The change in bacterial community structure during the anaerobic digestion of medium density fiberboard as determined by T-RFLP

By

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Abstract

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Jyeyoun Kim

Medium Density Fiberboard (MDF) is a material commonly used to construct furniture and is an engineered product made up of wood particles bonded with the adhesive resin, urea-formaldehyde. In the presence of the resin, MDF is not recyclable; therefore, it is normally burned or thrown in a landfill. The compound is easily hydrolyzed into urea and formaldehyde that potentially leach toxins. However, it can be decontaminated with microorganisms under the anaerobic condition, converting the components into biogas such as methane, carbon dioxide, and renewable energy sources. This study aims to characterize the microbial community changes during the anaerobic degradation of MDF. Specifically, observing any decreased or increased key taxonomic groups of bacteria through out the period when MDF was being digested under anaerobic conditions, producing gases including methane and carbon dioxide. DNA extraction of the first sample (sample A) was solely from diluted cow manure, then additional samples (Sample B-D) were taken from an inoculum consisting of MDF, cow manure, and water. According to the general total gas production trend, time specific samples were selected for DNA extraction from samples B-D. DNA extraction of each sample was replicated to limit random bias. Then the PCR-amplified 16S rRNA target genes that belonged to different taxonomic groups were examined to study bacterial community structure and dynamics by using terminal restriction fragment length polymorphism (T-RFLP). Finally, Phylogenetic Assignment Tool (PAT) was used to interpret the peaks from the profile to identify the groups of bacteria community. As expected, there were changes in structure of microbial communities and it was characterized that there were close relationships between sample B and sample D that were located in the high rate of gas production.

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CHAPTER 1 **INTRODUCTION**

1.1 Fiberboard and urea formaldehyde adhesive resins

Landfills are common repositories for solid waste from both municipal and industrial activities (US EPA, 2011). A number of components of solid waste such as paper, food scraps, plastics, glass, metals, rubber, leather, textiles, wood, and yard trimmings can be identified from the landfill (US EPA, 2011). Among all these solid wastes, this study focuses on fiberboard wood.

Fiberboard is a type of engineered wood product made out of wood fibers, often from mill waste. There are different types of fiberboard that depend on the density of the wood fibers. The types of fiberboard, in order of increasing density, are particleboard, Medium Density Fiberboard (MDF), and hardboard. MDF is widely used in the furniture industry (FAO, 1958). MDF is preservative-treated wood and wood particles bonded with adhesive resins (urea formaldehyde) to produce a strong, uniform building material.

In the presence of urea-formaldehyde, MDF is not readily recyclable. Therefore, it is either burned or ends up in the landfill where it occupies space and potentially leaches toxins (Dunky, 1998; Padgett, 2009). Urea-formaldehyde resin is most widely used for the manufacture of MDF and easily hydrolyzes into its constituent compounds, urea and formaldehyde, under warm, humid and slightly acidic conditions (Park & Jeong, 2011). Microorganisms rapidly mineralize urea into ammonia, which is one of the most toxic factors of landfill leachate (Bernard et al., 1997). The other compound of the adhesive resin is formaldehyde that is also known to be a human carcinogen (Cagliano et al., 2004). Formaldehyde can pollute the environment through its presence in

groundwater. It can also escape to the air by volatilization which has been cited as the largest contributor to cumulative cancer risk from typical residential air (Sax et al., 2006). Therefore, the presence of the MDF bonded with adhesive resin in the landfill is an environmental concern that potentially leaches toxins into the broader environment.

1.2 Decontamination of urea formaldehyde bonded MDF by anaerobic digestion

Urea and formaldehyde are the constituent components that have been hydrolyzed under warm, and slightly acidic water conditions from urea-formaldehyde. In the presence of microbial inoculum, under anaerobic conditions, the urea will be quickly mineralized to carbon dioxide and ammonia or ammonium depending up on pH level (Borja et al., 1996b and Garrido 2000). The other hydrolysis product, formaldehyde will be transformed into formate and methanol under anaerobic conditions (Gonzalez-Gil et al, 2002). Both formate and methanol can then be further converted into methane and carbon dioxide by degradation of methanogens (Ferry 2010).

The most commonly used treatment for industrial, agricultural, and municipal wastes is anaerobic digestion (Mata-Alvarez et al., 2000). It is very efficient treatment because of the low operational costs and the potential acclimation ability of anaerobic microorganisms to the exposure of toxicants (Alexandersson, 1982). It also involves the biodegradation and stabilization of organic materials under anaerobic conditions by microbial organisms and leads to formation of biogas such as methane, carbon dioxide, and renewable energy sources (Kelleher et al., 2000). Also there are two stages of anaerobic treatment of complex organic wastes, which are divided into acid fermentation and methane fermentation. The acid fermentation stage is the first stage where simply

organic material is converted to organic acid, alcohols and new bacterial cells. The second stage, called methane fermentation, involves several different species of strictly anaerobic bacteria converting the hydrolysis products to gases, mainly methane and carbon dioxide, which are first generation biofuels (Kellehar et al., 2002; Mohseni et al., 2012). Anaerobic digestion offers some major advantages over environment as it has low sludge production, requires low energy, and can result in possible energy recovery. (Ghosh and Pohland, 1974; van Staikenburg, 1997). As part of other solid wastes that degrade under the anaerobic conditions in the landfill, the degradation of resin in fiberboard helps make the landfills the largest single source of anthropogenically produced methane (Thompson et al., 2009)**.**

This study assessed the anaerobic digestion process to decontaminate waste MDF, which produced commercially valuable methane and carbon dioxide in the process. Specifically, I tested if the efficiency of this treatment could be enhanced through enrichment with particularly efficient anaerobic microorganisms.

1.3 Microorganisms leading to anaerobic degradation

Bacteria are the most common wood degraders under anaerobic conditions (Bjordal et. al., 1999). As various wastes are placed to landfills, one can also expect bacteria community structure and activity to vary as well. Despite this variation, bacteria communities can be divided into two general groups: anaerobic and aerobic communities. Four well-known general functional anaerobic bacteria groups are cellulolytic, acidogenic, acetogenic and methanogenic groups (Semrau, 2010). To obtain anaerobic

microorganisms and make the inoculum for this study, cattle manure was collected, as it is a readily accessible source and it contains a portion of organic solids, which are fats, carbohydrates, proteins and other nutrients that are available as food and energy for the growth and reproduction of anaerobic bacteria (Fulhage et al, 1993). Uncalculated numbers of different bacterial species are expected to be present in manure that will utilize different substrates, produce different fermentation products, and have different growth optima. Therefore, a bacteria community change can be well expected during the anaerobic digestion of MDF.

1.4 Potential toxicity of urea formaldehyde to anaerobic microorganisms

1.4.1 ***Ammonium toxicity***

Ammonium is produced during the hydrolysis stage of the degradation of proteinaceous organic materials, which in this study is urea, at pH levels of 6.8-7.2. At the appropriate concentration (below 200mg/L;Liu and Sung 2003), the nitrogen source from the ammonium is an essential nutrient for many organisms in the process of anaerobic digestion (Andualem Abate Woldeyohannis, 2012 and Liu et al. 2002). But high concentrations of free ammonia can be toxic to the microorganisms, and it has been suggested to be the key cause of inhibition of anaerobic digestion. Because it is freely membrane-permeable where the hydrophobic ammonia molecule can diffuse passively into the cell, producing proton imbalance, and/or potassium deficiency (Kroeker et al., 1979; de Baere et al., 1984 and Chen et al. 2008). As the pH increases, ammonium changes its form into more free ammonia (Appels et al., 2008; Walker et al., 2011; Lei, et

al., 2007). The levels of free ammonium can be controlled by maintaining the pH within the process of digestion at the optimal range (i.e. below 7.0) for methanogenesis. If needed, addition of dilute acid can maintain the pH level in an optimal range throughout the digestion process. The microorganism can become evolve resistance to the high levels of free ammonia by exposing them to slowly increasing concentrations over time (Melbinger and Donnellon 1971;Hashimoto, 1986; Angelidaki & Ahring, 1993). The difference in substrates, environmental conditions such as temperature, and pH level, and acclimation periods in inocula attribute to the significantly different inhibiting ammonia concentration (van Velsen et al., 1979; de Baere et al., 1984; Hashimoto, 1986; Angelidaki and Ahring, 1994). Among the four types of anaerobic microorganism, the methanogens are the least tolerant and most likely to cease growth due to ammonia inhibition (Kayhanian, 1994). As ammonia concentrations are increased, acidogenic populations were less affected than the methanogenic population (Koster & Lettinga, 1988).

1.4.2 ***Formaldehyde toxicity***

Allen et al. (1980) proposed that urea formaldehyde resin might contain as much as 36% free formaldehyde hydrate. Omil et al (1999) mentioned that even though there is no information about the inhibition pattern, formaldehyde has been reported to be the strong inhibitor of all microorganisms involved in anaerobic degradation. Bhattacharya et al. (1988) has summarized the first works carried out with formaldehyde under anaerobic conditions:

Chou (1977) reported that among all saturated aldehydes present in petrochemical

wastewaters, formaldehyde is the most toxic to anaerobic bacteria, exerting significant inhibitory effects at concentrations higher than 6.67 mM (200 mg l^{-1}); however, Hovious et al. (1973) found that toxic effects can be detected at much lower formaldehyde concentrations such as 1.67 mM (50 mg l^{-1}). This last conclusion was confirmed by the similar results from Pearson et al (1980). (p.531)

Recent studies have also concluded that formaldehyde is a carcinogen and a mutagen that can also be toxic to microorganisms (Gonzalez-Gil et al. 2002; Gómez et al. 2010).

Microbial populations can be acclimated over time to tolerate relatively high concentrations of both ammonia and formaldehyde if the addition is incremental (Gonzalez-Gil 2002 and Parking et al. 1982)

1.5 PCR-based bacterial community fingerprinting techniques

Analysis of the 16S rRNA gene can be used to identify species and assess the bacterial community structure and dynamics. The DNA analysis techniques such as Denaturing Gradient Gel Electrophoresis (DGGE)/Temperature Gradient Gel Electrophoresis (TGGE), Restriction Fragment Length Polymorphism (RFLP)/Amplified Ribosomal DNA Restriction Analysis (ARDRA), Single Strand Conformation Polymorphism (SSCP) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) have been applied over the last decade to analyze the microbial community diversity in various environments (Muyzer et al., 1993;Lee et al., 1996; Liu et al., 1997: Torsvik and Ovreas, 2002). T-RFLP is a molecular biology technique for producing profiles of microbial communities based on the placement of a restriction site closest to a labeled end of an amplified gene. The initial steps of DNA isolation, PCR amplification

and restriction of T-RFLP are similar to the initial steps of RFLP/ARDRA except one PCR primer is labeled with a fluorescent dye such as 6-FAM(phosphoramidite fluorochrome 5-carboxyfluorescein) (Liu et al.,1997). Many studies have used T-RFLP analysis to characterize complex microbial communities in the environment because the use of capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection made such analysis automated and sensitive (Clement 1998). Osborne et al. (2000) mentioned that T-RFLP analysis is a highly reproducible and robust technique that yields high quality community fingerprinting which is a method that can be used to quickly profile the diversity of a microbial community. Moeseneder et a. (1999) compared T-RFLP fingerprinting to DGGE by using these techniques to compare complex marine bacterial community samples collected at different sites in the Mediterranean Sea and concluded that T-RFLP had higher resolution than DGGE. Kaplan et al. (2001) used T-RFLP to successfully characterize the changes in fecal bacterial community structure caused by the probiotic dietary supplements. T-RFLP has the ability to detect not only spatial but also temporal changes in bacterial community structure by comparing Terminal Restriction Fragment (TRF) patterns taken at different times. Liu et al. (1997) used T-RFLP to reveal high species diversity in activated sludge, bioreactor sludge, aquifer sand, and termite guts and concluded that their results confirmed that T-RFLP is a powerful tool for analyzing complex bacterial communities and for rapid comparison of the community structure and diversity of different environments. Dunbar et al. (2000) indicated that T-RFLP is an excellent method to rapidly compare the similarity relationships between communities of microbial diversity in soil samples from four southwestern Unites States and/or good detection sensitivity. Consequently, 16S rRNA

T-RFLP was rewarded as the most appropriate method for studying the change in bacterial community structure during the anaerobic digestion of medium density fiberboard.

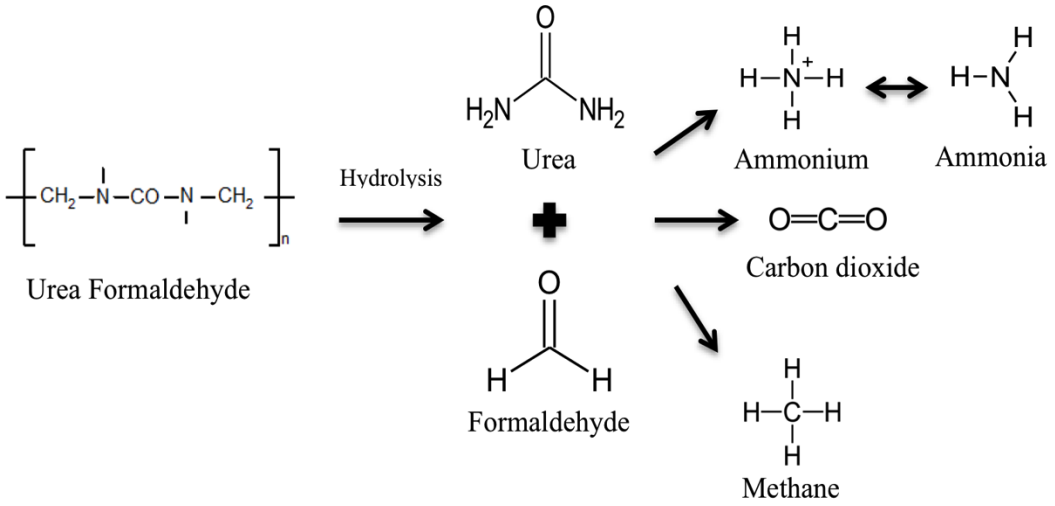
1.6 Phylogenetic analysis of T-RFLP profiles by phylogenetic assignment tool (PAT)

DNA fingerprint methods, such as T-RFLP, can be used to obtain estimates of the diversity of a microbial community. Although the generation of TRF profiles is an excellent tool to compare richness of species in different communities or treatments, they do not assess directly the composition of the community.

Therefore, for further analysis, the software Phylogenetic Assignment Tool (PAT) can be used which compromises a rapid, automated approach for phylogenetic analysis of TRFs. PAT is used to indicate complex community profiles where there are intersections of many species in an individual TRF that is represented by each peak from T-RFLP profiles (Kent *et al.* 2003). With this tool, the task of phylogenetic assignments from TRF files generated by multiple digests is done automatically, making such analyses readily accessible for analysis of complex communities. (He, 2010)

The objectives of this study were to generate DNA fingerprint profiles for bacteria communities in different stages of anaerobic digestion of medium density fiberboard. This was done in an effort to compare the profiles and identify any increased and decreased bacterial groups during anaerobic digestion of medium density fiberboard.

Figure 1 Pathways from urea formaldehyde to methane and carbon dioxide under anaerobic conditions



*Model based on information derived from; Effects of acid hydrolysis on microstructure of cured urea formaldehyde resins using atomic force microscopy (Park and Jeong 2011), Estimation of the hazard of landfills through toxicity testing of leachates: 2. Comparison of physico-chemical characteristics of landfill leachates with their toxicity determined with a battery of tests (Bernard et al., 1997), and The chemical biology of methanogenesis (Ferry 2010)

CHAPTER 2 **METHODS**

2.1 Sample collection

According to the general trend of total gas production from the anaerobic degradation of MDF (Figure 2) (Lee, 2014), four samples were picked for total DNA isolation. Sample A was a diluted fresh cattle manure (1:1 ratio of dilution) pooled from Noel Shore Maitland Hants Country NS Farm and it was expected to contain the most various numbers of different microbial communities. Sample B was an inoculum containing 400ml diluted cow manure, 10g MDF, and 500ml water after 12 days. It was selected on that day because it represented the expected time of first of gas production and the distinct change in structure of microbial communities was also expected at this point. Sample C was the same inoculum after 34 days where the trend was again at a low rate of gas production. Sample D was the same one after 53 days, where the trend increased to show a high rate of total gas production. The inocula were incubated in a water bath at 37°C (under anaerobic condition) (Lee, 2013). In case of errors or mistakes while bench working, additional samples were taken and stored at -80°C to be used if needed.

2.2 DNA extraction

The samples were cleaned manure that has been washed with deionized water before any extraction. The manure and deionized water were mixed together then

centrifuged to discard the supernatant that is the washed water. Power Soil DNA isolation Kits (MO BIO Laboratory, Inc., Solana Beach, CA) were used to extract DNA from 0.25g of each sample. Experienced User Protocol, offered by MO BIO Laboratories, Inc. was followed. (see below) Except for sample A, the rest of the samples were high in water content; therefore I followed the protocol of treating the wet samples. The beads and solutions from the Powerbead tube were transferred to sterile microcentrifuge tubes. Then the wet sample was added into the Powerbead tube, followed by centrifugation for 30 seconds at 10,000xg. The supernatant was discarded, weighed the sample to 0.25g then the beads and solution were transferred back to the Powerbead tube and vortexed to mix. For sample A, the 0.25g of sample was added to the 2ml Bead solution directly and vortexed to be mixed. Sixty microliter of solution C1 containing SDS (sodium dodecyl sulfate) which aids cell lysis was added and then horizontally vortexed at maximum speed for 10 minutes. A centrifugation for 30 seconds at 10,000xg was followed, then the supernatant (about 400-500ul) was transferred to a clean 2ml collection tube. Two hundred fifty microliters of solution C2 containing a protein precipitation reagent was added and vortexed for 5 seconds and then the tube was incubated at 4°C for 5 minutes. The tube was centrifuged at 10, 000xg for 1 minute after incubation. The supernatant (up to 600ul) was transferred to a clean 2ml collection tube, then 200ul of solution C3 containing a protein precipitation reagent was added and vortexed for 5 seconds and then the tube was incubated at 4°C for 5 minutes. The tube was centrifuged at 10, 000xg for 1 minute after incubation. The supernatant (up to 750ul) was then mixed together with 1.2ml solution C4 (making DNA bind to silica in the presence of high salt concentration) in a fresh 2ml collection tube. To yield the desired DNA binding to silica, the mixture of

supernatant (about 675ul) and solution C4 was loaded onto a spin filter and centrifuged at 10, 000xg for 1 minute. The flow through was discarded and an additional 675ul of supernatant was added to the spin filter then centrifuged at 10, 000xg for 1 minute. The remaining supernatant was loaded onto the spin filter and centrifuged at 10, 000xg for 1 minute. The harvested DNA was further cleaned by loading 500ul solution C5, an ethanol based wash solution, and additional centrifugation at 10, 000xg for 30 seconds. After the flow through was discarded, the spin filter was centrifuged a second time for 1 minute at 10, 000xg. The spin filter is placed in a clean 2ml collection tube. One hundred microliter of solution C6, a sterile elution buffer was added to the center of the white filter membrane and the collected DNA was eluted from the filter membrane into the flow through (about 100ul DNA extraction) after a centrifuged at 10, 000xg for 30 second. The spin filter was discarded. DNA nucleic acid concentration and purity were measure by nanodrop. If needed, DNA extraction was tested by running 5µl of flow through in 1.0% agarose gel and observed if any bands were shown. Two replicated DNA extractions were pooled together to limit random bias although systematic biases always persist.

2.3 PCR of 16S rRNA Gene

The DNA for each sample was amplified with a pair of bacterial universal primers that amplify a 527 base pair (bp) region of the 16S rRNA(Zhang et al., 2009). Each 50µl reaction mixture consisted of 30.78ul dH_2O, 5µl of 10X ThermolPol Reaction Buffer (New England Biolabs LTd., Whitby, On), 0.2mM (4µl of 2.5mM) dNTP (dATP, dCTP, dGTP, dTTP) (New England Biolabs LTd., Whitby, On), 0.4mM (2µl of 10mM) 6-FAM-5'-BSF8/20 and BSR534/18 (bacterial universal primers) (Applied Biosystems, Foster

City, CA), and 0.008U/µl (0.4µl of 1U/µl) Taq DNA polymerase (New England Biolabs LTd., Whitby On), 0.3mg/ml (1.5µl of 10mg/ml) Bovine serum albumin (BSA), 3.0mM (2.17µl of 25mM) $MgCl_2$ then 1µl of DNA templates. DNA amplification was done in the Applied Biosystems 2720 Thermal Cycler with cycling conditions of 3 minutes of denaturation at 94°C; 35 cycles of 75 seconds at 94°C, 45 seconds at 53°C for annealing and 45 seconds at 72°C for extension; and a final cycle of extension was at 72°C for 10 minutes. Ten replicated PCR reactions were performed per sample then the DNA from 10 tubes were mixed and averaged together to minimize PCR-induced random bias. Qiaquick PCR Purification Kit (Qiagen Inc. Mississauga. CA) was used to purify PCR products.

2.4 Generation of TRF Profiles and Data Sets

After PCR purification, four restriction enzymes (RE) were selected from the Activity Chart For Restriction Enzyme according to the Phylogenetic Assignment Tool (PAT) (New England Biolabs LTd., Whitby On, CA). They were chosen to fit into the most optimal condition such as if they have 4 base pair recognition sites, the appropriate temperature and the % of buffer that they react with. Therefore the selected enzymes; HaeIII, MspI, AluI and HinP1I were used to obtain four separate TRF profiles for each sample. Approximately 320ng of purified PCR product was digested with 10U (20U for MspI) of each selected restriction enzyme in 50µl reaction, which also contained about 36-39ul of dH_2O and 5µl of CutSmart buffer (10X). Each 50µl reaction mixture was incubated overnight in 37°C. For each restriction digestion, three replicates were set up and the DNA from tree replicates was combined together to minimize the artificial biases.

Digested PCR products were then purified with the QIAquick Nucleotide Removal Kit (QIAGEN Inc., Mississauga, CA). Finally, 6-FAM labeled terminal restriction fragments were separated and recoded at University core DNA services, Faculty medicine, University of Calgary, Calgary, AB, Canada (Zhang, 2006).

Once the TRF profile had been generated, the TRFs were outputted using the GeneMarker V 1.4 software (Zhang, 2006). Each TRF profile defined the peak height at apex, the area under the peak in fluorescence units (FU), and fragment length in nucleotides (the apex position of each peak on a base pair scale relative to a DNA size ladder, GeneScan 500 LIZ Size Standard, Applied Biosystems, Foster City, CA). The area of any one peak was measured by reflecting the amount of fluorescence detected under that peak, and the amount of the areas of all peaks between 50nt and 500nt (nucleotides) in one profile is the total area for that profile (Zhang, 2006). It operated to analyze the data to compare fragments obtained from the profiles to the fragment sizes predicted from known 16S rRNA gene sequences.

The presence of small peaks that result as a consequence of noise or the amount of DNA could have brought the possibility of inadvertently influencing the comparative analysis of the profiles (Osborne, 2005). Therefore, all TRF profiles were standardized by the application of the constant percentage threshold method. The TRFs that contributed less than this constant percentage threshold were removed before analyzing relationships between the profiles in the data set. Sait et al (2003) applied this method and showed that at a threshold of 5%, the effect of area on the number of TRFs per profile was minimal, while still leaving sufficient TRFs to analyze the microbial community.

2.5 Comparison of TRF Profiles

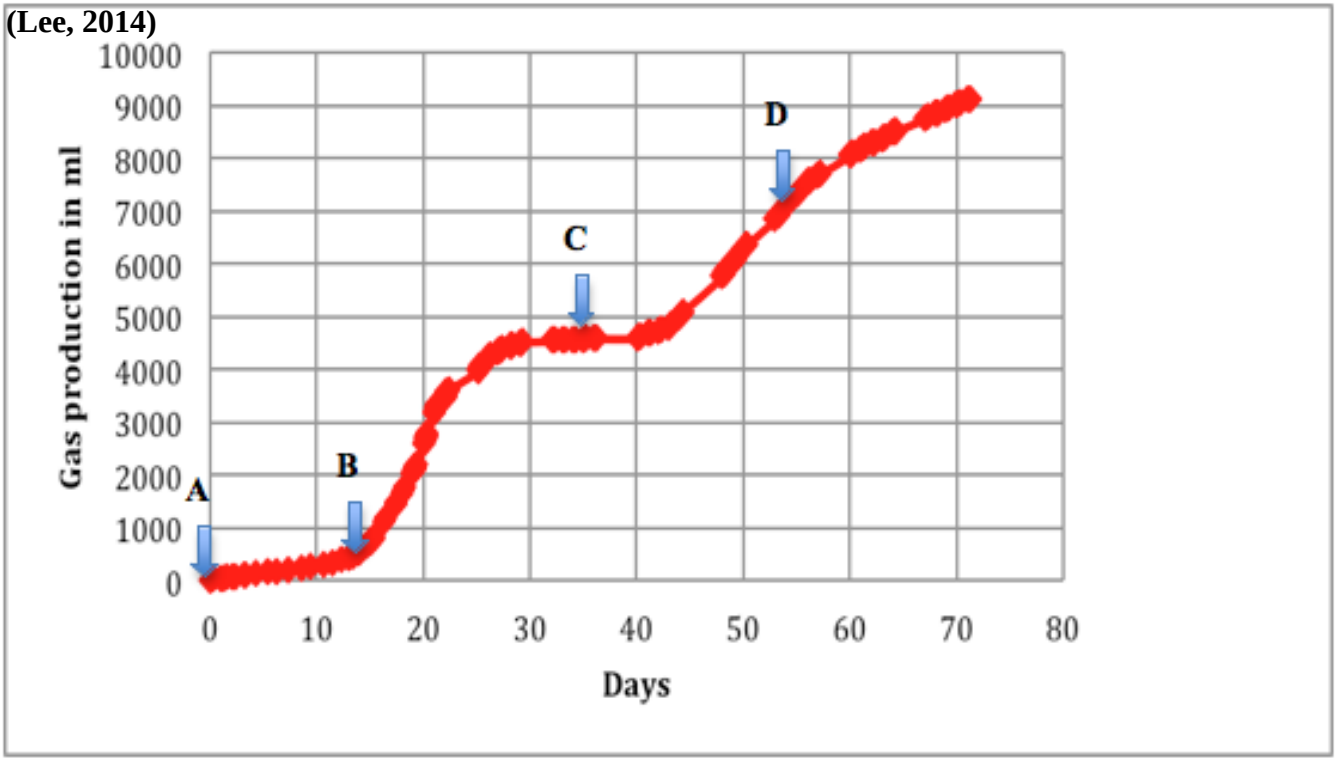
Followed Zhang's (2006) method for comparison of TRF profiles: Among the standardized TRF profiles within a data set, the TRFs that have synonymous fragment sizes were binned together according to the function of Bin table report in the GeneMarker V01.4 software. The average of the sizes of all TRFs within a bin represented by the peak which was fixed within ±0.4bp and was found among all samples within a data set. The presence or absence of the binned peaks in the composite list for each sample was represented by a binary vector: present (1), or absence (0). The data set was transformed into a binary matrix whose rows represented binned peaks and columns were samples. Pdist in Matlab7.1 was used to calculate the similarity of binary TRF profiles by Jaccard coefficient (Jaccard, 1908). Then under the rule of unweighted average distance (UPGMA), agglomerative hierarchical clustering was carried out based on the Jaccard coefficients. Finally, the dendrograms were plotted based on the hierarchical binary cluster tree created by the linkage function.

2.6 Phylogenetic analysis of T-RFLP profiles by Phylogenetic Assignment Tool (PAT)

The Phylogenetic Assignment Tool (PAT) software can be used to analyze the peaks from the profile to identify the groups of bacterial communities (He, 2010). The standardized TRFLP data sets, including sizes and area generated by two restriction

enzymes (AluI and HinP1I) from all samples, were compiled into Microsoft Excel files separately by different enzymes. The PAT only used data from columns one, three, and five from the excel files contained six columns to analyze them. The columns analyzed were sample and fragment ID (column one), size (bp) (column three), and peak area (column five). The excel files were transferred into tab-delimited formatted text files before being uploaded to the PAT website: https://secure.limnology.wisc.edu/trflp/: Following the instruction from the website, the data was analyzed automatically. The phylogenetic assignments were organized at different taxonomic levels; some were classified into genera, and some were identified by family. The TRF profiles of samples were compared in pairs and differences of TRF intensity were acquired. Any major increases or decreases in peaks and their percentages of total area were detected. Then they were reorganized to distinguish what taxonomic groups were located in the major peaks. The taxonomic groups were categorized into uniform taxonomic levels, classes and sorted by abundance.

Figure 2 A general trend of total gas production from the anaerobic degradation of MDF (Lee, 2014)



CHAPTER 3 **RESULTS**

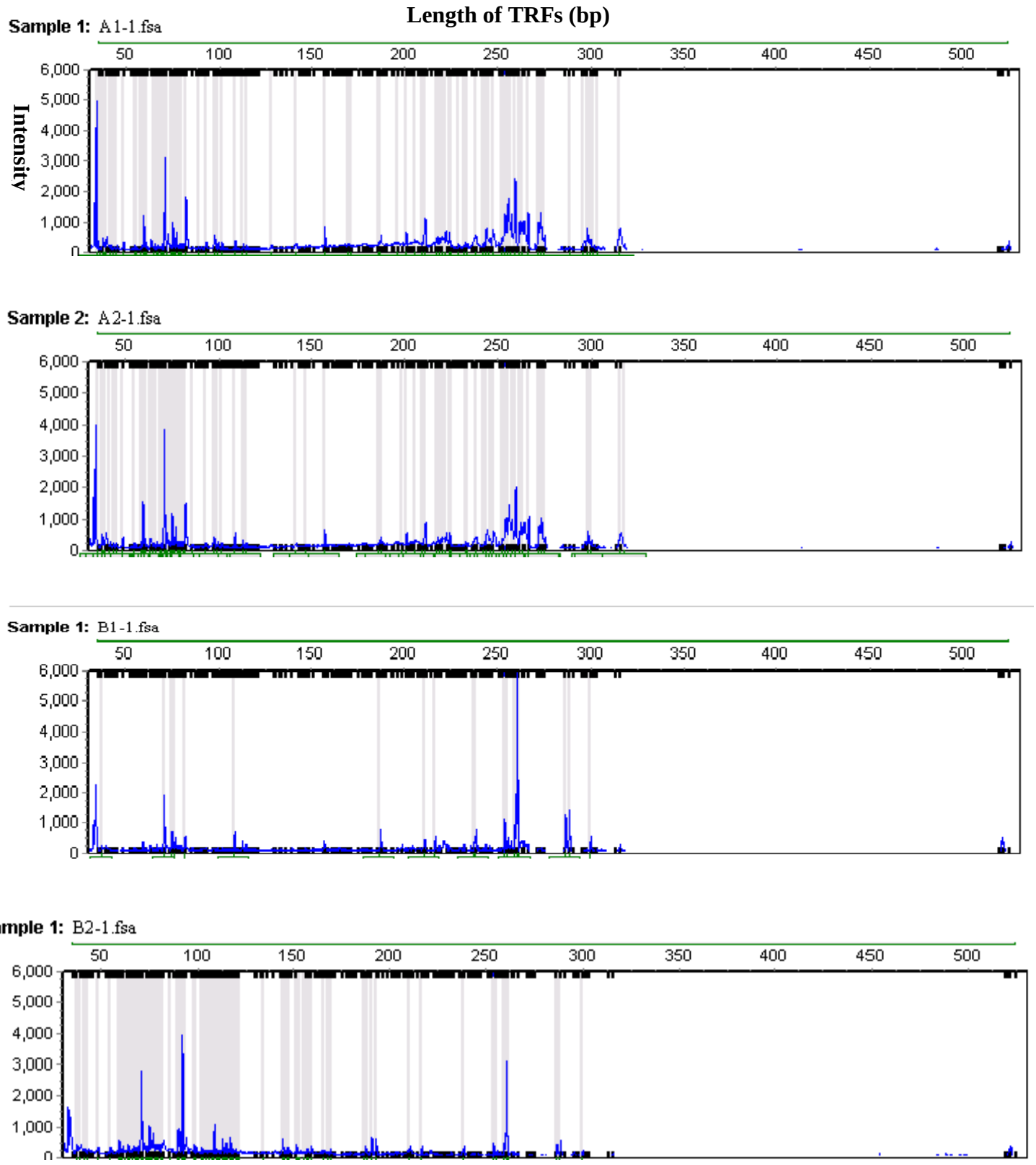
3.1 Generation of TRF profiles

DNA extracted from all four samples (A,B,C, and D) and their replicates showed a sharp band above 10kbp and smeared bands below 10kbp in 1.5% agarose gels. This indicates that the size of the most of the DNA fragments were bigger than 10kbp. This result qualified the extracted DNA as templates for amplifying 16S rRNA genes.

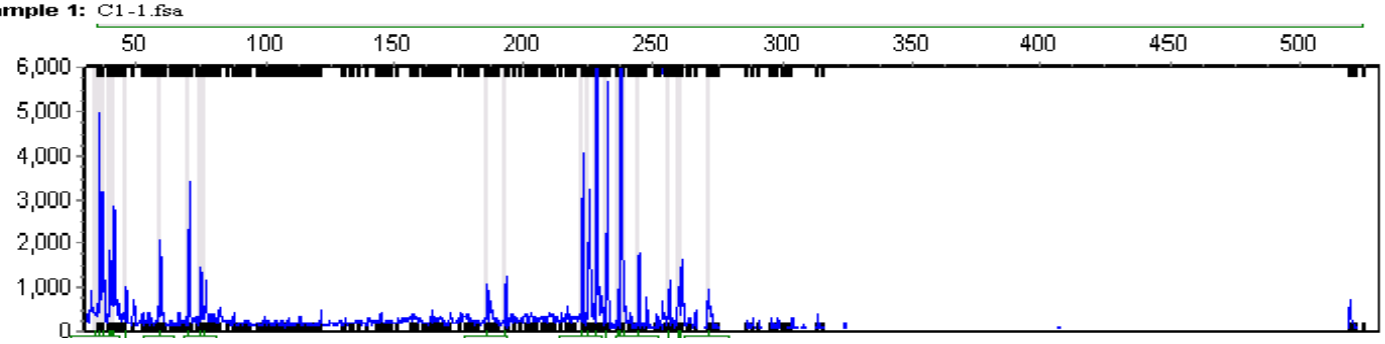
Most of the PCR products, amplified from the qualified DNA fragments of the four different samples, formed a sharp band around 500bp in 1.5% agarose gels, and some smeared bands located between 500bp and above it in 1.5% agarose gels. Therefore, most PCR products were considered as copies of 16S rRNA genes due to having the same size as estimated PCR products of 16S rRNA genes.

A pair of TRF profiles for one sample and its replicate were checked by manually scanning the electropherograms for all samples in each REs and they were mostly similar to one another. (Figure 3-6) These results ensure that the generating TRF profiles were analyzed successfully. Also, there were differences in the peak sizes when comparing different samples. For example, a peak from sample A that indicates 50bp increased or decreased as compared to sample B, and so on to, which indicated where a change of bacterial community structure occured. The data sets of each enzyme also showed different peaks indicating that applying more than one enzyme assisted to identify more bacterial communities.

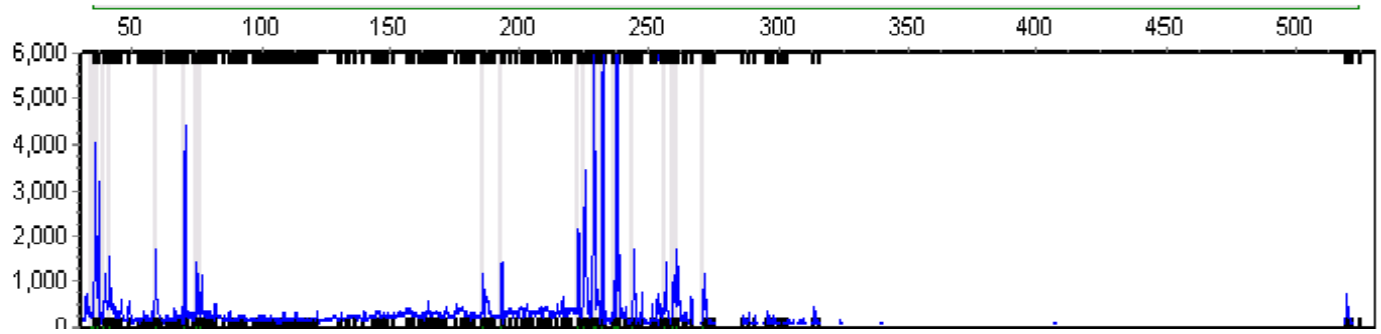
Figure 3 Electropherogram of HaeIII-derived TRF profiles from samples (A1&A2,B1&B2,C1&C2, and D1&D2)



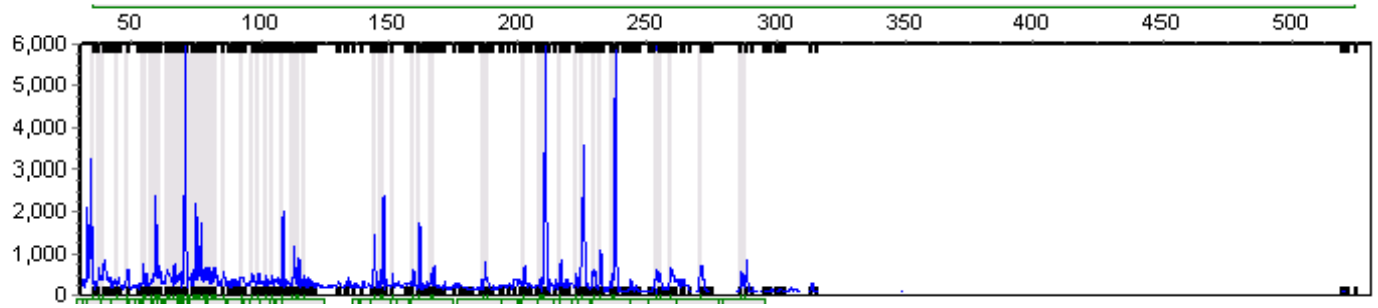
Sample 1: C1-1.fsa



Sample 2: C2-1.fsa



Sample 1: D1-1.fsa



Sample 2: D2-1.fsa

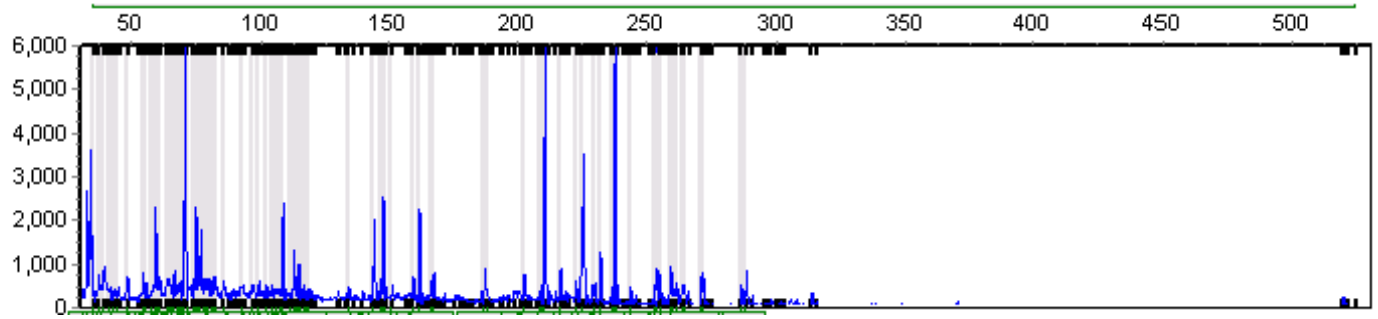
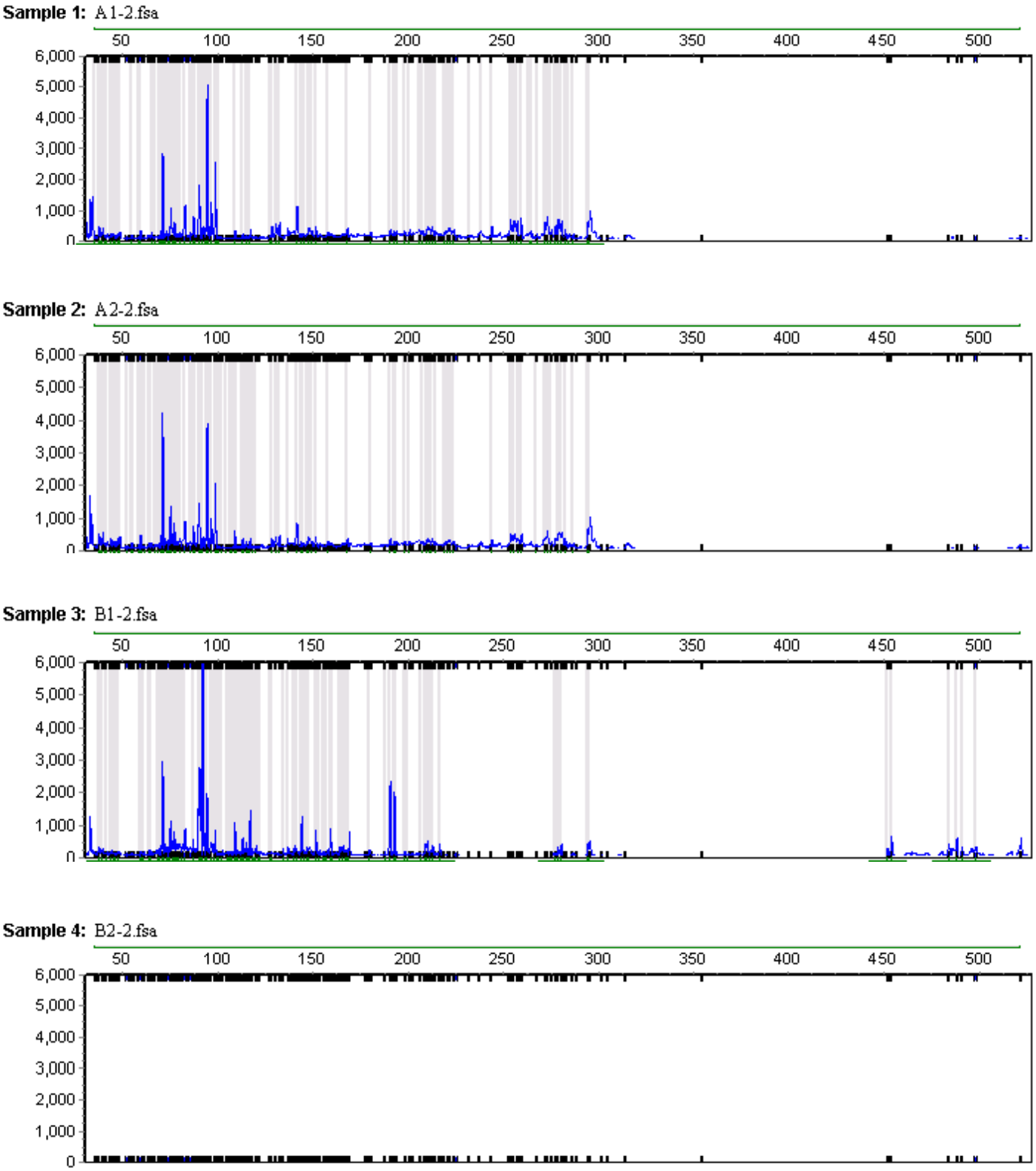
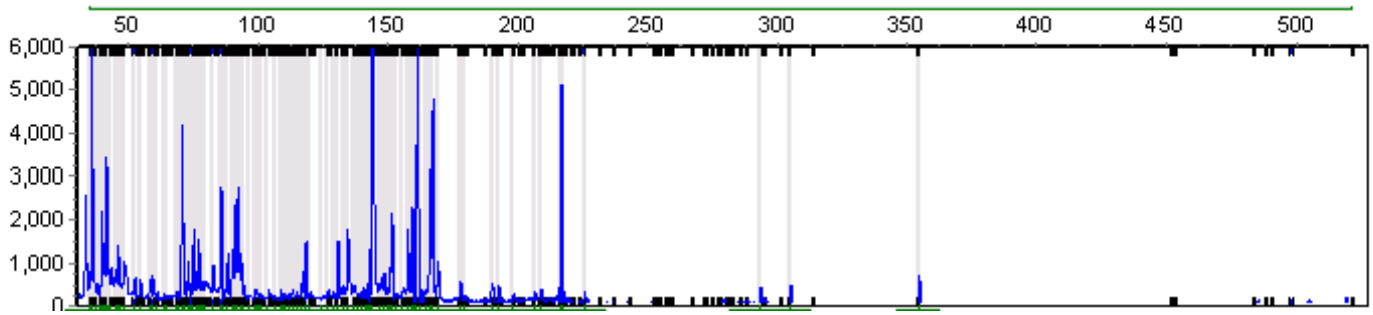


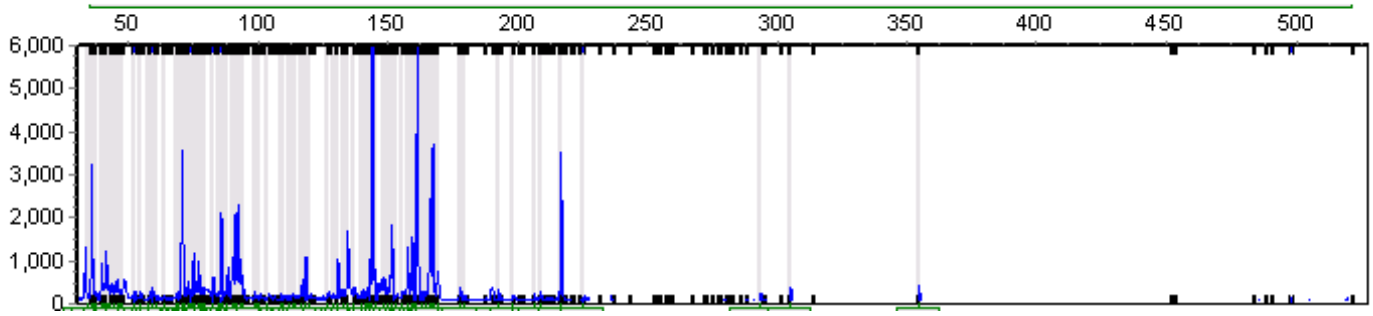
Figure 4 Electropherograms of MspI-derived TRF profile from samples (A1&A2,B1&B2,C1&C2, and D1&D2)



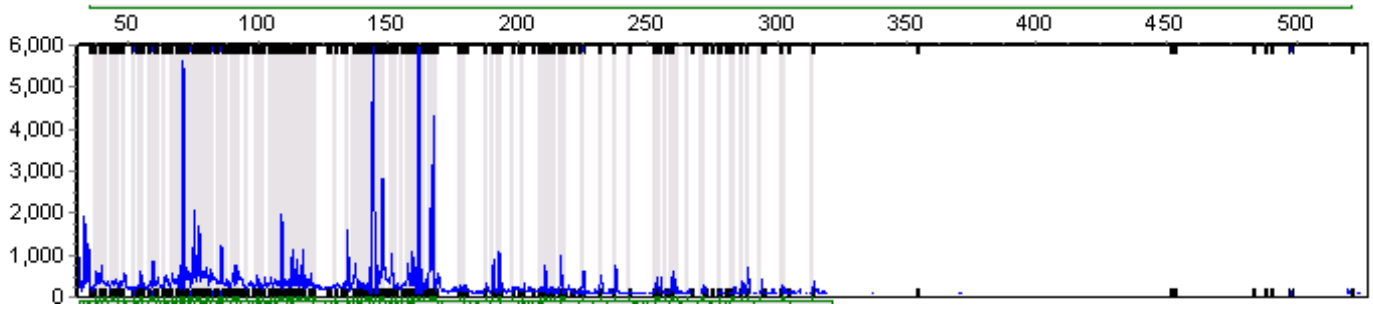
Sample 1: C1-2.fsa



Sample 2: C2-2.fsa



Sample 3: D1-2.fsa



Sample 4: D2-2.fsa

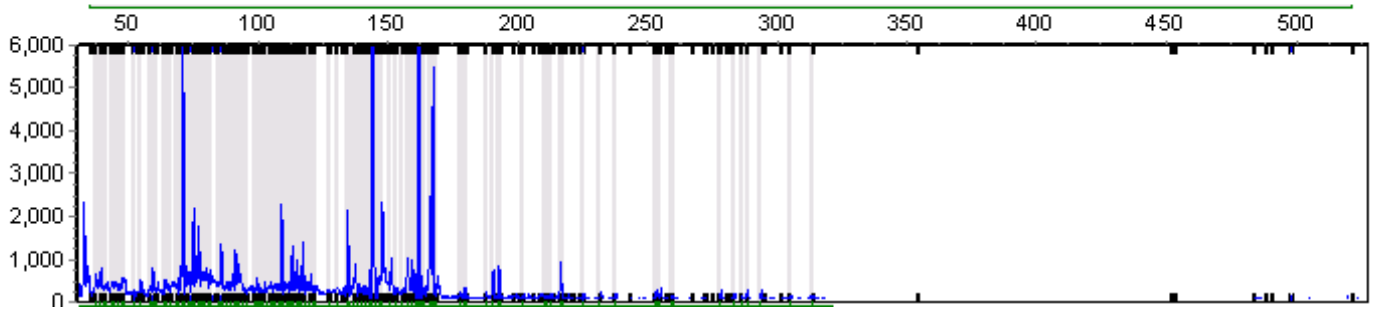
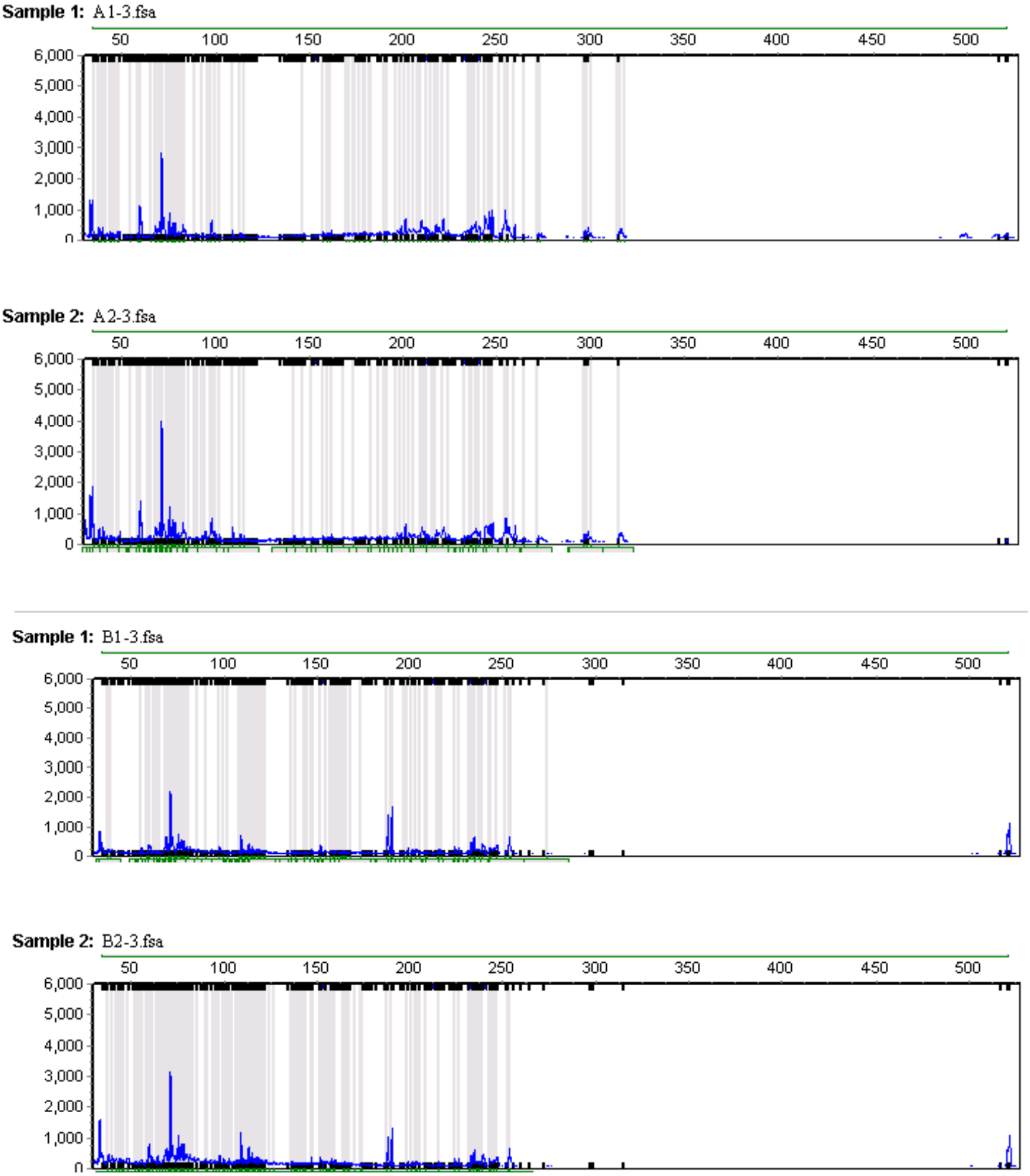
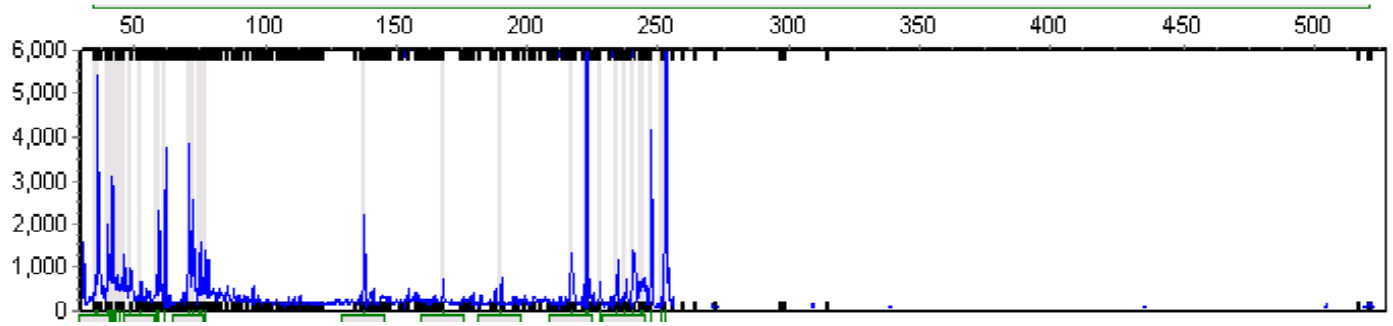


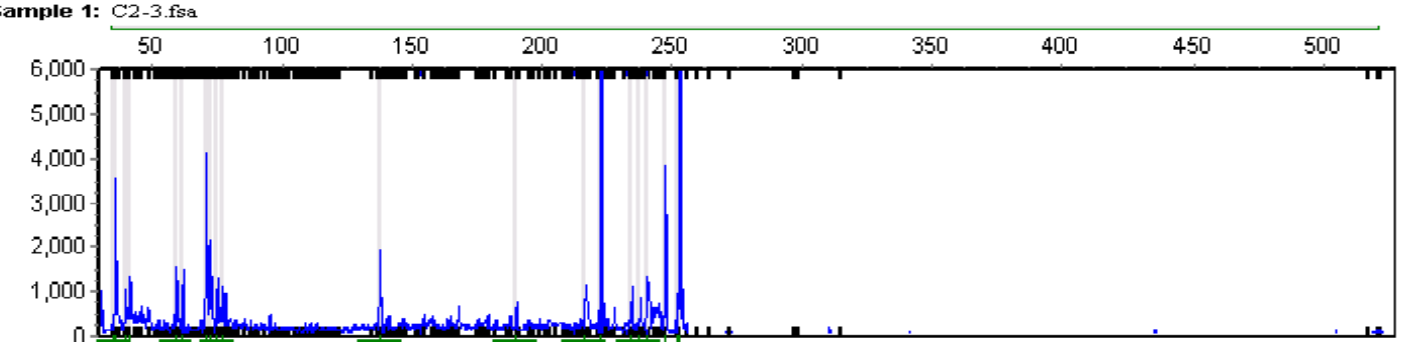
Figure 5 Electropherograms of AluI-derived TRF profile from samples (A1&A2,B1&B2,C1&C2, and D1&D2)



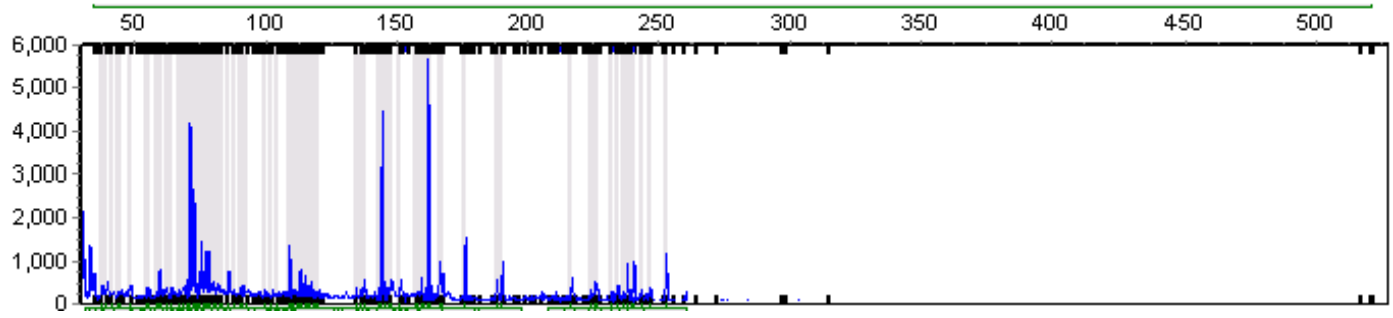
Sample 1: C1-3.fsa



Sample 1: C2-3.fsa



Sample 1: D1-3.fsa



Sample 2: D2-3.fsa

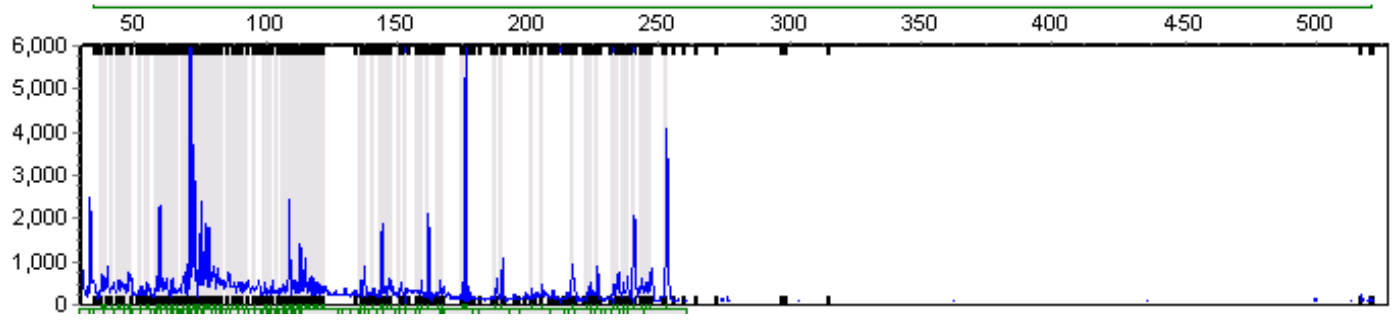
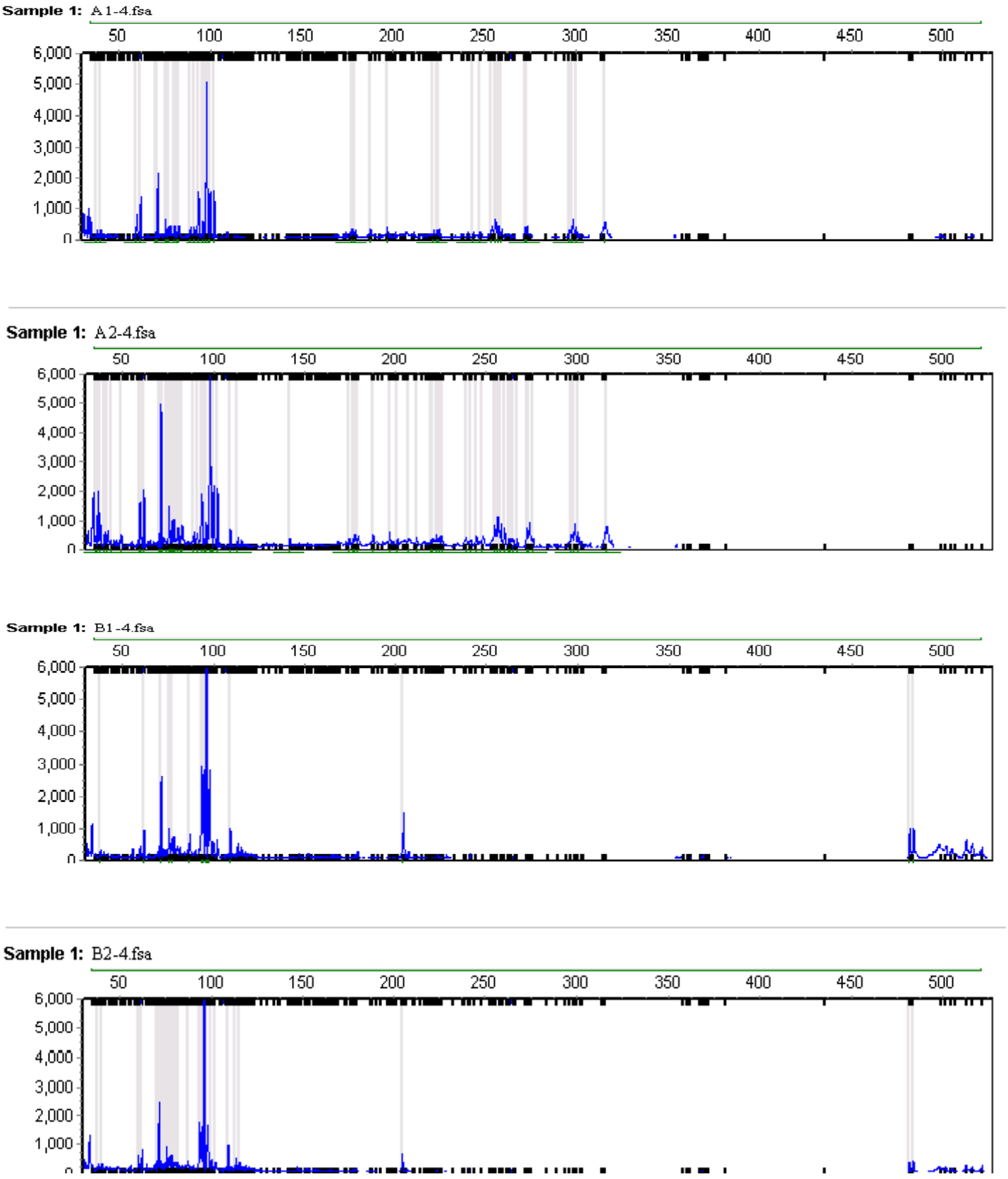
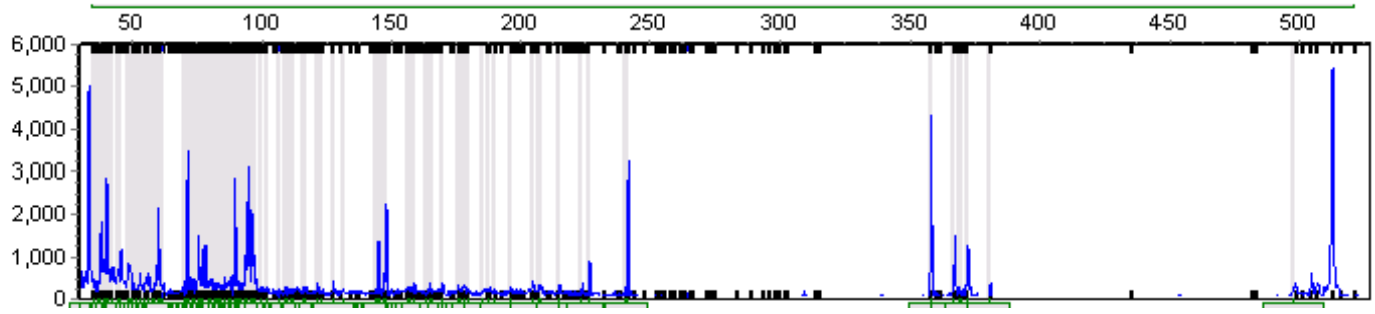


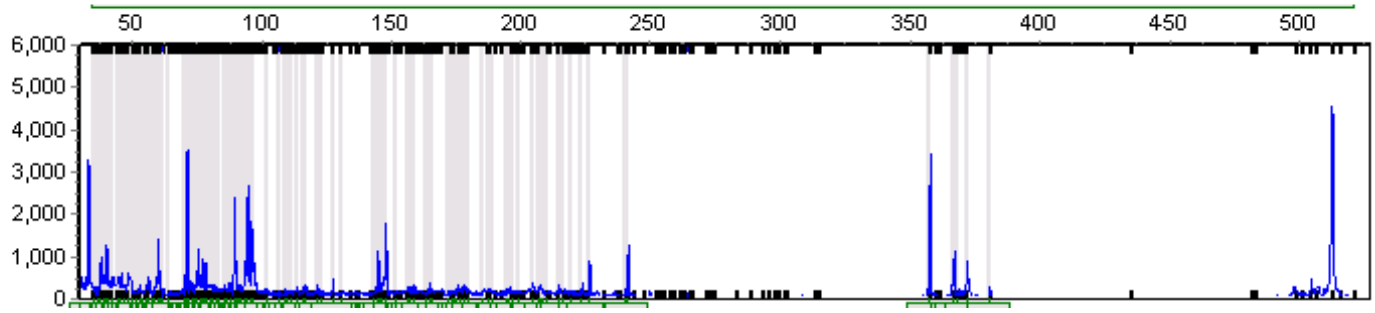
Figure 6 Electropherograms of HinP1I-derived TRF profile from samples (A1&A2,B1&B2,C1&C2, and D1&D2)



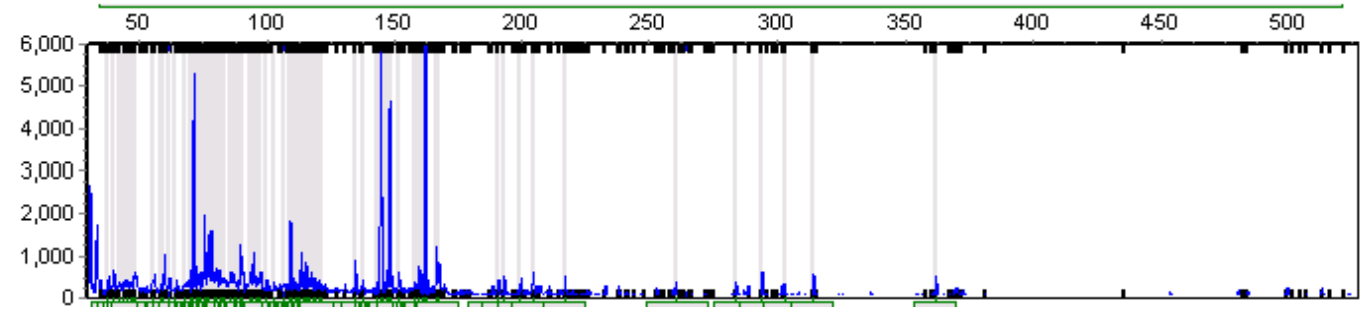
Sample 1: C1-4.fsa



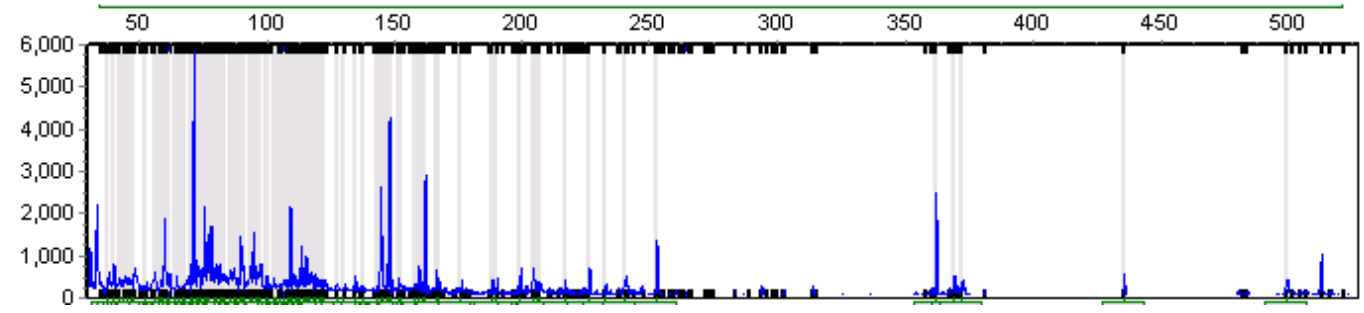
Sample 2: C2-4.fsa



Sample 1: D1-4.fsa



Sample 2: D2-4.fsa



3.2 Similarities between TRF profiles from different samples

The eight standardized TRF profiles (4 samples with 2 replicated) generated with each RE were compiled into one data set. Then all data sets were combined together to create a combined data. Dendrograms were constructed to display the similarities between TRF profiles of different samples (Figure 7-13). All dendrograms of the four REs (HaeIII, MspI, AluI, and HinP1I) had the ability to group most of the replicates in pairs. One hundred percent of replicates were paired by AluI and HinP1I and 75 percent of replicates were paired by MsPI and Hae III. The dendrogram for combined data also had low ability to group the replicates that might have been affected by enzymes MspI ad HaeIII. Therefore, another combined data analysis was formed excluding data from MspI and HaeIII. The more similar samples were, the more possible they were grouped together in one dendrogram. According to the final combined dendrogram (Figure 13), it represented that sample C and D were closely related than sample A and C but sample C seemed exclusively dissimilar to other samples.

Figure 7 Dendrogram structures of AluI-TRF profile comparisons four different samples (A, B, C, and D)

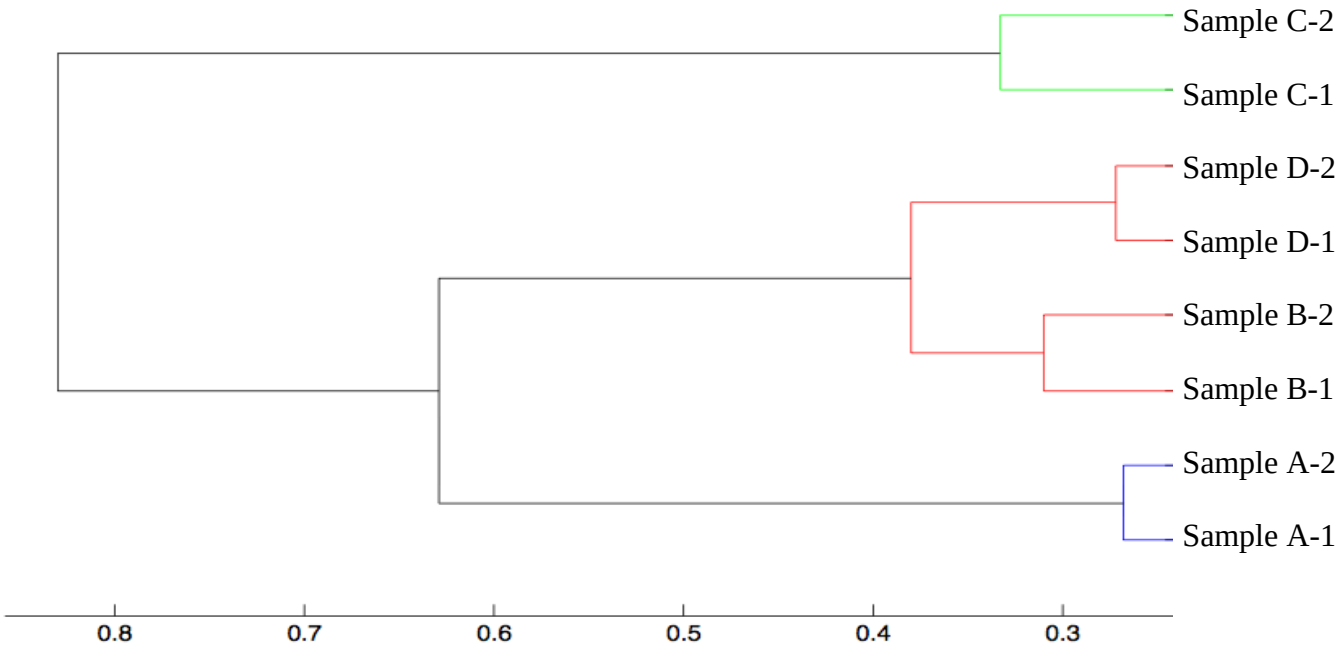


Figure 8 Dendrogram structures of HinP1I-TRF profile comparisons four different samples (A, B, C, and D)

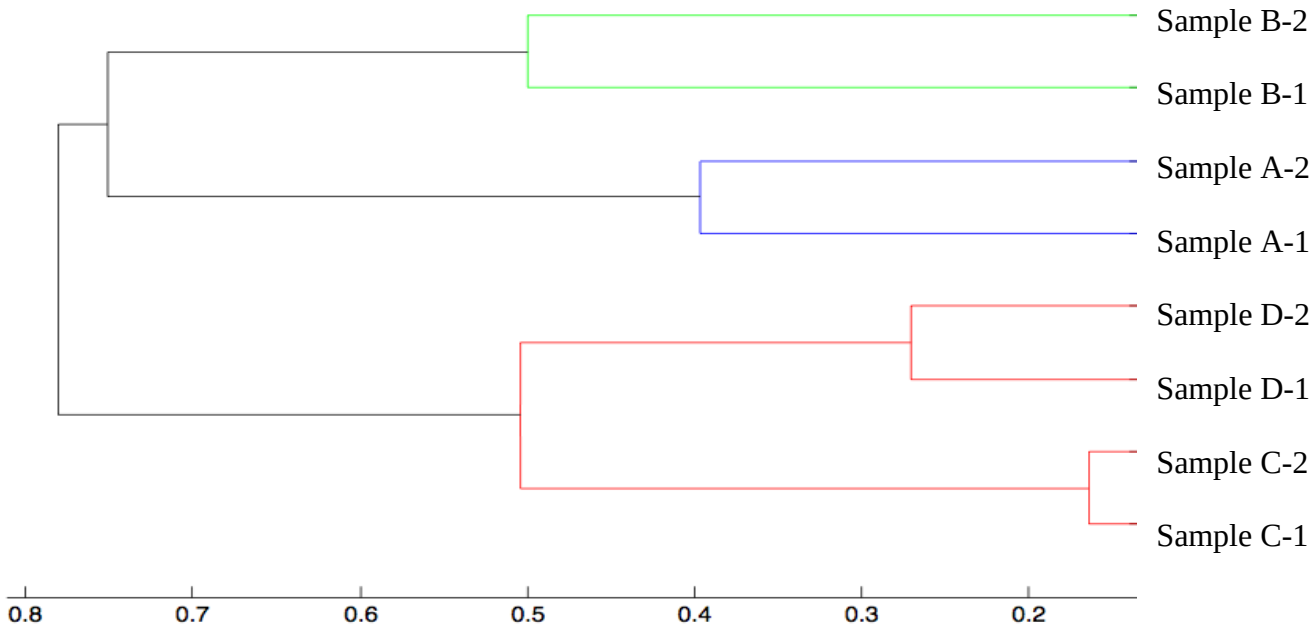


Figure 9 Dendrogram structures of HaeIII-TRF profile comparisons four different samples (A, B, C, and D)

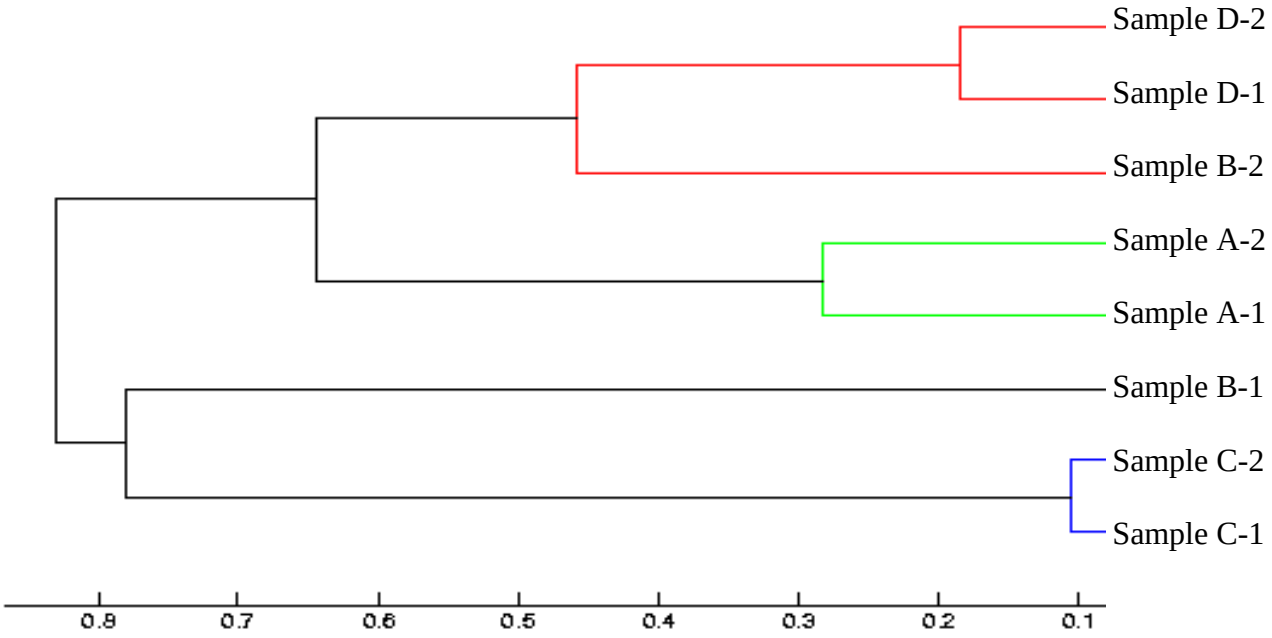


Figure 10 Dendrogram structures of MspI-TRF profile comparisons four different samples (A, B, C, and D)

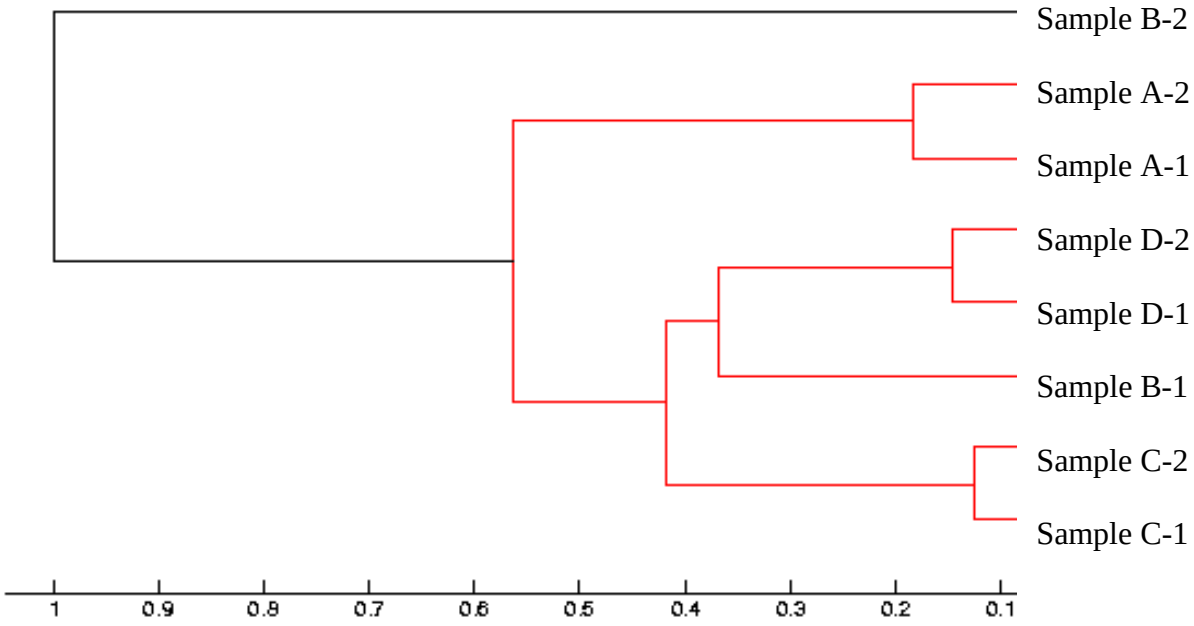


Figure 11 Dendrogram structures of Combined-TRF profile comparisons four different samples (A, B, C, and D)

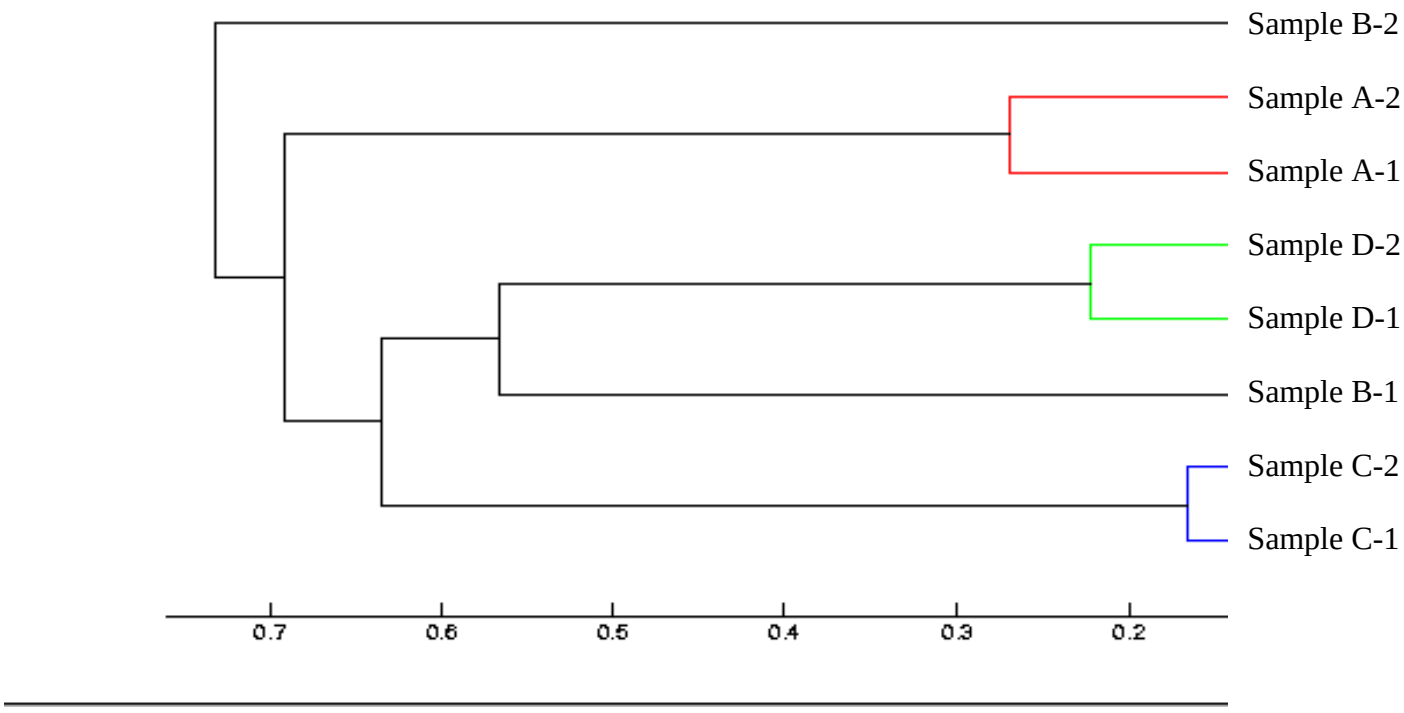


Figure 12 Dendrogram structures of Combined without MspI-TRF profile comparisons four different samples (A, B, C, and D)

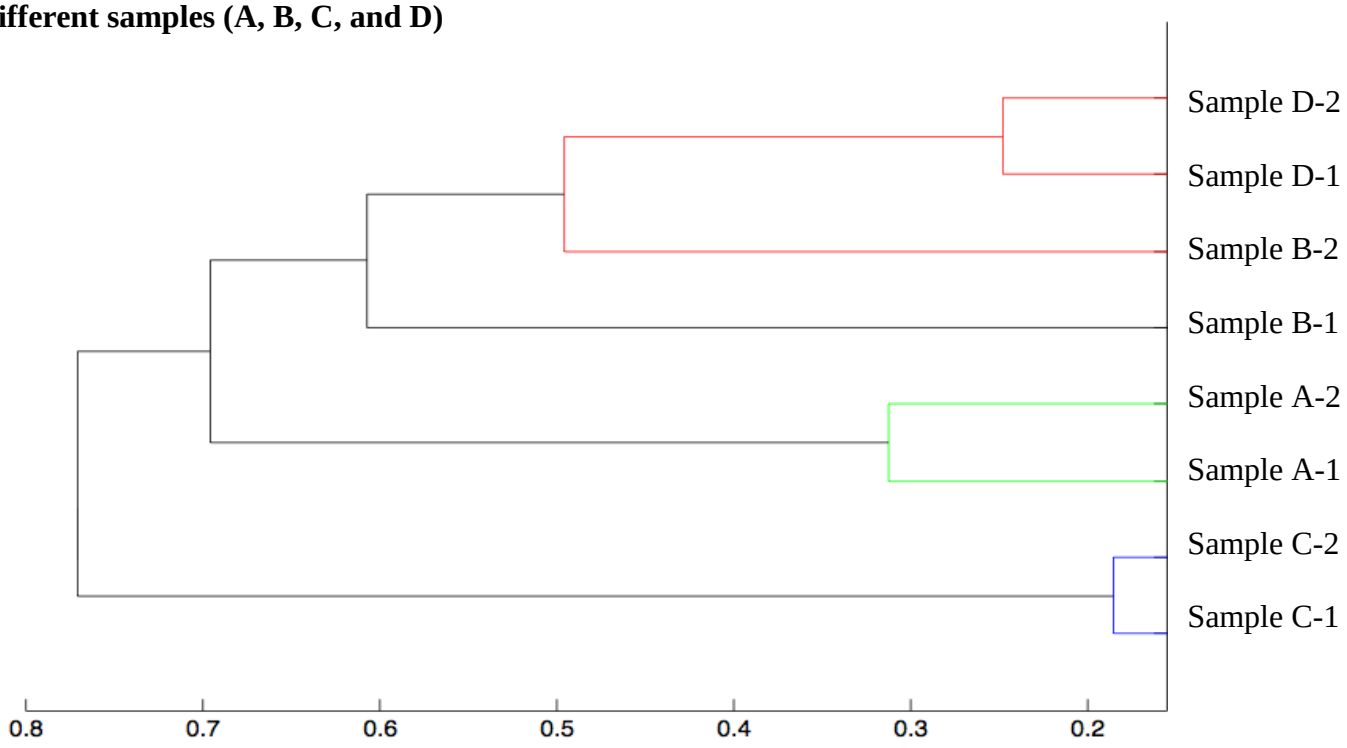
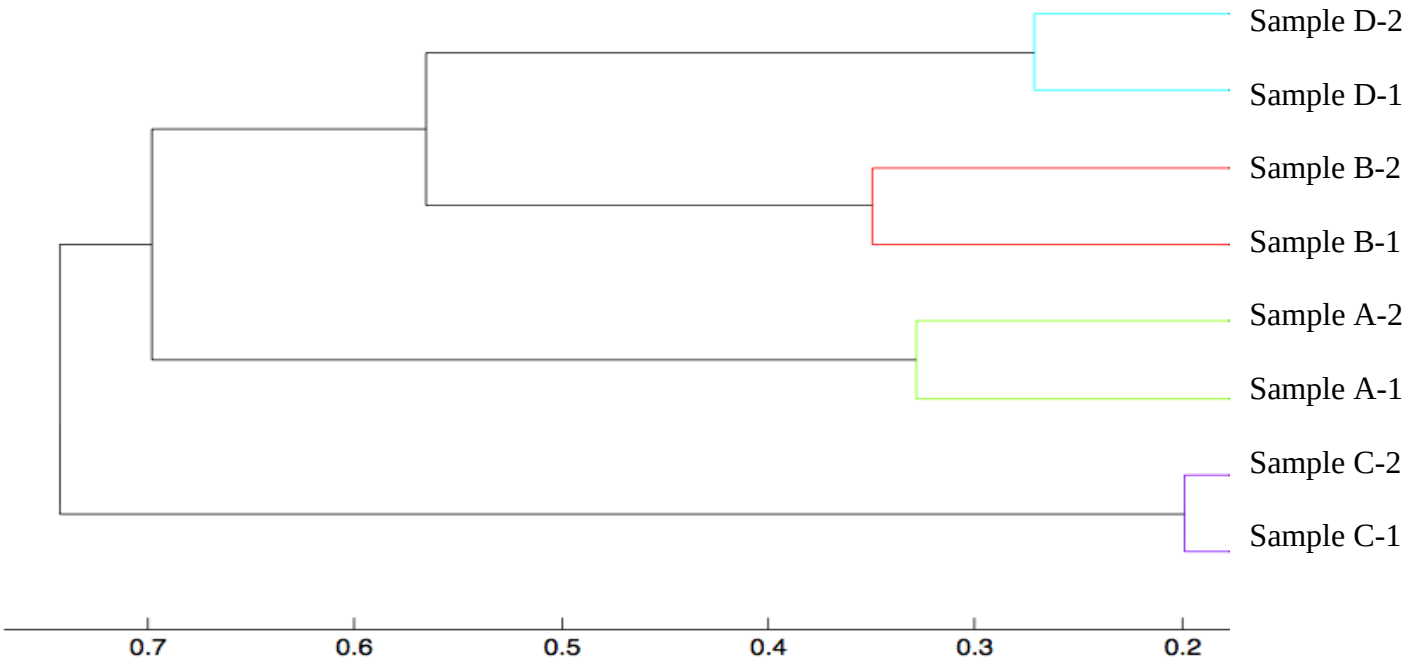


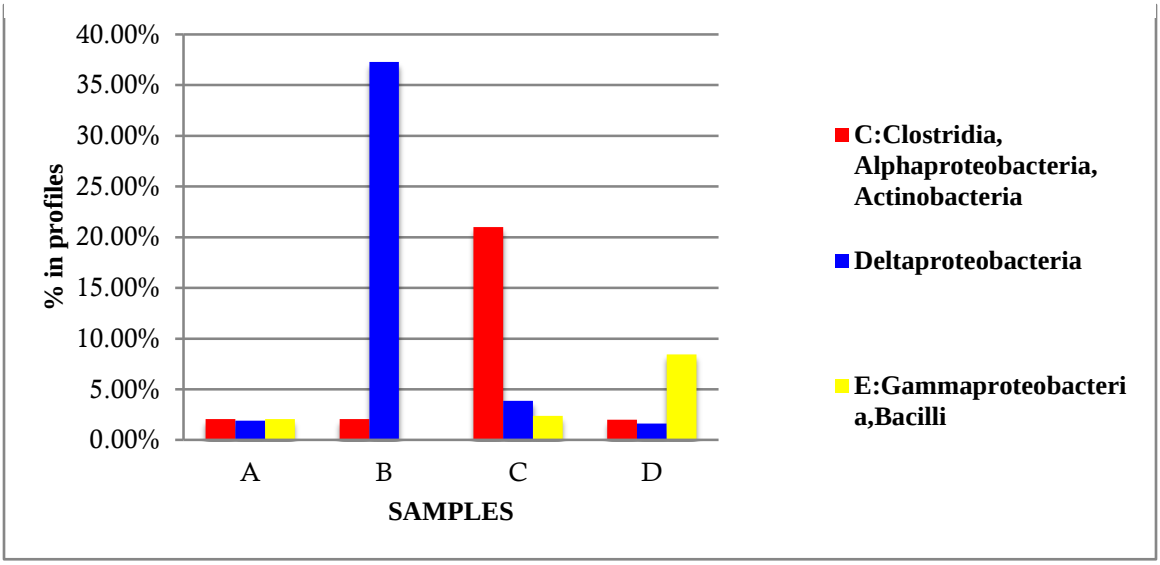
Figure 13 Dendrogram structures of Combined without MspI and HaeIII-TRF profile comparisons four different samples (A, B, C, and D)



3.3 Phylogenetic analysis of T-RFLP profiles by Phylogenetic Assignment Tool (PAT)

The data sets of enzyme HinIII and MspI were omitted for the phylogenetic analysis. The TRFs profiles comparisons of the samples, A and B, B and C, C and D, A and D, and B and D for both enzyme AluI and HinP1I showed change in some of the major taxonomic groups but the two enzymes did not have much in common. Figure 14 shows that Deltaproteobacteria increased dramatically from sample A to B then they decreased again. Most of Clostridia, Alphaproteobacteria, and Actinobacteria (C) were observed in sample C and Gammaproteobacteria and Bacilli (E) were mostly observed in sample D. These results indicate that there were changes in the bacteria community structure under anaerobic conditions.

Figure 14 Change in key bacterial community structures



CHAPTER 4 **DISCUSSION**

4.1 Generation of TRF profiles

Once the TRF profiles were generated, they always had number of small peaks resulting from either amplification of noise or the amount of DNA loaded on a gel which could not be removed. They could affect negatively on the similarity analysis of bacterial community structures in different samples based on the binary (presence: 1/absence: 0) TRF profiles. To try to minimize the negative effect of false peaks on similarity analysis, the TRF data set was standardized by applying an artificial threshold. Previous study introduced the constant percentage threshold method designed for normalizing data set (Sait *et al.,* 2003). Peaks below the threshold were considered as false peaks and removed from the data set. The error this method could have brought was that false peaks whose percentage were higher than constant percentage threshold might not have been detected in profiles with higher total area, while more valuable peaks whose percentage were lower than constant percentage threshold were removed in profiles with lower total area.

This could have been improved by the method based on the variable percentage threshold reported by Osborne *et al.* 2005. It is considered as more accurate method for normalizing TRF profiles because of the broad variation in total area of each profile within a data set. The percentage thresholds are calculated by dividing the total area of each profile with different values (divisors). For each divisor, all peaks that placed less than the percentage threshold calculated for that profile are removed then the remaining number of peaks are plotted against the total area, so that each profile contributed one point on that plot. The divisor that has the weakest relationship between the number of

peaks remaining and the initial total area are the optimal divisor. The unique percentage threshold for each profile is calculated by dividing the total area of that profile by the optimal divisor. Normalization with this threshold can achieve reasonable results by removing false peaks and keeping peaks with useful information.

4.2 Similarities between TRF profiles from different samples

Comparing the dendrograms manually was easy to notice errors such as not paired replicates from enzyme MspI and HaeIII. However, this does not confirm that the results are not acceptable. The results could have been more accepted by calculating the cophenetic correlation coefficient. The cophenetic distances of the cluster tress was compared with the original distance data generated by the pdist function to measure how faithfully a dendrogram generated by the linkage function reflects the data. The closer the value of the cophenetic correlation coefficient is to 1, the more accurately the clustering solution reflects the data.

Moreover, the results from dendrogram that showed a close relationship between sample B and D was not shown in the results of PAT (Fig.14). This is because of the use of binary matrix (unweighted) method, which might have shown limited results as it only represented the presence of the peaks or absence of the peaks. Therefore even though there was a dramatic change in the size of the peak, it will still read two presences of the peaks, not indicating how much the peak has changed. Consequently, although the dendrogram showed some relationships between the samples, they could not be found in the PAT results.

CHAPTER 5 **CONCLUSIONS**

The objectives of this study were to generate DNA fingerprint profiles for bacteria communities in different stages of anaerobic digestion of medium density fiberboard and to identify any changes in bacterial groups.

As sample A (only cattle manure) contained the most variable numbers of microorganisms, this could be the reason for its closer relationship to sample B and D than sample C which showed least similarities to other samples conferring to the dendrogram results. The numbers of some microorganisms could have been decreased from sample A to B due to the introduction of a new environment (MDF added) consisting of toxin from resin and because they do not intake any substrates from that inoculum. However, some bacteria could tolerate the entrance of toxins and due to the existence of required substrates, they could have increased. The different substrates from each stage where sample B and D were extracted, could have been degraded by common microorganisms to produce biogas. This was shown by the general trend of total gas production where stages of sample B and D were producing most of carbon dioxide and methane. Sample C seems to be a transition state of components from the adhesive resin where the microorganisms were degrading different substrates or not functioning.

For the future study, the researchers can apply the characterized microbial community changes from this study to find appropriate microorganism to enhance the decontamination of the MDF bonded with urea formaldehyde from the landfill.

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