulse length	~5-6 msec
Chamber gap	2 mm

After 1 hour incubation at 37 °C, the transformed *E. coli* cells were plated on to the LB-agar ampicillin plates. Before adding the cells on to the plates, 100 µl of IPTG (100 mM) and 50 µl of X-Gal (20 mg/ml) were spread on them. After that, 100 µl transformed cell mixture were added and spread on to the each plates. The plates were incubated over night at 37 °C in a laboratory incubator (Termaks B805).

Archaeal 16S rRNA gene fragments were amplified from randomly selected clones by PCR with pGEX-T primer set T7-SP6. PCR was performed in a 96-well plate. 25-µl reaction mixture contained single positive transformed *E. coli* colony as a template, 2.5 µl

of 10 x PCR buffer, 0.2 mM dNTPs, 0.25 U of DyNAzyme™ II DNA polymerase (Finnzymes), and 0.5 μM of each primer. PCR was performed by MBS PCR system (Thermo Fisher Scientific). PCR reaction conditions were as follows: Initial denaturation at 94°C for 1 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. The PCR products were checked by AGE (1% agarose gel) and ethidium bromide staining and visualised by the Gel Doc 2000 gel documentation system (BioRad, UK).

The positive colony PCR products were chosen for sequencing. Before sequencing reaction, the colony PCR products were purified with Exonuclease I (Exo I) (Fermentas) and Shrimp Alkaline Phosphatase (SAP) according to the instruction manual (Fermentas). The composition of the enzyme mixture is shown in Table 3. 10 μl of colony PCR products and 4 μl of Exo-SAP enzyme mixture were mixed. The PCR machine was used as a heater in the purification reactions. First, the PCR machine (MBS PCR system, Thermo Fisher Scientific) was heated to 37 ℃ for 60 minutes, which breaks down excess primers and dNTPs. Second, the PCR machine was heated to 80 ℃ for 15 minutes in order to break down the excess polymerase.

Table 3. The composition of Exo-Sap enzyme mixture.

Reagent	Concentration	Proportion
H_2O		0.5:99.5
Exonuclease I	20 u/ μl	89:99.5
Shrimp Alkaline Phosphatase	1 u/ μl	10:99.5

After purification of the colony PCR products, actual sequence reactions were performed using ABI BigDye® terminator v3.1 cycle sequence kit (Applied Biosystems). The reaction master mixture is shown in Table 4. Two separate reaction master mix were prepared: One with forward primer (Ar109F) and the other with reverse primer (Ar912rt). Thus, both strands of the DNA were sequenced. 3 μl of purified colony PCR product was mixed with 16 μl sequencing reaction mixture. The samples were run in the PCR apparatus (MBS PCR system, Thermo Fisher Scientific) and the cycle reaction conditions were following: Initial

denaturation at 96°C for 1 min followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and the final extension at 60°C for 4 min.

Table 4. Sequencing reaction mixture. Ar109F was used as a forward primer and Ar912rt was used as a reverse primer.

| Reagent	Volume
H_2O	11.75 μl
Primer: Ar109F or Ar912rt (3.2 μM)	1 μl
5x Buffer	3.75 μl
25x Big-Dye sequencing premix	0.5 μl
Total	17

Before running the sequenced samples on the ABI Prism® 3130xl, the samples were purified. 2 μl of 125 mM EDTA, 2 μl of 3 M Sodium Acetate and 50 μl cold 100 % ethanol were pipetted on the samples in the PCR plate. The reagents were mixed with the samples by inverting and left to incubate at ambient for 15 minutes. The samples containing PCR plate was centrifuged at 1109 rcf for 45 min in cold (+4 °C) Beckman plate centrifuge and liquid reagents were removed by centrifuging the PCR plate upside down on a paper towel at 100 rcf for 10 seconds. 70 μl cold 70 % ethanol was added to the samples and the PCR plate was centrifuged at 1109 rcf for 15 minutes. The ethanol was removed by centrifuging the PCR plate again upside down on a paper towel at 100 rcf for 30 seconds and the PCR plate was dried at 37 °C for 10 min in the incubator.10 μl of Hi-Di™ formamide (Applied Biosystems) was added to the samples and let to incubate for 10 minutes at room temperature. After the incubation the samples and formamide were mixed by pipetting up and down, and the samples were run in the ABI Prism® 3130xl.

3.7 Comparative sequence analysis

Comparative sequence analysis was done by Washington University Basic Local Alignment Search Tool Version 2.0. (WU-BLAST2) at http://www.ebi.ac.uk/blast2/nucleotide.html?embl. The query of the sequencies was done from the EMBL Nucleotide Sequence Database.

4. Results

Archaeal 16S rRNA gene was amplified from the DNA extraction retrieved from different fractions at each time point. Amplification was successful only for the samples S17 (solid fraction on day 17), L10 and L17 (leachate fraction on days 10 and 17) and W10 and W17 (loosely attached fraction on days 10 and 17) (Fig. 4). The amplicons were further subjected for terminal restriction fragment length polymorphism analysis using restriction enzyme TagI. The two diluted digestion mixtures provided the best results in the T-RFLP analysis.
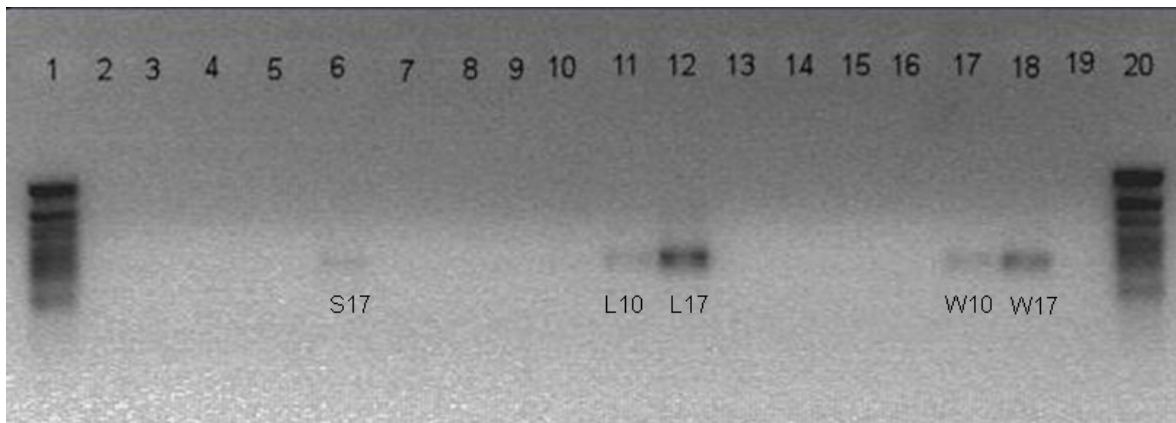
Figure 4. PCR for T-RFLP. Sample order: 100 bp ladder (1,20) (GeneRuler™, Fermentas), S1 (2), S3 (3), S6 (4), S10 (5), S17.1 (6), S17.2 (7), L1 (8), L3 (9), L6 (10), L10 (11), L17 (12), L49 (13), W1 (14), W3 (15), W6 (16), W10 (17), W17 (18), and negative control (19). In all the positive samples the band was observed in 800 bp.

4.1 T-RFLP profiles

The T-RFLP fingerprints revealed total 8 different T-RFs as shown in Fig. 5. A T-RF with size of 89 bp dominated in all profiles. In the profiles derived from L10, W10, W17 and S17, the T-RF of 89 bp accounted for 74-92% of the profile wheras in the profile from L17, the amount was 40%.

Change on the composition of T-RFs and their abundance occurred in the leachate profiles. On day 10 there were also T-RFs of 184, 88, and 80 bp present in the profile besides the T-RF of 89 bp. On day 17, T-RF of 184 bp was more abundant than day 10. In contrary, the

amount of T-RF of 88 bp in the profile decreased and the T-RF of 80 bp was disappeared on day 17. In addition, a T-RF of 281 bp emerged on day 17. The T-RFs of 184 bp and 281 bp were most abundant in the leachate profile on day 17 (Fig. 5).

In the loosely attached biomass on day 10, five different T-RFs in addition of the T-RF of 89 bp were observed. They are the T-RFs of 389, 281, 88, 84 and 80 bp. On day 17, the T-RFs of 376 and 281 bp appeared, in the profile that showed the most diverse T-RFs in the present study.

There were six T-RFs with size of 389, 281, 89, 88, 84 and 80 bp detected in the solid profile on day 17. The amount of the T-RF of 84 bp was the highest in solid fraction on day 17.

The T-RF of 281 bp appeared to emerge into the reactor on day 17. The T-RF of 376 bp was unique to the loosely attached fraction on day 17 and it accounted circa 3% of the total fraction. All together, the diversity of different T-RFs was highest in the loosely attached fraction and lowest in the leachate fraction.
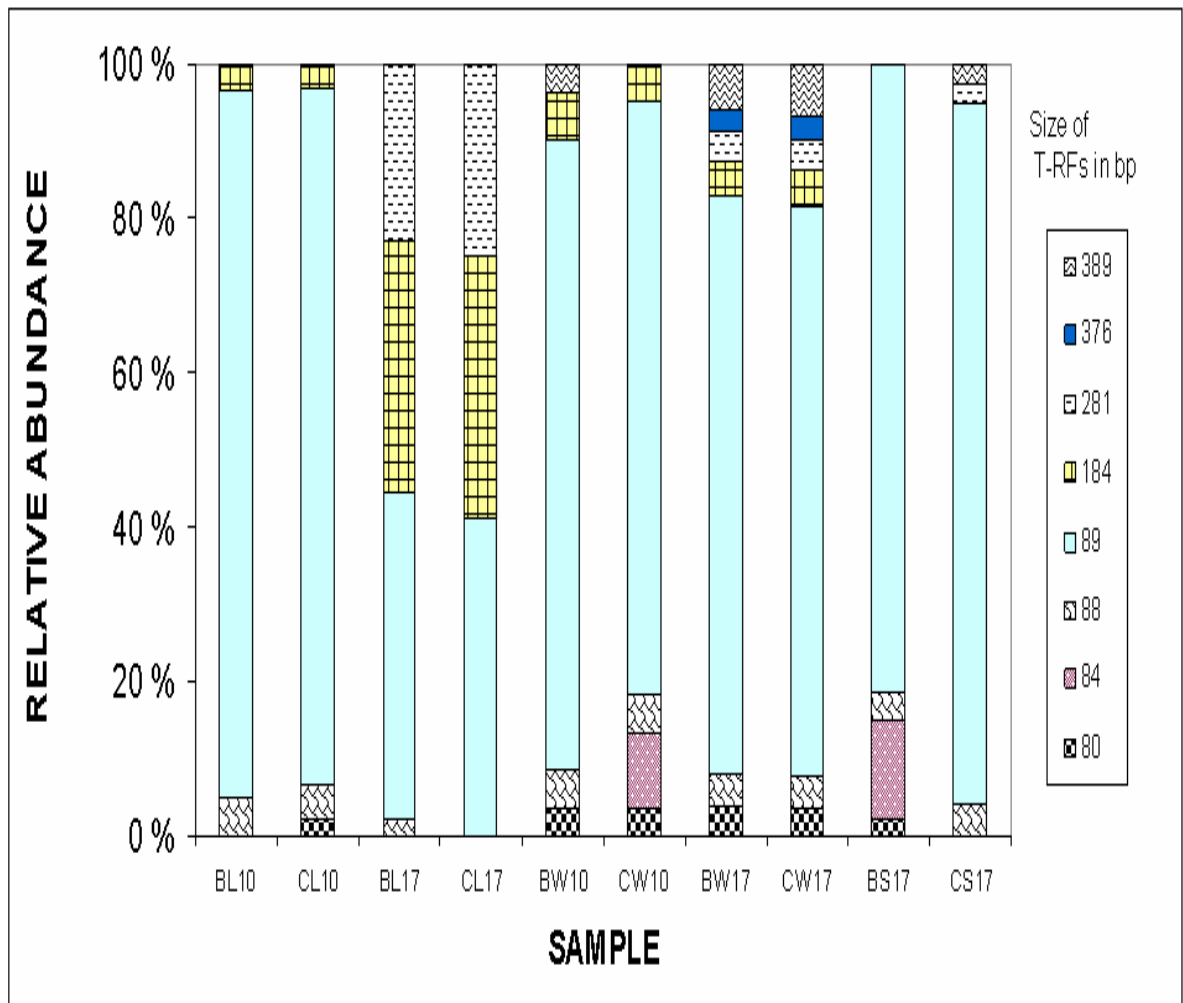
Figure 5. T-RFLP profiles from the days 10 and 17. B and C indicate different dilution of the digestion products in the water (1:1 and 1:1, 5).

4.2 Sequence analysis of the archaeal 16S rRNA genes

Totally 7 clone groups (SEQ1-SEQ7) were retrieved from the sequence analysis of the clone libraries from the samples S17 and W17. Homology search against the EMBL Nucleotide Sequence Database with the seven sequences provided information about closest relatives. Five best alignments are shown for each sequence (Table 5). The majority of phylotypes in the reactors were closely related to the members of *Methanobacterium,* a group of hydrogenotrophic methanogenic archaea.

For sequencing, 16S rRNA genes were first amplified by PCR, amplicons were ligated to the plasmid, and formed recombinant plasmids were transformed into the *E. coli* cells, which were finally plated to form 16S rRNA gene library. The number of colonies occupied identical sequences, could offer indicative information about the abundance of the sequences.

To correlate the sequence data and the T-RFLP data, virtual digestion of the sequences by TaqI was performed by RestrictionMapper version 3 at http://www.restrictionmapper.org/ . TaqI recognition site was detected only from SEQ1, SEQ6 and SEQ7. Terminal fragments of these sequences were 93 bp for SEQ1, 187 bp for SEQ6 and 93 bp for SEQ7. These T-RFs did not match the T-RFs of the T-RFLP data. However, it is possible that there is a bias in T-RFLP analysis, which shortens the T-RFs by couple of bases. Thus, the T-RF of 89 bp can correspond to the T-RF of 93 bp produced by virtual digestion of the sequences SEQ1 and SEQ7. Furthermore, the T-RF of 184 bp may correspond to SEQ6, which produced T-RF of 187 bp.

Table 5. The results of the comparative analysis of the sequences. High score value indicate the suitability of the alignments. The greater value indicates the better match between the query sequence and the database entry.

Sequence name	S17 library % n = 23	W17 library % n = 34		Sequencies producing five best alignments	High Score
SEQ1	47.8	64.7	1	*Methanobacterium curvum* 16S ribosomal RNA gene, partial sequence	3961
			2	*Methanobacterium congolense* 16S ribosomal RNA gene, partial sequence	3936
			3	Uncultured *Methanobacteriaceae* archaeon gene for 16S rRNA, partial sequence, clone:AR-H2-B	3885
			4	Uncultured *Methanobacteriaceae* archaeon gene for 16S rRNA, partial sequence, clone:LF-Eth-A	3858
			5	Uncultured *Methanobacteriaceae* archaeon gene for 16S rRNA, partial sequence, clone:LF-For-B	3849

SEQ2	26.1	8.8	1	*Methanobacterium subterraneum* strain 9-7 16S ribosomal RNA gene, partial sequence	3970
			2	Uncultured bacterium clone QHO-A15 16S ribosomal RNA gene, partial sequence	3970
			3	*Methanobacterium formicicum* strain FCam 16S ribosomal RNA gene, complete sequence	3961
			4	*Methanobacterium* sp. F gene for 16S rRNA, partial sequence	3961
			5	Uncultured bacterium clone QHO-A13 16S ribosomal RNA gene, partial sequence	3587

SEQ3	13.0	8.8	1	*Methanobacterium* sp. C5/51 16S rRNA gene, strain C5/51	3970
			2	*Methanobacterium* sp. OM15 16S rRNA gene, strain OM15	3970
			3	*Methanoculleus* sp. M06 gene for 16S rRNA, partial sequence	3970
			4	*Methanobacterium* sp. T01 gene for 16S rRNA, partial sequence	3970
			5	*Methanobacterium formicicum* strain S1 16S ribosomal RNA gene, partial sequence	3970

SEQ4	4.3	14.7	1	*Methanobacterium* sp. 169 gene for 16S ribosomal RNA, partial sequence	3903
			2	Uncultured archaeon clone 1G1 16S ribosomal RNA gene, partial sequence	3876
			3	Uncultured archaeon gene for 16S rRNA, clone: CG-4	3876
			4	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: SwA77fl	3876
			5	*Methanobacterium formicicum* strain FCam 16S ribosomal RNA gene, complete sequence	3817

SEQ5	4.3	-	1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: HsA55fl	3844
			2	Uncultured archaeon partial 16S rRNA gene, clone OuI-11	3780
			3	*Methanobacterium beijingense* strain 4-1 16S ribosomal RNA gene, partial DE sequence	3704
			4	Uncultured archaeon clone MP104-1109-a25 16S ribosomal RNA gene, partial sequence	3704
			5	Unidentified archaeon clone vadinDC06 16S ribosomal RNA gene, partial sequence	3695

SEQ6	4.3	-	1	Uncultured archaeon clone ATB-KS-0088 16S ribosomal RNA gene gene, partial sequence	3929
			2	Uncultured *Methanosarcinales* archaeon gene for 16S rRNA, partial sequence, clone: TDS-J-r-A03	3917
			3	Uncultured *Methanosarcina* sp. partial 16S rRNA gene, clone KT17	3911
			4	Uncultured *Methanosarcina* sp. partial 16S rRNA gene, clone KT29	3902
			5	Uncultured *Methanosarcinaceae* archaeon partial 16S rRNA gene, clone LrhA83	3899

SEQ7	-	2.9	1	*Methanobacterium beijingense* strain 4-1 16S ribosomal RNA gene, partial sequence	3965
			2	Unidentified archaeon clone vadinDC06 16S ribosomal RNA gene, partial sequence	3956
			3	Uncultured archaeon 16S rRNA gene, clone GZK52	3956
			4	*Methanobacterium beijingense* strain 8-2 16S ribosomal RNA gene, partial sequence	3947
			5	Uncultured archaeon clone MP104-1109-a25 16S ribosomal RNA gene, partial sequence	3884

5. Discussion

According to the PCR amplification of the 16S rRNA genes, archaeal population seemed to emerge into the leach bed reactors on day 10 (Fig. 4). In our laboratory was also discovered earlier that the methane concentration in the gas of the leach bed reactors started to increase at the same time (Lehtomäki et al., 2008). These results suggested that methanogens emerged into the reactor around day 10 and started to produce methane. This data is also consistent with the food chain model described earlier this thesis (p. 9). Methanogenesis will not occur until appropriate substrates, produced by other microbes, are present for the methanogenic archaea.

The production of CH_4 was correlated to the production of CO_2 in the UASB reactor (Lehtomäki et al., 2008). Though, the correlation does not indicate directly causal connections one can make assumption that most of the methane was generated from CO_2. This means that the main substrates available for archaeal methanogens were H_2/CO_2 and the members of *Methanobacterium* are known to be able to reduce CO_2 to methane by using H_2 as an electron donor. The concentration of acetate did not correlate with methanogenesis and concentrations of the other substrates for methanogenesis were not determined.

The T-RFLP analysis produced 8 different T-RFs and the sequencing of the gene library produced 7 different sequences. In theory, one T-RF is assumed to represent one ribotype or 16S rRNA gene (Marsh, 1999). Thus, the number of the sequences recognized by T-RFLP analysis was close to the number of sequences produced by sequencing. This indicates that the methods were quite reliable because they produced similar result.

One 16S rRNA gene sequence is thought to correspond to one species. Therefore, it seems that the diversity of the archaeal community is relative low i.e. 7-8 species. Nevertheless, according the T-RFLP profiles (Fig. 5) the diversity of the community was increasing in the time. For instance, the diversity of the loosely attached sample on the day 10 (W10) was 5 sequences. On day 17 (W17), the diversity of the sample has increased to 7 sequences. One can speculate that the archaeal diversity should increase in time when the

essential resource and environmental condition were favourable for the archaea growth. However, the PCR of the leachate sample on day 49 (L49) was not successful (Fig. 4). This indicates that there was no archaeal DNA present or the amount of it was very low. Thus, the archaeal population depleted during the late operational phase of the reactors. This could be because of the insufficient substrates and inhibitor for the archaeal species. If there would be continuous supply of substrates, higher archaeal diversity could be probably detected in longer operation times of the reactor.

According to the sequence analysis, all the sequences except the SEQ6 affiliated to the genus *Methanobacterium*. Instead, the SEQ6 showed homology to the genus *Methanosarcina*. SEQ1 indicated best alignment with the 16S rRNA gene of *Methanobacterium curvum* and the second best alignment was with the 16S rRNA gene of *Methanobacterium congolense*. *M. curvum* has morphology of curved rod and it uses H_2/CO_2 for methane production (Sun et al., 2001). However, there is limited number of information concerning biology of *M. curvum* available. The species was first isolated and characterised from the anaerobic digester processing wastewater from Beijing beer factory (Sun et al., 2001). More information was available concerning *M. congolense,* which was isolated and characterised from the anaerobic digester treating cassava-peel waste (Cuzin et al., 2001). Morphologically it is rod shaped and it stains Gram-positive. *M. congolense* is able to grow and produce methane from H_2/CO_2. If CO_2 is present and either 2-propanol, 2-butanol or cyclopentanol are available for hydrogen donation, *M. congolense* can produce methane without ability grow. The temperature range for this species is between 25 - 50 °C and the optimal temperature is between 37 - 42 °C. The optimum pH of the *M. congolense* is 7. 2 and it is unable to grow outside pH-range 5.9 to 8.2. The temperature of the reactor was 35 (±1) °C and the pH of the LB effluent was 7.5 on day 17 (Lehtomäki et al., 2008). Thus, the temperature and the pH of the leach bed reactors were suitable for the growth of the *M. congolense*-like archaea. Furthermore, SEQ1 was the most abundant sequence comprising 48% of total clones in the S17 gene library and 65% in the W17 gene library. It seems that the SEQ1 was more abundant in the loosely attached fraction than in the solid fraction. In addition, according to virtual digestion, the T-RF of 89 bp can correspond to the SEQ1. Thus, the SEQ1 might be the dominant sequence in all T-RFLP profiles (Fig. 5).

SEQ2 comprised 26% of total clones in the S17 library and 9% in the W17 library being the second abundant sequence. SEQ2 aligned best with 16S rRNA gene of *Methanobacterium subterraneum.* It also indicated high similarity with uncultured bacterium clone QHO-A15 and *Methanobacterium formicum. M. subterraneum* has been isolated from deep granitic ground water as deep as 420 m (Kotelnikova et al., 1998). *M. subterraneum* is morphologically non-motile rod. It can grow and produce methane using H_2 and CO_2 or formate. It is able to grow even in very low temperatures (3.6 - 45°C) and its optimum temperature is between 20 - 40 °C. *M. subterraneum* is alkaliphilic (pH optimum 7.8 - 8.8) and halotolerant. It has been shown to grow autotrophically in mineral medium without any organic additions (Kotelnikova et al., 1998). *M. subterraneum* is thought to be one of the methanogens, which represent chemoautolitotrophic organism that initiate food chains in the oligotrophic deep subsurface habitats at the expense of geologically produced hydrogen.

SEQ3 aligned equally with *Methanobacterium* sp. C5/51, *Methanobacterium* sp. OM15, *Methanoculleus* sp. M06 and *Methanobacterium* sp. T01. However, these strains are not well characterised. Of the better characterised strains, SEQ3 showed high similarity with *Methanobacterium formicum*. SEQ4 aligned mostly with 16S rRNA gene sequences of incompletely characterised organisms, but showed also high similarity with *Methanobacterium formicum*, which has ability to use formate for its growth and methanogenesis (Jones et al., 1987; Schauer and Ferry, 1980). *M. formicum* has morphology of rod and ideal conditions for growth are at 37 °C and pH 7.0. The temperature and pH conditions were suitable for the *M. formicum*-like archaea on day 17 in the leach bed reactors (Lehtomäki et al., 2008). SEQ3 consisted 13% of the S17 gene library and 9% of the W17 gene library and SEQ4 comprised 4% of the S17 gene library and 15% of the W17 gene library.

SEQ7 aligned best with 16S rRNA gene of *Methanobacterium beijingense*. Also SEQ5 indicated some homology with *M. beijingense*. SEQ7 comprised 3% of the W17 gene library and SEQ5 4% of the S17 gene library. *M. beijingense* has been isolated from anaerobic digester and it is able to grow and produce methane by reducing CO_2 or formate

with molecular H_2. The species is non-motile rod and it has been observed to grow occasionally in chains (Ma et al., 2005). The optimum growing temperature is 37 °C and it is able to grow between 25 - 50 °C. Its pH range is 6.5 - 8.0 and the optimum is 7.2. The temperature and pH in the the leach bed reactors on day 17 were suitable for *M. beijingense*-like archaea. Considering the possible bias of the T-RFs in T-RFLP analysis, the T-RF produced by *in silico* restriction digest of the SEQ7 can correspond to the 89 or 88 bp T-RFs observed in T-RFLP profiles.

SEQ6 comprised 4% of the S17 gene library. Unlike the other sequencies SEQ6, indicated homology with the genus *Methanosarcina*. The members of the genus are able to convert many substrates such as acetate, methanol, CO_2 and H_2, and carbon monoxide to methane (Rother et al., 2007). Characterised species *Methanosarcina mazei* and *acetivorans* have optimum temperature of 40 °C and *Methanosarcina thermophila* has optimum temperature of 50 °C (Jones W. *et al.* 1987). *Methanosarcina barkeri* has best growing conditions at 35 °C, which is close to the temperature of the reactor and therefore it is possible that SEQ6 represents *M. barkeri*-like archaea. The species has morphology of coccus and it is able to utilize H_2/CO_2, methanol, methyl amines, and acetate (Hippe et al., 1979). Furthermore, according to the *in silico* restriction digestion, SEQ6 might correspond to the T-RF of 184 bp in T-RFLP profiles.

The information concerning the archaeal community obtained from this research is dependent on DNA extraction, PCR reaction, and the used primers. The extraction of DNA is a critical phase and may lead to errors in the results (Forney et al., 2004; Frostegård et al., 1999; Martin-Laurent et al., 2001). To obtain a detailed knowledge about the microbial community, the entire microbial DNA (in this study the archaeal DNA) should be collected. Nevertheless, there are no methods available that will certainly collect the entire microbial DNA. Therefore, in the next step, when the DNA is replicated by PCR (in this case the PCR for T-RFLP and sequencing), the amount of DNA present is unequal between different species. This may lead to the situation, where the DNA of the species that is present in the sample, is failed to be extract and consequently not shown in the results. It would be important that the method for extraction would lyse all the cells in the

same way allowing to collect information from all possible populations in the sample (Forney et al., 2004).

PCR is competitive enzymatic reaction and the concentrations of the reactants affect to the yield of the reaction. This means that the 16S rDNA templates are amplified according to their concentration. Thus, the greater the amount of certain template DNA, the better amplification is achieved. On the contrary, the very low concentration of 16S rRNA gene template may elude observation. According to the Forney et al. (2004), populations, which comprise ~1% of the total community, may include even $>10^5$ cells per g and the population of this size may not be seen in the T-RFLP analysis. Hence, PCR is affecting significantly to the results. There may also occur errors in the PCR reaction and this may lead alterations in the sequences. Furthermore, when the domain specific universal primers are used, it has been showed that some templates may replicate more effectively than others (Polz and Cavanaugh, 1998). In other words, although the concentration of two templates would be the same, the reaction might favour the other template more and create a greater amount of amplicons of it.

When analyzing community structure of certain microbial domain in the sample by using the 16S rRNA genes as fingerprints, the primers used in the PCR should be both specific and universal for the domain (Baker et al., 2003). It is important to realize the fact that although the number of sequences in the databases has increased greatly in recent years, they represent only a fraction of the entire diversity of prokaryotes. For example, bacterial sequences in the RDP (Ribosomal Database Project, Cole et al., 2007) are estimated to represent only 1% of the total number of the bacterial species (Curtis et al., 2002; Forney et al., 2004). Hence, the design of the universal primer is based only on a fraction of the all sequences and thereby it is impossible to design specific and universal primers. In addition, the design of the universal primers is complicated by the fact that although the certain sites of the 16S rRNA genes are highly conserved, they may still have diversity between species. To enhance the universality of the primers, degenerate primers, which have more than one nucleotide in certain site or inosine containing primers whit ability to pair all the bases in the DNA, can be used (Forney et al., 2004).

Archaeal 16S rRNA genes were amplified using primer set Ar109f-Ar912rt for sequencing and T-RFLP. For T-RFLP reverse primer was labelled by 6-carboxyfluorescein (FAM). The primer set was enhanced from earlier primers (Lueders and Friedrich, 2002). The set was shown to allow a more efficient discrimination between archaeal target and bacterial non-target templates. However, SEQ5 aligned best with a bacterium sequence, which indicated unspecific binding of the primers. Nevertheless, the best possible primer set available was used and most of the T-RFs should represent archaeal population in the present study.

Although, nucleic acid-based methods bias the information about the community structure, they bias it less than culture dependent methods (Hugenholtz, 2002). All the above mentioned problems are needed to take in to consideration before making any conclusions about phylotypes present in the samples. To get valid information about the archaeal community structure in the biogas reactor, several different methods should be used. Furthermore, molecular methods in combination with microbial cultures can provide realistic information (for example see Kisand and Wikner, 2003). Unfortunately, the culturing of anaerobic archaea is complex and laborious (for example see Cuzin et al., 2001 and Kotelnikova et al., 1998).

As a conclusion, knowledge concerning archaeal community structure in the hydrolytic reactors of two-stage anaerobic digestion of grass silage was achieved by T-RFLP analysis and comparative sequence analysis. According to the PCR of 16S rRNA genes, archaeal species emerged into the biogas reactor not before day 10. According to the T-RFLP analysis, community structure was different in the leachate, in the solid and in the loosely attached fractions. The diversity of the archaeal community was also increasing during the operational day 10 to 17. According the PCR, the archaeal DNA was absent on day 49. Closest cultured relatives of the sequences retrieved from the solid fraction and from the loosely attached fraction on day 17 were *Methanobacterium curvum* (SEQ1), *Methanobacterium subterraneum* (SEQ2), *Methanobacterium formicum* (SEQ3, SEQ4), *Methanobacterium beijingense* (SEQ5, SEQ7) and *Methanosarcina barkeri* (SEQ6).

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Appendices: The sequences

SEQ1
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1    ACGGCTCAGT AACACGTGGA TAACCTACCC TTAGGACTGG GATAACCCTG GGAAACTGGG
61   GACAATACCA GATACATGGA GATGCCTGGA ATGGTTCTCC ACTTAAATGT TCCGACGCCT
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721  GGAAGCTGTT AAGTCCACCG CCTGGGAAGT ACGGTCGCAA GACTGAAACT TAAAGGAATT
781  GGCGGGGGAG CAC
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