İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

POPULATION DYNAMICS IN TWO-STAGE ANAEROBIC DIGESTER TREATING SOLID WASTES

M.Sc. Thesis by
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Programme : Environmental Engineering

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FOREWORD

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This thesis is dedicated to my family and people who are believe in me.

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Arda Gülay
Environmental Engineer

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ABBREVIATIONS

AB : Anaerobic Bacteria
AMA : Acetate Methanogenic Archaea
ASRB : Acetate Sulphate-Reducing Bacteria
COD : Chemical Oxygen Demand
sCOD : Soluble Chemical Oxygen Demand
DHW : Dinner hall wastes
DGGE : Denaturating Gradient Gel Electrophoresis
EAB : Ethanol Acetogenic Bacteria
ESRB : Ethanol Sulphate-Reducing Bacteria
FAAB : Facultative Aerobic and Anaerobic Bacteria
FAB : Formate Acetogenic Bacteria
FB : Fermentative Bacteria
FISH : Fluorescence In Situ Hybridization
FMA : Formate Methanogenic Bacteria
GFB : Glucose Fermentative Bacteria
HMA : Hydrogenophilic Methanogenic Archaea
HRT : Hydraulic Retention Time
I.T.U : Istanbul Technical University
SRT : Solid Retention Time
LFB : Lactate Fermentative Bacteria
LSRB : Lactate Sulphate-Reducing Bacteria
MMA : Methanol Methanogenic Archaea
MPN : Most Probable Number
PAB : Propionate Acetogenic Bacteria
SMA : Specific Methanogenic Activity
SRB : Sulphate-Reducing Bacteria
OFUSW : Organic Fraction Urban Solid Waste
PCR : Polymerase Chain Reaction
RFLP : Restriction Fragment Length Polymorphism
SIP : Stable Isotope Probing
SSCP : Single-strand conformation polymorphism analysis
VFA : Volatile Fatty Acids
VOL : Volumetric Organic Load
TS : Total Solids
TVS : Total Volatile Solids
VHW : Vegetable hall wastes

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SUMMARY

Today, global problems associated with depleted natural sources and energy insecurity, changes research efforts toward sustainable techniques to eliminate environmental pollution. For achieving an effective anaerobic process for energy recovery, adequate understanding of process microbiology and dynamics are playing a key-role. The objective of this study was to monitor the chemical gradients and population dynamics that occur during anaerobic treatment of two different organic wastes, and compare them according to system performance and microbial community structure. Archaeal and bacterial population dynamics were examined in two-stage anaerobic digester system that was separated as acidification and digestion, to identify those organisms associated with organic waste degradation and to assess patterns in microbial response across environmental variables. Samples were taken monthly from each reactors that were operated under different conditions (pH, substrate, and loading rate) and were fed with dinner hall and vegetable hall wastes. The microbial diversity and changes in the microbial composition were analyzed by molecular microbiological techniques based on the 16S rRNA gene: cloning and sequencing, denaturing gradient gel electrophoresis. From each reactor, clone libraries were constructed using universal primers for either the class Archaea and Bacteria. Sequencing of 145 bacterial clones that 84 in fermenter, 61 in digester from 7 libraries and 65 archaeal clones from 4 libraries for dinner hall wastes, 147 bacterial clones that 96 in fermenter, 51 in digester from 6 libraries and 25 archaeal clones from 4 libraries revealed a diverse anaerobic sludge community and distinct differences among reactors for both substrates. The DGGE and clone analysis indicated that the archaeal community structure was closely correlated with the volatile fatty acid (VFA) concentration and pH, while the bacterial population was impacted by pH. Members of the class *Lactobacillus* species were dominant after 30 days operation in fermenter and *Thermotogae*, *Firmicutes*, *Synergistetes*,

Synergistetes, Bacteroidetes phylum's in digester of dinner hall waste's reactors. The archaeal community of fermenter consisted mainly of *Methanobrevibacter acididurans sp*. from *Methanobacteriales* phylum and *Methanofollis liminatans* from *Methanomicrobiales* phylum. Digester community were consisted mainly of *Methanosarcinaceae sp*. then changed to *Methanosaetaceae* sp. after 3-month operation. Bacteria corresponding to prominent DGGE bands in vegetable hall reactor's sludge were belong to the class *Lactobacillaceae* and *Veillonellaceae*, together with *Prevotellaceae* in fermenter, *Desulfobacteraceae*, *Syntrophaceae* class in digester. Raw substrate contains archaeal communities such as *Methanobacteriaceae* and *Methanosarcinaceae* that could be linked to micro-anoxic zones inside raw waste. *Methanobacteriaceae sp*. was also dominant in fermenter sludge, *Methanobacteria* and *Methanomicrobia* phylum and *Methanococci* in minor amounts in digester sludge were detected. Despite similar reactor performance with respect to chemical parameters in digester of different substrates, the underlying community structures were different, which may have an influence on energy recovery period.

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KATI ATIK ARITAN İKİ KADEMELİ HAVASIZ REAKTÖR SİSTEMİNDEKİ POPULASYON DİNAMİKLERİ

ÖZET

Günümüzde ortaya çıkan doğal kaynakların tükenmesi ve enerji yetersizliği gibi global problemler, bilimsel araştırmaların yönünü çevrel kirliliğini de önleyecek sürdürülebilir tekniklere yöneltmiştir. Bu bağlamda, etkili bir enerji geri kazanımı için, proses mikrobiyolojisinin ve dinamiklerinin yeterli şekilde anlaşılması anahtar bir rol üstlenmektedir. Bu çalışmanın amacı, iki farklı organik atığın anaerobik arıtımı sonucu oluşan kimyasal gradyanların ve populasyon dinamiklerinin izlenmesi, bunların sistem performansı ve mikrobiyal topluluk yapısına göre karşılaştırılmasıdır. Arke ve bakteri populasyon dinamikleri, çevresel değişkenlerin sonucundaki mikrobiyal izlerin değerlendirilmesi ve organik atık parçalanmasıyla ilişkili organizmaların tanımlanmasını sağlamak için asitleştirici (fermentör) ve çürütücü(metan reaktörü) şeklinde ayrılan iki kademeli anaerobik sistemde incelenmiştir. Örnekler, yemekhane ile sebze atıklarıyla beslenen ve farklı işletme koşullarında işletilen (pH, substrat ve yükleme oranı) reaktörlerden aylık olarak alınmıştır. Mikrobiyal çeşitlilik ve mikrobiyal komposizyondaki değişimler 16S rRNA geni tabanlı klonlama ve sekanslama ile DGGE moleküler mikrobiyolojik teknikler kullanılarak incelenmiştir. Her bir reaktörden alınan numunelerden, genel primerler kullanılarak arke ve bakteri sınıflarının klon kütüphanesi oluşturulmuştur. Yemekhane atıkları için 84'ü fermentörden, 61'i çürütücüden olmak üzere toplam 145 bakteri klonu 7 kütüphaneden, toplam 65 arke klonuda 4 farklı klon kütüphanesinden, sebze atıkları için 96 bakteri klonu asitleştiriciden(fermentör), 51'I çürütücüden, toplamda 145 bakteri klonu 6 farklı kütüphaneden, toplamda 25 arke klonu da 4 farklı kütüphaneden sekanslanmıştır. Bu sayılar anaerobik çamurdaki mikroorganizma türlerinin çeşitlilik gösterdiğini ve her bir substrat için reaktörlerdeki açık farklılıkları ortaya koymaktadır. DGGE ve klon analizleri arke tür yapısının pH ve uçucu yağ asit (UYA) konsantrasyonuyla yüksek korelasyona sahip olduğunu, bakteri türleri içinde pH'ın etkili olduğunu göstermektedir. Yemekhane

atıklarıyla yapılan çalışmada, fermentörün 30 günlük işletilmesi sonunda *Lactobacillus* türü dominant hale gelmiş, çürütücüde ise *Thermotogae, Firmicutes, Synergistetes, Synergistete*s, *Bacteroidetes* filumlarının baskın olduğu görülmüştür. *F*ermentörde baskın olan arke türü *Methanobacteriales* filumundan *Methanobrevibacter acididurans'*tır, *Methanomicrobiales* filumundan *Methanofollis liminatans'*ta reaktör çeşitliliğinde yer almaktadır. Çürütücüde ilk olarak görülen *Methanosarcinaceae* türünün baskınlığı 3 ay işletmeden sonra *Methanosaetaceae* türüyle değişmiştir. Sebze atıklarıyla yapılan çalışmada DGGE bant verilerine göre fermentördeki baskın bakteri populasyonu *Lactobacillaceae, Veillonellaceae* ve *Prevotellaceae* türleridir, çürütücüde ise *Desulfobacteraceae ve Syntrophaceae* türleri baskındır. Prosese girmemiş atıkta mikro-anoksik boşluklar sebebi ile *Methanobacteriaceae* ve *Methanosarcinaceae* türlerine rastlanmıştır. Fermentör çamurunda *Methanobacteriaceae* dominant olup, çürütücü çamurunda ise *Methanobacteria* ve *Methanomicrobia* filumu ve az miktarda *Methanococci* filumuna rastlanmıştır. Farklı subtratlarla farklı fiziko-kimyasal şartlarda işletilmesine rağmen, birbirine benzer arıtma preformansları elde edilmiş olan çürütücü reaktöründe mikrobiyal topluluk yapısı önemli ölçüde farklı bulunmuştur. Söz konusu mikrobiyal populasyon farklılığı,, enerji geri kazanım sürecini etkileyebilmektedir.

1. INTRODUCTION

1.1 Aim of the Study

Today, global problems associated with depleted natural sources and energy insecurity, changes research efforts toward sustainable techniques to eliminate environmental pollution. To overcome, these problems, anaerobic technology become an important technique with its sustainability, recovery of valuable byproducts and renewable biofuels from low-value feedstock such as waste streams. (Figure 1.1). Development and the application of high rate anaerobic bioreactors are very crucial for the successful application of anaerobic biotechnology and the conversion of biosolids [178]. Moreover, biological processes like anaerobic digestion are the sum of complete microbial-dependent processes. In this context, adequate understanding of process microbiology and dynamics are playing a key-role to achieve a more effective anaerobic process performance. Both culture dependent and culture independent molecular approaches are used to have more knowledge about microbial communities in bioreactors [1]. Narihiro and Sekiguchi stated that; especially, because of analysis focused on 16S rRNA gene, compositions of them are recorded. Furthermore, the characterizations of very important anaerobes are done [2]. Developments in molecular techniques that uses 16S rRNA database, enable us to study ecology of microbial communities and understand the complex structures of anaerobic environments. So that, design and operation of engineered systems like anaerobic digesters can be improved and tested [179]. The aim of this research is to get a deeper and better insight into the population dynamics of microbial consortia in anaerobic bioreactors feeding with dinner hall and vegetable hall wastes. The use of combination of molecular techniques (PCR-RFLP-Cloning and Sequencing, PCR-DGGE) enabled us to identify several species in an anaerobic syntrophic degrading consortium and biomethanization.

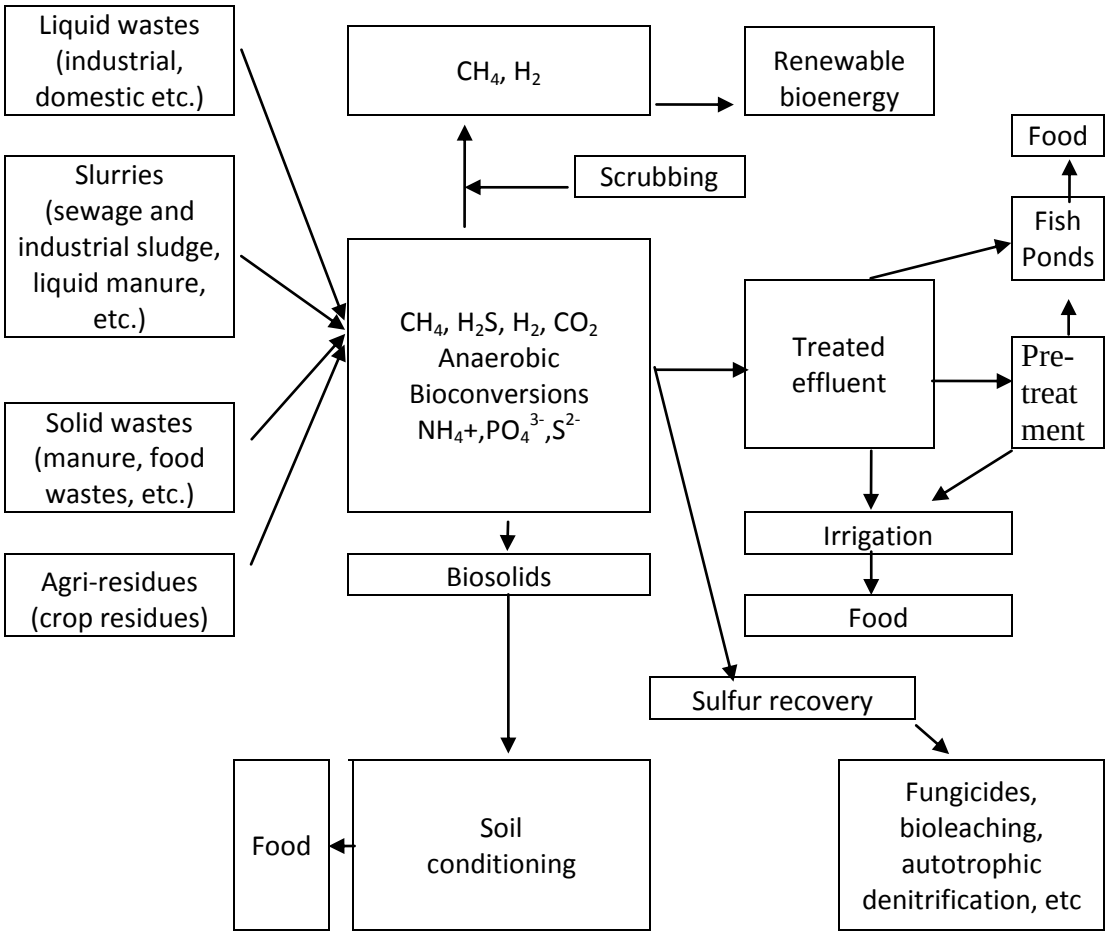


Figure 1.1 : Anaerobic bioconversion processes in recovery of resources from wastes [177]

1.2 Outline of the Thesis

The first 3 chapters cover the fundamental aspects of anaerobic process and molecular approaches. The remaining six chapters were focus on experimental procedure, performance and results of anaerobic reactors.

Chapter 2 presents an overview of anaerobic degradation and anaerobic microbiology including definitions, biochemical reactions, and major process considerations. In Chapter 3, an extensive review on the applications, the advantages, and the drawbacks of molecular techniques used in ecological studies were given. In Chapter 4, the materials and the methods used in this thesis were given in detail.

In Chapter 5, performance and monitoring results of a two-stage system, which was operated for six months, and fed with dinner hall wastes were given in detail. COD, sCOD, TS, TVS, VFA parameters were shown on graphs with pH values in detail. In Chapter 6, for monitoring diversity changes and to find out dominant species in sludge communities`which was fed with dinner hall wastes, molecular uncultured methods, polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE) and cloning-sequencing, was applied to characterize the reactor sludges. Population dynamics of anaerobic sludge was characterized. Archaeal and bacterial population shifts, which were affected by substrate composition and pH, monitored and evaluated by using DGGE. In Chapter 7, performance and monitoring results of a two-stage system, which was operated for five months and fed with vegetable hall wastes, were given in detail. COD, sCOD, TS, TVS, VFA parameters were shown on graphs with pH values in detail. Chapter 8, for monitoring diversity changes and to find out dominant species in sludge communities`which was fed with vegetable hall wastes, molecular uncultured methods, polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE) and cloning-sequencing, were applied to characterize the reactor sludges.. Archaeal and bacterial population shifts, which were affected by substrate composition and pH, monitored and evaluated. In Chapter 9 Archaeal and bacterial population shifts of two different sludges, which are from reactors fed with dinner hall, wastes and vegetable hall wastes were compared and evaluated. In Chapter 10 the major findings described in the previous chapters were stated.

2. LITERATURE REVIEW ANAEROBIC DIGESTION

2.1 Microbiology of Anaerobic Digestion Treating Solid Wastes

Digestion is a method by which organic material is solubilised and chemically transformed.There for it can be absorbed by the cells of an organism and used to maintain body functions [100]. In anaerobic degradation process, degredation of organic chemicals in a completely mixed reactor usually involves several consequent degradation phases such as hydrolysis, acidogenesis, and then methanogenesis [101]. Complex organic compounds, such as polysaccharides, proteins and lipids are hydrolyzed to monomers like sugars, amino acids and fatty acids. These intermediate products are then degraded by acidogens, forming volatile fatty acids, which are further degraded by acetogens, forming acetate, carbon dioxide, and hydrogen. Finally, both acetate and H_2/CO_2 are converted to methane by methanogens [101]. (Figure 2.1). Anaerobic degradation is not always suitable for all substrates. According to Gerardi, treatment of organic waste and wastewater with less cost needed and production of biogas is anaerobic digestion which has the optimum conditions when the oxidation-reduction potential (ORP) is between 200-400 mV [3].

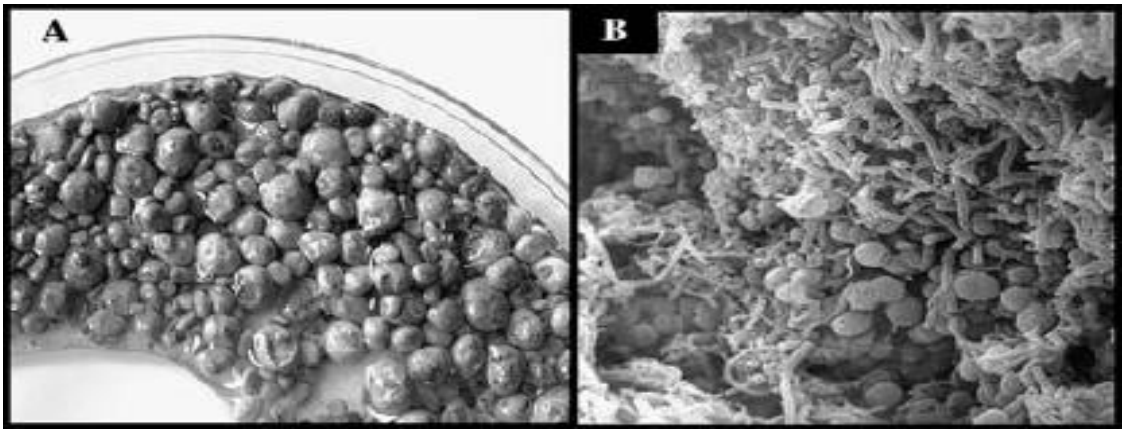


Figure 2.1 : (A) Anaerobic granules removed from a laboratory-scale anaerobic bioreactor. (B) Scanning electron micrograph (SEM) of anaerobic granules (·2.9 K) [5].

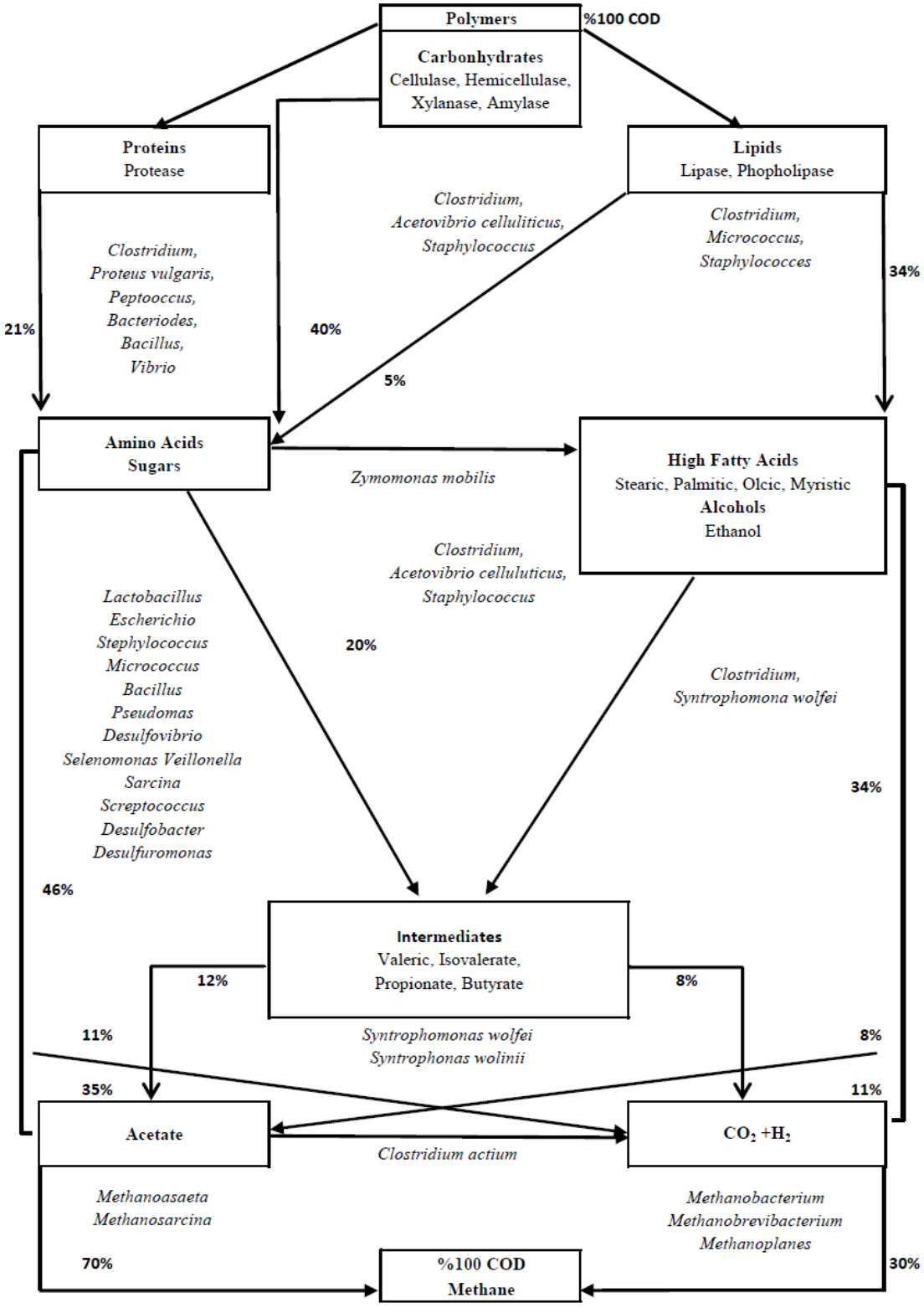


Figure 2.2 : Carbon flow to methane in anaerobic digesters with the microorganisms responsible for each step [9].

2.1.1 Hydrolytic Bacteria

Either methanogic or acetogenic organisms need complex polymeric substrates to become soluble. Therefore, hydrolysis is the most important step in the anaerobic degredation [4]. Moreover, organic waste stabilization does not occur during hydrolysis; the organic matter is simply converted into a soluble form that can be utilized by the bacteria [102]. Hydrolysis is a chemical process in which a molecule is cleaved into two parts by the addition of a molecule of water. One fragment of the parent molecule gains a hydrogen ion (H^+) from the additional water molecule. The other group collects the remaining hydroxyl group (OH^-).

Complex substrates have no ability to enter microorganism's cell wall because of its size. So, it needs to degrade into smaller sizes. Lipids, proteins, polysaccharides, nucleic acids, insoluble organic material are degraded by hydrolysis by exoenzymes and endoenzymes [6]. All microorganisms's have to utilize substrates, which appropriate sizes for cell membrane. Polprasert and Speece assumes that complex organic molecules such as proteins, cellulose, lignin, lipids are turned into soluble monomer molecules (e.g.,amino acids, glucose, fatty acids, glycerol) by anaerobic bacteria communities. After that, they got ready for the next bacteria community to use. Enzymes have the key role in hydrolization for degrading polymers. Different enzymes have different specific sites for different substrates. Gerardi points out that bacterial enzyme are used to degrade the substrate by catalyzing biochemical reaction. While substrate is degradated, endoenzymes and exoenzymes get into reaction [3]. As it can be observed on table 2.1, a group of specific substrates can be degraded by either exoenzyme or endoenzyme. Thus, to make sure that all the types of exoenzymes and endoenzymes, which are suitable for the current substrates, obtainable; various bacteria communities are needed [3]. While extracellular enzymes like celluloses, lipases, proteases catalyze hydrolysis process, the phase is can be considered as slow and also can limit anaerobic digestion of wastes that contain lignin and lipids [7].

Exoenzymes and substrates [3].

Substrate to be degraded	Exoenzyme needed	Example	Bacterium	Product
Polysaccharides	Saccharolytic	Cellulase	Cellulomonas	Simple sugar
Proteins	Proteolytic	Protease	Bacillus	Amino acids
Lipids	Lipolytic	Lipase	Mycobacterium	Fatty acids

Mara and Horan declare that hydrolytic genera (e.g., *clostridium*, *peptococcus*, *vibrio*, *micrococcus*, and *bacillus*) carry out anaerobic digestion, which manufactures many hydrolic enzymes. These enzymes play the trigger role in attacking the complex substrates. Total number of hydrolytic bacteria, involving facultative and obligate anaerobes, in an anaerobic digester is about 108-109 [9]. Table 2.3 shows the diversity of the hydrolytic species.

2.1.2 Acidogenic Bacteria

In acidogenesis, the hydrolyzed compounds are fermented into volatile fatty acids (acetate, propionate, butyrate, and lactate), neutral compounds (ethanol, methanol), ammonia, hydrogen and carbon dioxide. Mara et al. declare intermediate products such as acetate, propionate, butyrate, hydrogen generated throughout the second acid-forming stage by fermentation of the monomers which hydrolytic bacteria produced [9]. Syntropic microorganisms in anaerobic habitats need specific substrates for their metabolism. These substrates are generally product of another organism like acidogens, acetogens and methanogens. The fermentative bacteria are usually separated into groups based on one or several fermentation products, which reflect their metabolic pathways (Table 2.4) Bacteria, which process in fermentation, affect the products. Thus, the concentration of the products such as acids and alcohols differs according to the operational conditions change, which affects the dominant bacteria. The existing substrate used by bacteria, which form methane, their activity, digester performance affected by the changes in these concentrations [3]. Gerardi states that sugars, amino acids, fatty acids are converted to organic acids such as acetic, propionic, formic, lactic, butyric, succinic acids; alcohols; ketones such as ethanol, methanol, glycerol, acetone; acetate; CO_2, H_2 by acidogenic bacteria.

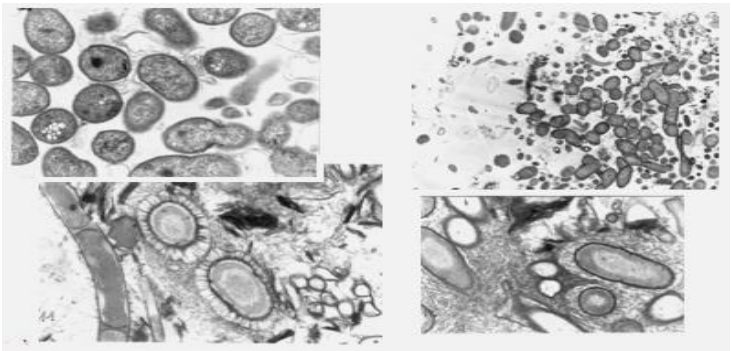


Figure 2.3: Fermentative Bacteria [10]

Table 2.1: Bacteria participating in the hydrolysis process [11]

Taxonomy	Species	Description	Metabolism
Genus: Bacteroides	B.uniformis B.acidifaciens B.vulgatus B.splanchnicus B.ruminicola	The genus Bacteroides consists of immobile, Gram-negative rods	They take as substrate carbonhydrates, peptones, and metabolic products of other micoorganisms like sugar, aminoacids, and organic acids, and organic acids. The metabolic products og the *Bacteriodes* are succinate, acetate, formate, lactate, and propionate. Butyrate is mostly not a main product of the fermentation of carbohydrates and occurs normally with iso butyrate and isovalerate.
Genus: Lactobacillus	L.pentosus L.plantarum L.agilis L.aviarius L.lindneri	The genus Lactobacillus consists of Gram-positive, catalase-negative rods, which do not generate endospores.They are normally immobile	They ferment glucose to lactate and other organic acids either homofermentatively or heterofermentatively. Lactobacilli are known for their need of additional nutrients like vitamins, aminıacids, purines, and pyrimidines.
Genus: Propioni-bacterium	P.microaerophilum P.granulosum P.lymphophilum P.acnes P.avidum P.propionicus P.combesii P.thoenii P.freudenreichii P.cyclohexanicum	They are immobile Gram-positive rods, which do not form spores. Propionibacterium	Propionibacteria are catalase-positive. They are chemoorgantrophic and produce much propionate and acetate during fermentation of carbonhydrates.

Table 2.2: (continued) Bacteria participating in the hydrolysis phase [11]

Taxonomy	Species	Description	Metabolism
			By products of the fermentation are isovalerate, formate, succinate, lactate, and CO_2
Genus: Sphingomonas	S.aromaticivorans S.subterranea S.stygia	They occur in deep sediments.	Sphingomonas are able to degrade aerobically a wide spectrum of substituted aromatics.
		Sphingomonas on Xanthos	The can utilize anaerobically the methoxyl groups of trimethoxybenzoate without splitting the aromatic ring.
Genus: Sporobacterium	Sp. Olearium		Sporobacterium is able to degrade stoichiometrically trimethoxybenzoat yo acetate and butyrate by splitting the aromatic ring.
Genus: Megasphaera	M.elsdenii	They occur in the rumen.	The Megasphaera use the acrylate pathway.
Genus: Bifidobacterium			Bifidobacteria ferment glucose to lactate and acetate. The decomposition of hexoses occurs via a special pathway.

The major product of carbohydrate fermentation is acetate. Both culture conditions such as tempeture, pH, redox potential and variety of the bacteria have an effect on the products [3]. These differentiations in products are due to the microorganisms that have different pathways and substrates. Mara et al. claim that clostridum, bacteroides, ruminococcus, butyribacterium, propionibacterium, eubacterium, lactobacillus, streptococcus, pseudomonas, desulfobacter, micrococcus, bacillus and Escherichia are the genera and species contained by the acidogenic stage. The facultative organisms use the trace of oxygen that may consist in the reaction. This process helps to protect the oxygen-sensitive methanogens [9].

Table 2.3: Groups of fermentative bacteria able to grow under anaerobic conditions, and their fermentation products [103]

Fermentation characterizing bacterial groups	Typical Species	Substrate	Fermentation product	
			Major	Minor
Ethanol fermentation	Zymomonas mobilis	Glucose	Ethanol	CO_2
Lactate fermentation: Homofermentative	Lactobacillus casei	Glucose	Lactate	
Heterofermentative	Leuconostoc mesenteroides	Glucose	Lactate	Ethanol, CO_2
Heterofermentative	Bifidobacterim bifidum	Glucose	Acetate	Lactate
Butyrate fermentation	Clostridium butyricum	Glucose	Butyrate	Acetate+H_2+CO_2
	Clostridium acetobutylicum	Glucose	Butyrate,butanol	Acetone, z2-propanol
	Clostridium kluyveri	Ethanol+ Acetate	Butyrate	Caproate,H_2
Homoacetate fermentation	Clostridium aceticum	Fructose	Acetate	
Propionate and succinate fementation	Propionibacterium pentosaceum	Sugars, lactate	Propionate	Succinate
	Veillonella alcalescens	Lactate	Propionate	Acetate, H_2, CO_2
	Bacterioides numinicola	Sugars	Propionate	
Mixed acid and butanediol fermentation	Escherichia coli	Glucose	Lactate, ethanol, acetate	Formate, H_2+CO_2 succinate
	Eterobacter aerogenes	Glucose	2,3-Butanediol, ethanol	Formate, H_2+CO_2
Nitrogenous compounds fermentation	Clostridium tetanomorphium	Glutamate	Butyrate	Acetate, CO_2,NH_3
	Clostridium sticklandii	Lysine	Butyrate	Acetate, NH_3
	Clostridium oroticum	Orotate	Acetate	CO_2,NH_3

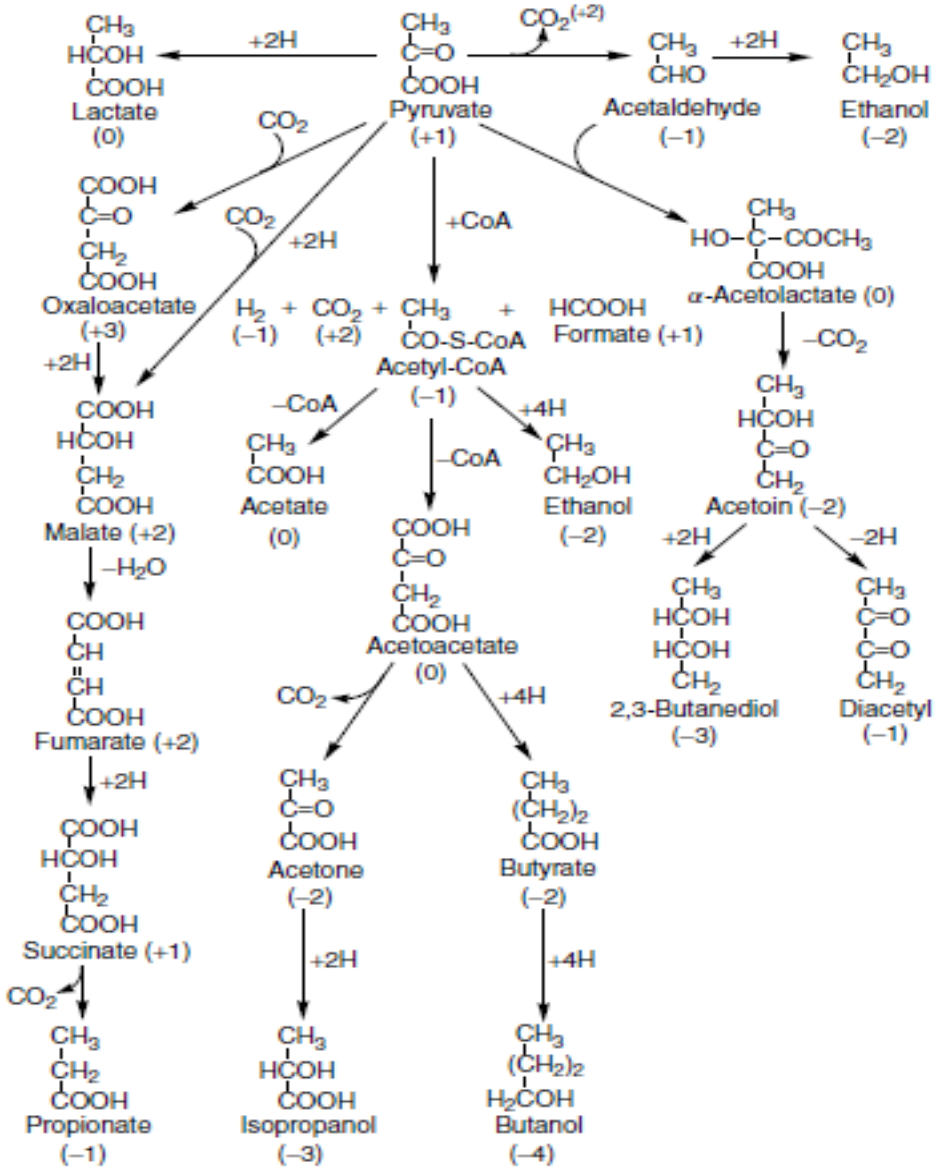


Figure 2.4 : Schematic view of major pathways of fermentation product formation from pyruvate. Numbers in parentheses are the oxidation values [176]

2.1.3 Acetogenic Bacteria

Acetogenesis is a process through which acetate is produced from a variety of energy and carbon sources by anaerobic bacteria. Acetogenic bacteria produce acetate, H_2 and CO_2 from the organic acids and alcohols. Methanogens can only use acetate, CO_2, hydrogen competently. Acetogenic bacteria produce these during the anaerobic digestion [9].

Acetic acid, carbon dioxide, hydrogen are produced from the major fatty acid intermediates (propionate and butyrate), alcohols and other higher fatty acids (valerate, isovalerate) by obligate hydrogen-producing acetogens. This group plays a scientific role in both β-oxidation of longer-chain fatty acids beginning from lipid hydrolysis and the anaerobic degradation of aromatic compounds [9].

Fermentation end products produced by a bacterium depend on the environmental conditions in which it grows. Partial pressure of H_2 has a huge effect in this change and in a natural environment or an anaerobic digester; hydrogenotrophic microorganisms such as methanogens keep the hydrogen partial pressure low[104]. Moreover, Hawkees stated that acetogens and lactic acid bacteria were inhibited by the high CO_2 partial pressure and substrate conversion to microbial biomass was reduced [104]. Mc.Inernay states that volatile fatty acids such as propionic acid, butyric acid and alcohols turned into acetate, hydrogen, and carbon dioxide by acetogenis bacteria like as Syntrobacter wolinii and Syntrophomonas wolfei. These products are used by the methanogens [13]. Also Lowe claims that syntrophomonas wolfei was the first of them. It is secluded from anaerobic digestor sludge in a syntrophic coculture with methanospirillum hungatei [14].Table 2.5 show important syntrphic acetogenic bacteria in anaerobic digestion.

Acetic acid is produced by acetogenic bacteria using ethanol, propionic acid, butyric acid as it is shown in the following reactions:

$$\overset{\textbf{ethanol}}{CH_3CH_2OH}+H_2O \longrightarrow \overset{\textbf{acetic acidl}}{CH_3COOH}+2H_2 \quad \textbf{(3.1)}$$

$$\overset{\textbf{propionic acid}}{CH_3CH_2COOH}+2H_2O \longrightarrow \overset{\textbf{acetic acid}}{CH_3COOH}+CO_2+3H_2 \quad \textbf{(3.2)}$$

$$\overset{\textbf{butric acid}}{CH_3CH_2CH_2COOH}+2H_2O \longrightarrow \overset{\textbf{acetic acid}}{2CH_3COOH}+2H_2 \quad \textbf{(3.3)}$$

Björnsson says that for monitoring the hydrogen concentration properly, some group needs low hydrogen pressure for fatty acid conversion. For these groups H_2 partial pressure increases, formation of acetate is reduced. Thus, instead of methane substrate is turned into propionic acid, butyric acid and ethanol.

Table 2.4: Syntrophic acetogenic bacteria [14]

Substrates	Fermentation Products	Isolation/Habitat	Growth	Syntrophic Partner
Ethanol	Acetate, H_2	Methanobaclillus omelianskii	37°C	Methanobacterium sp.
Butyrate	Acetate, H_2	Digester sludge	30-37°C	Desulfovibrio sp., Methanospirillum hungatei, Methanobacterium formicicum
C_4-C_{18} straight-chain fatty acids	Acetate, CO_2	Digester sludge	30-37°C	Desulfovibrio sp., Methanospirillum hungatei
C_4-C_{18} linear saturated fatty acids	Acetate, H_2	Digester sludge	35°C, pH7.3	Desulfovibrio sp., Methanospirillum hungatei
Butyrate, 2-methylbutyrate	Acetate, H_2,propionate	Marine and freshwater mud	28-34°C, pH6,5-7,5	Desulfovibrio sp., Methanospirillum hungatei
Propionate	Acetate, H_2	Sewage digester	37°C, pH6,8-7,2	Desulfovibrio sp.,
Benzoate	Acetate, H_2,CO_2,formate	Sewage digester	37°C, pH7,2	Desulfovibrio sp.,
Fructose	Acetate, H_2	Rumen	35-42°C, pH6,4	Metanobrevibacter smithii
Ethanol, 1,2-propanediol, 2,3-butanediol	Acetate	Anaerobic digester, marine sediments	35°C	Metanobrevibacter arboriphilus
Ethanol	Acetate, CO_2	Freshwater and brackish water mud, marine sediments	34-37°C	Methanosarcina barkeri

The relationship between acetogenic bacteria and methanogens can consider as symbiotic. Low hydrogen pressure, which is needed by acetogenic bacteria partly attained by methanogens [15]. Homoacetogens are the second group of acetogenic bacteria. In addition, these organisms are very strict and catalysis the process of hydrogen and carbon dioxide to produce acetate [9]. Methanogens grow much slower than acetogens. μ_{max} value of the former group is around 1 $h^2$1, while the same value for the latter is around 0.04 $h^2$1 [16].

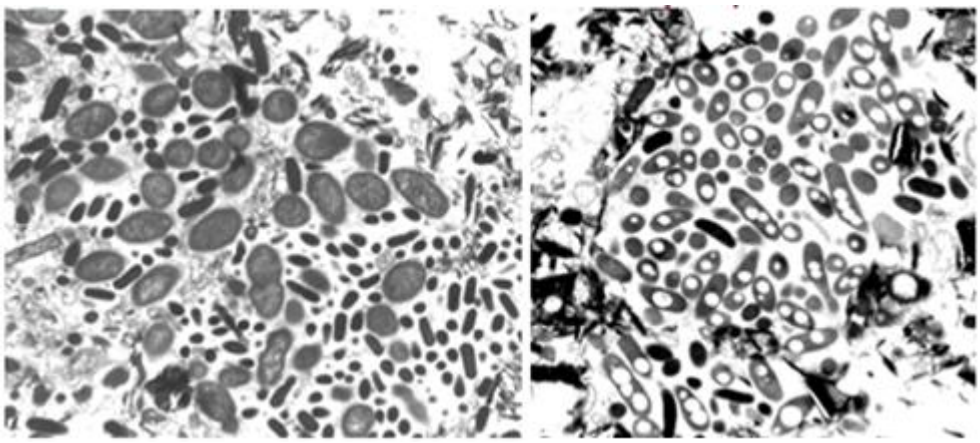


Figure 2.5 : Acetogenic Bacteria: Syntophobactrer with methanogen– Syntrophomonas[10]

Now, it is known that around 500 million tons of methane/year is discharged into the atmosphere by the anaerobic digestion of organic matter in the environment. That signifies around 0.5 percent of the organic matter, which is gained from photosynthesis [17].

The methanogenic microbes are a large and diverse group that is combined by three features: They form large quantities of methane as the major product of their energy metabolism. Secondly, they are strict anaerobes and they are members of the domain Archaea, or archaebacteria. Methanotrophic bacteria, or methanotrophs, are a subset of a physiological group of bacteria known as methylotrophs.

Methanotrophic bacteria are special in their ability to utilize methane as a sole carbon and energy source. A small group of substrate consisting of acetate, H_2, CO_2, formate, methanol, methylamines is used by methanogens. The list of substrates (Table 2.6) for growth of methanogens can divided into three groups

In the .first group, the energy substrate (electron donor) is H_2, formate, or certain alcohols and the electron acceptor is CO_2, which is reduced to methane.

In the second group, the energy substrate is one of a variety of methyl-containing C-1 compounds, which can serve as substrates for a few taxa of methanogens.

In the third group, acetate is the major source of methane, but the ability to catabolize this substrate is limited to species of Methanosarcina and Methanosaeta ("Methanothrix").

Table 2.5: Reactions and standard energies for metahanogenesis[105]

Reaction	ΔG° (kJ/mol of methane)
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	-135.6
4 Formate $\rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	-130.1
4 2-Propanol + $CO_2 \rightarrow CH_4 + 4$ Acetone + $2H_2O$[b]	-36.5
2 Ethanol + $CO_2 \rightarrow CH_4 + 2$ Acetate[c]	-116.3
Methanol + H2 $\rightarrow CH_4 + H_2O$	-112.5
4 Methanol $\rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	-104.9
4 Methylamine + $2H_2O \rightarrow 3CH_4 + CO_2 + 4\ NH_4^+$	-75
2 Dimethylamine + $2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_4^+$	-73.2
4 Trimethylamine + $6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4NH_4^+$	-74.3
2 Dimethylsulfide + $2\ H_2O \rightarrow 3CH_4 + CO_2 + H_2S$	-73.8
Acetate $\rightarrow CH_4 + CO_2$	-31

[a]The standard changes in free energies were calculated from the free energy of formation of the most abundant ionic species at neutral pH. Thus, "CO_2" is HCO_3^- + H^+ and formateis $HCOO^-$ + H^+.
[b]Other secondary alcohols utilized include 2-butanol, 1,3-butanediol, and cyclopentanol.
[c]Other primary alcohols utilized include 1-propanol and 1-butanol.

First group includes hydrogenotrophic methanogens such as hydrogen-using chemolithotrophs, transform hydrogen and carbon dioxide into methane. H_2 and CO_2 are used by most of methanococcales and methanobacteriales [18]. :

$$CO_2+4H_2 \longrightarrow CH_4+2H_2O \quad \textbf{(3.4)}$$

Second group commonly found in the marine sediments, rumen of mammals. Kiene stated methyl-containing compounds like dimethylselenide and methane thiol used as a substrate for methanogenesis. But, these substrates do–not support growth of methanogens [105]

$$CH_3OH+H_2 \longrightarrow CH_4+H_2O$$
$$CH_3OH \longrightarrow 3CH_4+CO_2+2H_2O \quad \textbf{(3.5)}$$

Third group includes Acetotrophic methanogens, which can called as acetoclastic, or acetate-splitting methanogens. They transform acetate into methane and CO_2 as it is shown by the following reaction [18]:

$$CH_3COOH \longrightarrow CH4+ CO2 \quad \textbf{(3.6)}$$

Mackie and Bryant state that acetotrophic methanogens produce around two-thirds of methane, which is gained from acetate conversion. The rest of it is gained from the reduction of carbon dioxide by hydrogen [22]. The following features are the main dissimilarities of methanogens which belongs to a different domain, the archaea:

- The cell wall composition is different; for instance, methanogens have less peptidoglycan. Cell walls composed of protein, glycoprotein, or pseudomurein; murein is absent [105]
- The cell membrane, which is made of stemmed hydrocarbon chains fixed to glycerol by ether linkages, composition is different [105]
- According to Bitton environments, which are free from oxygen (freshwater sediments, marine sediments, swamps, landfalls, the rumen of cows, anaerobic digesters), are the best places for methanogens to flourish [28]
- Ribosomal RNA chains of methanogens are also different from bacteria and eukaryotes [28]
- Capability of extreme thermophily in some groups [105]
- Lipids composed of glycerol ethers of isoprenoids and tertraethers are common [105]
- Stereochemistry of lipids is 2,3-sn glycerol [105]
- Antibiotic sensitivity differs from that of eubacteria [105]

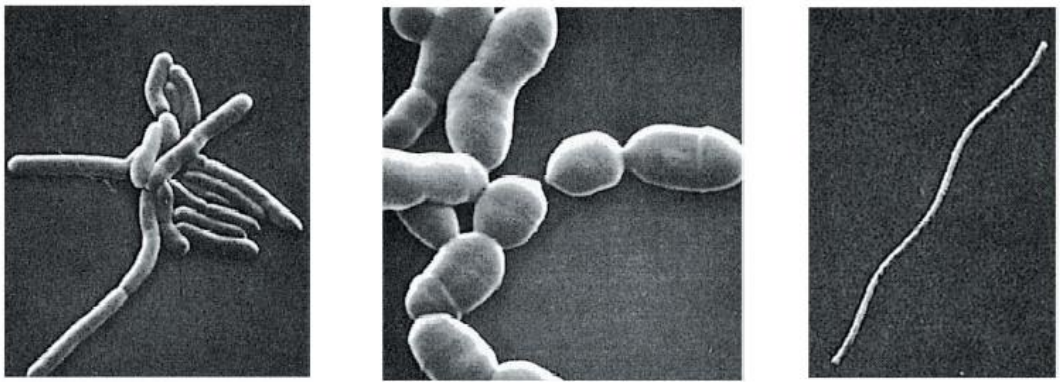


Figure 2.6 : Methanogenic communities (Methanobrevibacter ruminantium, Methanobrevibacter arborphilus, Methanospirillum hungati)[135]

Table 2.6: Methanogenic Classification [29].

Order	Family	Genus	Species	Morphology	Substrate
Methanobacteriales	Methanobacteriaceae	Methanobacterium	M.formicium	Long rods, filaments	H_2,CO_2
			M.bryanti	Short long rods	H_2,CO_2
			M.thermoautotropticum	Long rods, filametns	H_2,CO_2
			M.wolfei	Rods	H_2,CO_2
			M.alcaliphilum	Rods	H_2,CO_2
			M.uliginasium	Rods	H_2,CO_2
			M.thermoformicicicum	Rods	H_2,CO_2
		Methanobrevibacter	M.urbophilius	Short rods	H_2,CO_2
			M.ruminantium	and short	H_2,CO_2
			M.smithii	chains	H_2,CO_2
	Methanothermacease	Methanothermus	M.fervidus	Short rods	H_2,CO_2
			M.sociabilis	Rods	H_2,CO_2
Methanococcales	Methanococcaceae	Methanococcus	M.vannielli	Irregular	H_2,CO_2
			M.voltae	cocci	H_2,CO_2
			M.maripaludis	Single or pars	H_2,CO_2
			M.thermolithotrophicus		H_2,CO_2
			M.halophilius		Methnol, methylamines
			M.jamnaschi	Irregular cocci	formate
			M.deltae		formate
			M.frisisus	Irregular cocci	
Methanomicrobiales	Methanomicrobiaceae	Methanomicrobium	M.Mobile	Short rods single	formate
			M.paynter	Short rods single	
		Methanogenium	M.carinci	Irregular	H_2,CO_2, formate
			M.marisnigri	cocci,single or pairs	H_2,CO_2, formate
			M.olentangyl	cocci,single or pairs	H_2,CO_2, formate
			M.thermophilicum	Irregular cocci	H_2,CO_2
			M.aggregands	Irregular cocci	H_2,CO_2, formate
			M.bourgense	Irregular cocci	H_2,CO_2, formate
			M.tationis	Irregular cocci	H_2,CO_2, formate
		Methanospirillum	M.hungatei	Spirillum, regular rods and filaments	H_2,CO_2, formate
	Methanoplanaceae	Methanoplaneus		Plated shape	H_2,CO_2, formate
	Methanosarcinaceace	Methanosarcina	M.limicla	Pseudosarcina	H_2,CO_2, formate
			M.barkeri	Irregular cocci in large aggregates	
			M.mazei		
			M.thermophila	Pseudosarcina	Acetate
			M.acetivorans	Pseudosarcina, coccoid	Methylamins

Table 2.6: (continued) Methanogenic Classification [29].

Order	Family	Genus	Species	Morphology	Substrate
			M.vacuolate	Pseudosarcina	
		Methanococcoide	M.methylutents	Irregular cocci	Methanol, methylamins, Acetate
		Methanothrix	M.soehengenii	Irrgeular cocci sheat forming long filament	Acetate
			M.concilli	sheated rod	Acetate
		Methanolobus	M.tindarius	Irregular cocci single or loose	Methanol, methylamins

2.2 Anaerobic Digestion of organic solid wastes and Bio-methane recovery

Aerobic and anaerobic treatment techniques (composting, biomethanization) that convert and dispose of solid wastes, are used for decreasing volume, stabilization of wastes and removing of pathogens, process stability, low disposal cost of surplus sludge, low-nutrient requirement, high organic loading rates, net energy production, low production of greenhouse gases. These advantages of anaerobic treatment of solid wastes make this technique economical and effective for ecological point of view [84,106,107]. The treatment of organic fraction of the solid wastes with anaerobic digestion is getting more priority especially in Europe. More than 120 waste treatment plants have been constructed in Europe, which anaerobic digestion plants cover the significant parts [82]. Anaerobic digestion is more favourable compared to other treatment options due to the production of methane which can be used to generate energy [83]. Additionally, stabilized end products of methane which can be used as agricultural and soil conditioning purposes.

Anaerobic digestion processes can be operated under different conditions, which are wet or dry feeding, mesophilic or thermophilic temperatures and single or two phase digestions. Two-stage processes have advantages in terms of separating hydrolysis/acidogenesis and methanogenesis and optimizing each process separately, leading to a higher overall system performance and biogas yield [84]. Moreover, the fermentation products from first stage may provide external carbon stage for the denitrification of the central wastewater treatment plants. Wet anaerobic digestion processes have an operational advantage especially in the transferring the slurry materials between the process units.

2.3 Anaerobic treatment technologies for organic solid wastes

Some pre-treatment and post-treatment processes are requiring for the anaerobic treatment of solid wastes. Magnetic separation, rotary drum, grindering, screening, pulpering, settling and pasteurisation are important pre-treatment techniques. Moreover, dewatering and wet mechanical seperation are post-treatment processes that provide a better recovery

Anaerobic treatment plants, treating solid wastes are complex systems that consist of different processes. Although the substrate characterization and composition are the key factors for determining amount and the quality of the end-products, the design of the anaerobic reactors is also very crucial. Moreover, designs of the anaerobic reactors were also determine the pre and the post treatment requirements. Solid matter percentage (wet and dry systems), reactor numbers (one and two stages) and the operation temperature (mesophilic and thermophilic systems) parameters are used for classifying anaerobic reactors treating solid wastes. Table 2.9 is shows the design and operation limitations for some different processes.

Table 2.7: Design and Operational Conditions

Processes	**Design and Operational conditions**
Mesophilic Wet	HRT: 14-30 day OLR: 2.6-4 kg TVS/m^3.day HRT: 14-30 day OLR: 1-4 kg TVS/m3.day
Mesophilic, Half-Wet	HRT: 15-20 day OLR: 6-8 kg TVS/m3.day HRT: 12-14 day OLR: 3-4 kg TVS/m3.day
Thermophilic, Half-Wet	HRT: 6-15 day OLR: 6-20 kg TVS/m3.day HRT: 12-14 day OLR: 8-12 kg TVS/m3.day
Mesophilic, Dry	HRT: 17 - 30 day OLR: 6 - 9 kg TVS/m3.day HRT: 17-25 day OLR: 3 - 6 kg TVS/m3.day
Thermophilic, Dry	HRT: 12-20 day OLR: 9 – 15 kg TVS/m3.day HRT: 12-16 day OLR: 6 -9 kg TVS/m3.day HRT: 12-16 day OLR: 4 - 6 kg TVS/m3.day

HRT: Hydraulic retention time

OLR: Organic Loading Rate

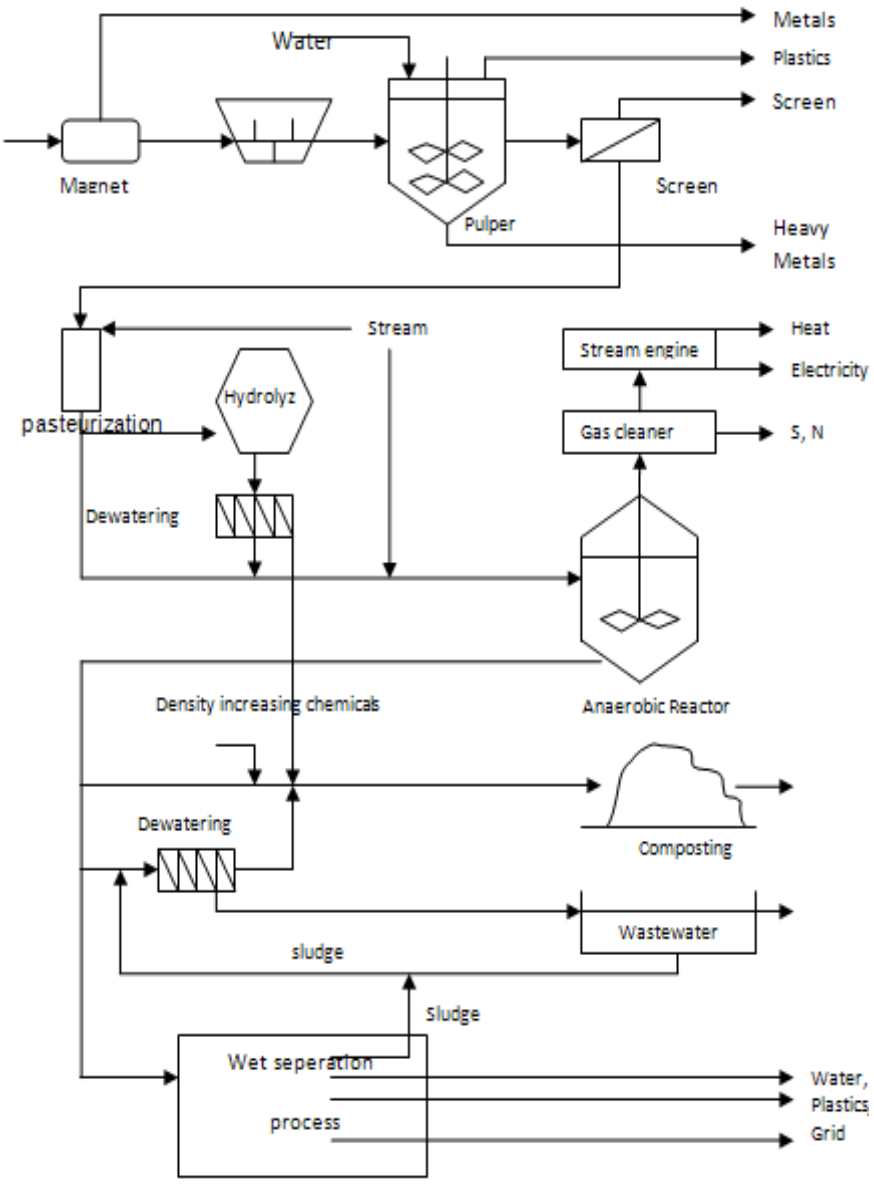


Figure 2.7: Common processes in solid waste treatment [107]

2.3.1 Single –Stage Systems

In single-stage digestion all biochemical processes such as hydrolysis, acidogenesis, acedogenesis and methanogenesis are achive in one reactor but, in two-stage systems reactions take place in two different reactors. Single-stage systems are classified according to their operating conditions into two: “wet (low solids matter) and dry (high solid content).

2.3.2 Two –Stage Systems

Main processes that are occurred in anaerobic treatment period require different optimum environmental conditions. As a result, two or more stage systems have been developed. According to Ghosh and others, optimizing anaerobic treatment processes in different reactors can increase the reaction speed and biogas production [86]. While in the first stage of two -stage systems, hydrolysis and acidification processes are occur. In the second stage acetate and methane production reactions in which slow growing rate of microorganisms have the key role in speed limiting step occur.

Because of these two main processes that occur in different reactors, it is possible to operate the second stage of two stage systems with high biomass concentrations and sludge ages [87]. The main advantage of two-stages is to have a stable performance while treating some substrates, which is not possible in single-stage systems. All two-stage systems provide some protection to the organic loading variations. Nevertheless, because of the high biomass concentrations and high sludge ages in two-stage systems, they are resistant to high nitrogen concentrations and other inhibitors.

Two-stage systems with low sludge age

Simple two- stage systems, especially operating in laboratories are made of two serial CSTR reactors [88]. Properties of these systems is close to single-stage “wet” digesters. Substrates are grinded and diluted to %10 TS content before feeding to first stage.

Schwarting-Uhde Process

Diluted, grinded and %12 TS content bio-waste is treating through perforated plates in Schwarting-Uhde process with two serial CSTR reactor. (Figure 2.10) Horizontal movements inside reactor provide the mixture of the substrate and collect biogas on the top. Because of the perforated plates, this systems are appropriate for non-inert wastes (no inert materials like glass, metal, plastic) and easily biodegradable substrates.

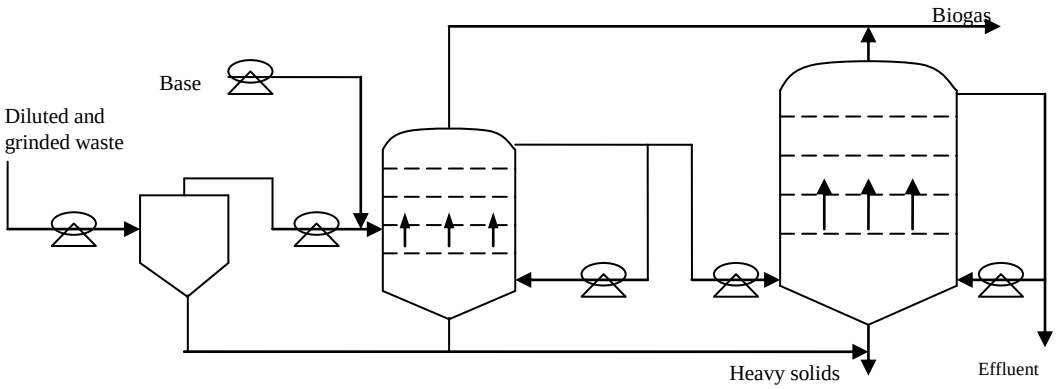


Figure 2.8 . Schwarting-Uhde Process

BRV Process

In first stage of the BRV process solid wastes approximately with %34 TS content fed into an aerobic reactor for hydrolysis process. In this process %2 of waste energy got lost by respiration. The main reasons of making hydrolysis process in micro-aerobic conditions are to solubilize (hydrolyze) particulate COD into soluble COD more than losses COD in process [89]. After 2 days hydrolysis period waste is taken into methane reactor. Methane reactor operate under thermophilic conditions (55^{o}C') and with %22 TS content waste, it proceed 25 days. Main advantages of the process is small reactor volumes because of the high TS content of feeding and complete hygienization because of plug flow in system.

Two-Stage systems with high sludge age

Recycling suspended solids inside the effluent flow of the second stage is a way to increase the sludge age of the two stage systems. This can done with integration of an internal settling inside the reactor or a membrane system. However, if the inert matter amount inside the substrate is more than 15% of all, accumulation of inert matters decrease the active biomass portion, increasingly with time.

As a result, this method is better in use with wastes that have high biodegradability properties and wastes that can hydrolyze easily [90]. Another way to increase sludge age is using filling materials in the second stage of system to provide biofilm formation. So, system can reach high sludge ages and high biomass concentrations. Two-stage systems with high sludge ages have a crucial disadvantage is that: the necessity of low suspended solid content in the influent flow of second stage. Thus, after hydrolysis stage, suspended solids have to be removed in the effluent. Two processes have developed according to this principle stage; suspended solids have to be removed in the effluent. Two processes have developed according to this principle.

BTA Process

In BTA process %10 TS content effluent wastes from pulper first pasteurized and dewatered. The supernatant part of the dewatered waste sent to methane reactor [91]. Solid part of the waste is saturated with process water and hydrolyses in a CSTR reactor operated with mesophilic conditions ad with 2-3 days hydraulic retention time.

For regulating hydrolysis reactor's pH to 6, 7, methane reactor's process water is recycled. The effluent flow of the hydrolysis reactor dewatered again and supernatant flow is directed to methanization reactor. Methanization reactor can be design as a biofilter, because of low TS content influents.

Bioercolat Process

Instead of BTA process, in Bioercloat process hydrolysis step is occur with "dry" and micro-aerobic conditions. Process water continuously recycled over substrate inside the reactor for increasing the process speed [92]. Obtained wastewater fed into a reactor that is design as a biofilter and has a plug flow characteristic. Having an aerobic hydrolysis reactor and a biofilter provides short hydraulic retention times in system like 7 days.

3. CHARACTERIZATION OF MICROBIAL COMMUNITIES

3.1 Molecular Ecology

Phylogenetic view, which is gained due to sequencing technology, is significant for microbiology. Proper nucleic acid sequence assessment is the most essential and clear method for categorizing and relating the organisms [30]. As long as the basic biochemical properties and potentials of the organisms are identified, members of the phylogenetic population can be examined [30]. The cloning and sequencing of 16S genes directly from the environment using specific or general PCR primers (16S surveys), it shown that microbial diversity is far more extensive than culture-based studies. However, conventional microbiological techniques, based on isolation of pure cultures and morphological, metabolic, biochemical and genetic assays, also have provided extensive information on the biodiversity of microbial communities [28].

Studies of microbial ecology, diversity, and evolution have always been intimately tangled. Carl Woese and his group showed that microbial forms were dominated the main lineages of life [32]. Researches on ribosomal RNA (rRNA) sequences indicated that all cellular life belonged to one of the three domains, Bacteria, Archaea and Eukarya [28]. According to the Woese, comparative sequence analyses allowed the definition of the major lineages (phyla or divisions) within the three primary domains. However, this is not providing the original phylogenetic structure that had been until now lacking in microbial [34, 35]. But there was a handicap, Brock states that ecological inferences based on the metabolic properties of cultivated bacteria are, unrepresentative of the natural populations from which they were obtained [35].

Although the biases of cultivation-based approaches were recognized by Winogradsky, uncultivated studies newly approaches as stated in Figure 3.1 [35]. Researchers like Zuckerkandl, Pauling and Woese combine molecular methods and microbial phylogeny for support the identity of uncultivated bacteria to be determined. Norman Pace's group, in India was one of the first researchers combine phylogeny with molecular biology and started to study as molecular microbial ecology is now known [38,39].

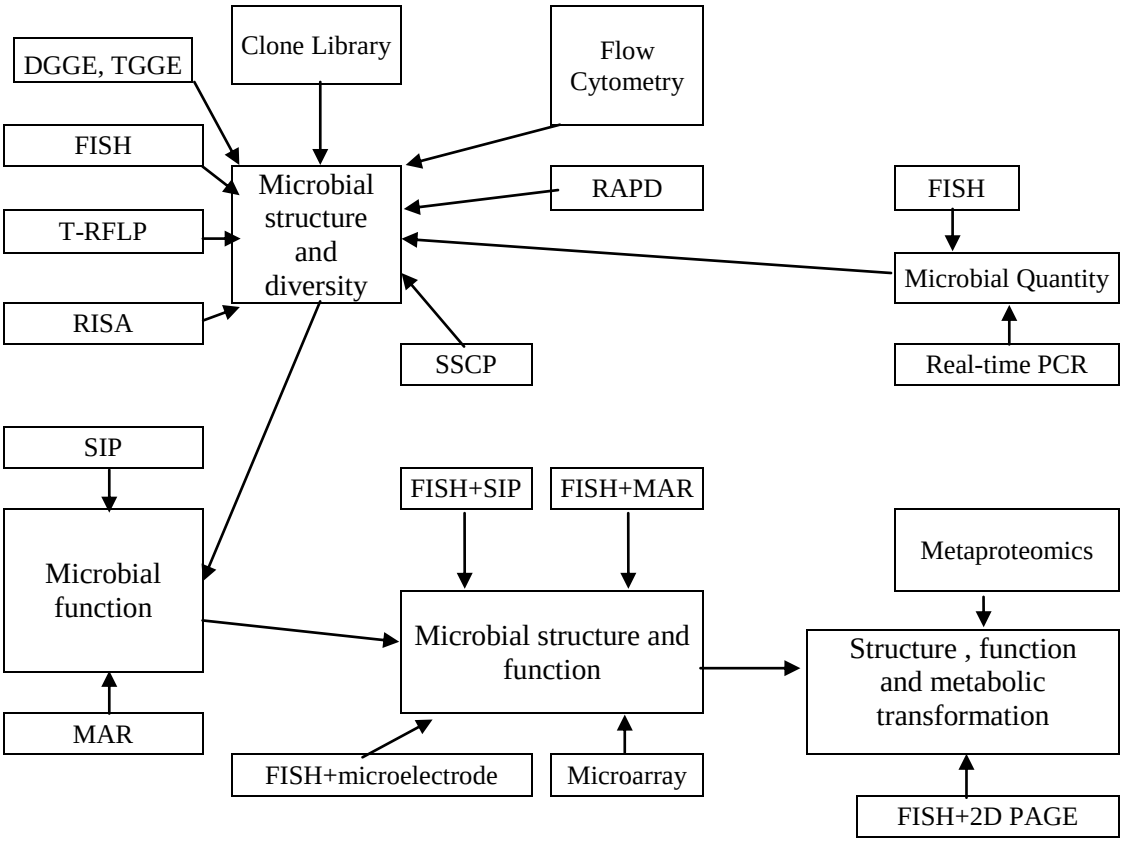


Figure 3.1 : Molecular and post genomic techniques for analysis of microbial community structure function and metabolic transformation used in microbial ecology

3.2 Microbial Phylogeny

16S ribosomal RNA (16S for short) holds a special place in the study of microbial evolution and ecology. 16S ribosomal RNA brought two important reforms into the microbial ecology. First, it brought a new point of view into the cellular life and its classification [32]. Second, is the cloning and sequencing of 16S genes directly from the environment using conserved broad-specificity PCR primers (16S surveys [41]. The three domains of life are bacteria, archaea, and eukarya (Figure 3.2) [42]. Bacteria, along with actinomycetes and cyanobacteria (blue-green algae) belong to the prokaryotes while eukaryotes or eukarya include fungi, protozoa, algae, plant, and animal cells.

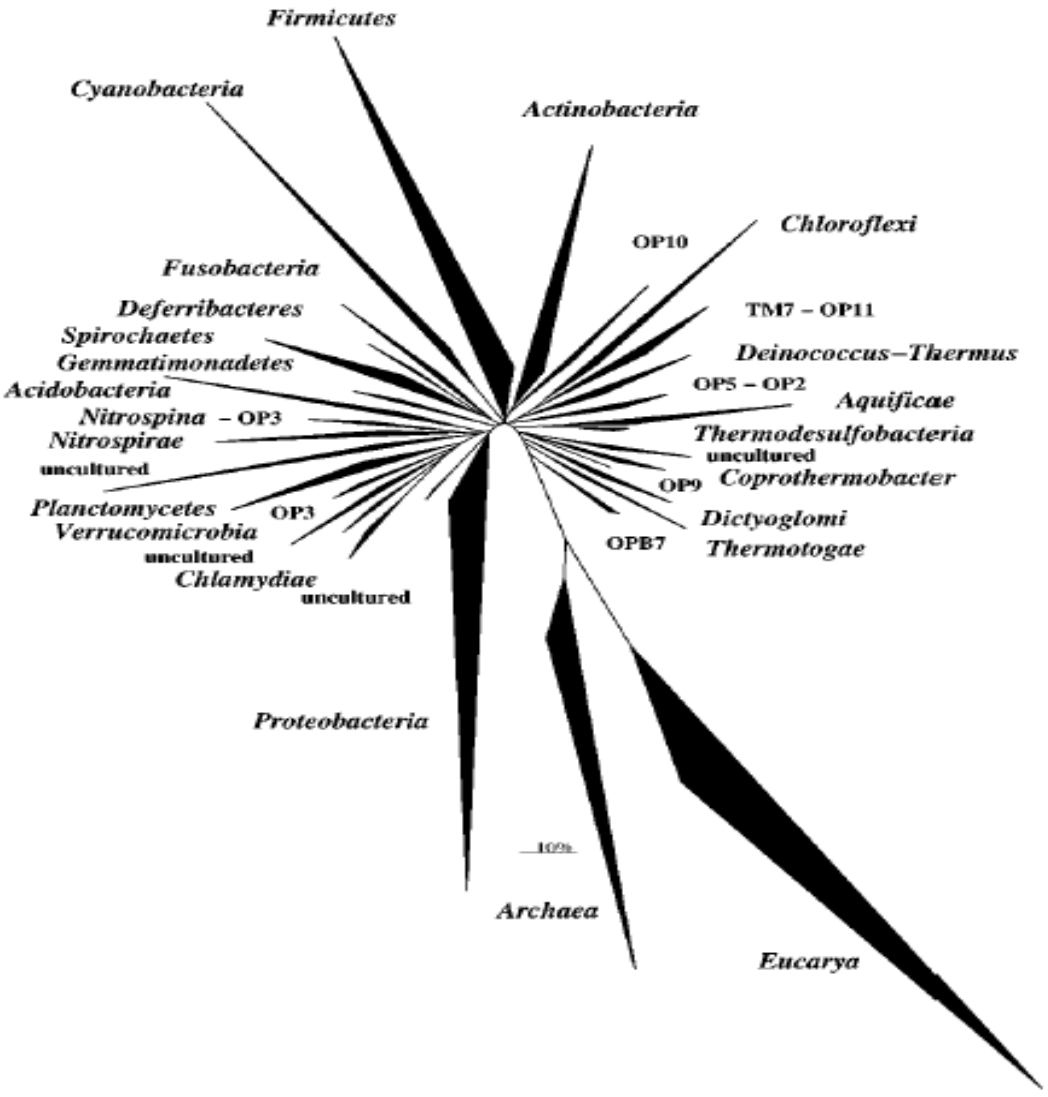


Figure 3.2 : Three domains of life are bacteria, archaea, and eukarya [44]

3.3 Molecular Tools

3.3.1 Ribosomal RNA gene sequences

Nucleotide sequence comparison of ribosomal RNAs (rRNAs) is phylogenetic, population members are related to known organisms in terms of their fundamental biochemical properties and potentials. Gary and his group maintains macromolecular comparisons may be based either on experimental measurements of "molecular match" (e.g. antibody cross reactivity, DNA-DNA hybridization, and ribosomal RNA-DNA hybridization) or on mathematical analyses of molecular sequence data [45]. Today, complex microbial communities can generally analyzed by rRNA targeted nucleic acid probes.

These tools are directly target the 16S/18S rRNA of the small subunit of the ribosome (SSU rRNA) or to the 23S/28S rRNA of the large subunit of the ribosome (LSU rRNA) [32].

Moreover, the 5S and 16S rRNAs have been used most frequently for rRNA based phylogenetic characterizations [49]. In addition, Olsen states that 5S (-120 nucleotides), 16S (-1600 nucleotides), and 23S (~3000 nucleotides) are the three rRNA’ s in all bacteria [50]. However, the lack of independently varying nucleotide positions in the 5S rRNA molecule has limited its phylogenetic usefulness [38]. Table 3.1 show the similarity of techniques, which are targeted 5S and 16S rRNA respectively.

The rRNAs are essential components of the protein synthesis machinery and are therefore widely distributed and functionally conserved in all organisms. They are antique molecules and are extremely conserved in overall structure, making them readily isolatable and identifiable. Madiak mentioned large amount of rRNA in most cells; the apparent lack of lateral gene transport; and a good length of about 1500 and 3000 nucleotides for 16S and 23S, respectively, with a range of very conserved to quite variable sites [46]. Furthermore, 16S rRNA sequences have been determined for a large fraction of the authentically described species and their natural amplification with high copy numbers per cell greatly increases the sensitivity of rRNA-targeted probing [49]. However, there is a limitation about rRNA-targeted analysis that rRNA's does not provide a direct relation to physiology. Wagner and his group state that populations that are identified from rRNA target sequences expected to share metabolic features with close relatives characterized by results of pure cultures. However, interference can be occurred for more distant relatives of the identified organism [110]

In the present day with the ARB, rRNA-targeted oligonucleotide probes can be designed and. organisms (database entries) or (phylogenetic) groups can be chosen by mouse click or search tools [47].

Table 3.1: Flow charts for (a) 5S rRNAs and (b) 16S rRNA genes from natural populations

Population analysis using 5S rRNAs genes	**Population analysis using 16S rRNA genes**
Biomass (Mixed population)	Biomass (Mixed population)
↓ break cells, phenol extract	↓ break cells, phenol extract
Bulk RNA	Total DNA
↓ low-resolution polyacrelamide gel eloctrophoresis	↓ "clone" into bacteriophage lambda
Mixed 5S rRNAs	Recombinant DNA Library
↓ 32p end-label RNAs, polyacrelamide gel electrophoresis	↓ screen by hybridization with "mixed kingdom" 16S rRNA probe
Purified 5S rRNAs	16S rRNA gene clones
↓ determine nucleotide sequence, compare with other 5S rRNA sequences	↓ Sequence with 16S rRNA-specific primers; cmpare with other 16S rRNA sequences
PHYLOGENETIC CHRACTERIZATION OF POPULATION MEMBERS	PHYLOGENETIC CHRACTERIZATION OF POPULATION MEMBERS

Advantages of rRNA according to other phylogenetic targets are:

The rRNAs, as key elements of the protein-synthesizing machinery, are functionally and evolutionarily homologous in all organisms.

The rRNAs are ancient molecules and are extremely conserved in overall structure. Thus, the homologous rRNAs are readily identifiable, by their sizes.

Nucleotide sequences are also conserved. Some sequence stretches are invariant across the primary kingdoms, while others vary. The conserved sequences and secondary structure elements allow the alignment of variable sequences so that only homologous nucleotides are employed in any phylogenetic analysis. The highly conserved regions also provide convenient hybridization targets for cloning the rRNA genes and for primerdirected sequencing techniques

The rRNAs constitute a significant component of the cellular mass, and they are', readily recovered from all types of organisms for accumulation of a data base of reference sequences

The rRNAs provide sufficient sequence information to permit statistically significant comparisons.

The rRNA genes seem to lack artifacts of lateral transfer between contemporaneous organisms. Thus, relationships between rRNAs reflect evolutionary relationships of the organisms.

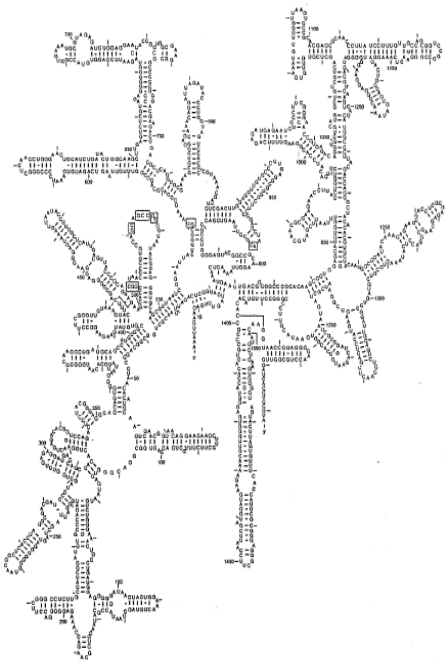


Figure 3.3 : Primary structure (base sequence) and secondary structure (hyrogen bonding and folding) of the 16S rRNA from E.coli.

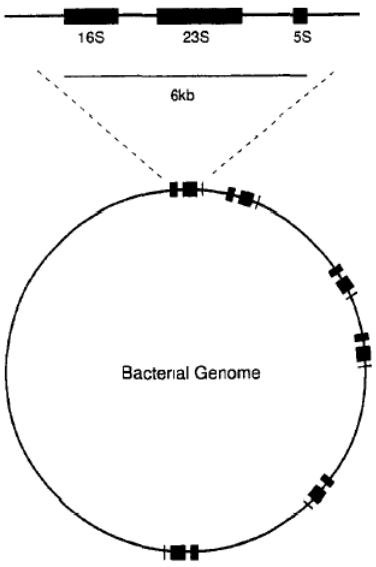


Figure 3.4 : Schematic diagram of a ribosomal RNA operon (rrn) showing the relative positions of the genes encoding 16S, 23S, and 5S rRNA [52]

3.3.2 Amplification of the SSU rRNA genes using Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a primer-based enzymatic amplification of specifically cloned or genomic DNA sequencer and is used to amplify the double stranded DNA [111]. In PCR amplification, buffered reaction is catalyzes by a thermostable DNA polymerase such as *Taq* DNA polymerse and an excess of an oligonucleotide primer pair and four deoxynucleoside triphosphates (dNTPs) are use to create millions of copies of the target sequence [112]. In this technique, the target DNA is first melted, then the set of primers are annealed and elongated. Repeating this cycle for about 20-35 times, millions of copies of DNA can be obtained at the end of process. Innis and Gelfand stated from 40– 45 cycles to amplify 50 target molecules, and 25–30 cycles to amplify 3 × 105 molecules to the same concentration [111]. Although PCR products are mainly used for successively performed molecular tools due to the requirements of a high number of copies of DNA, they are also used to check whether the presence of organisms even as a species level in the environmental samples. Specific primers have been used to amplify fragments of rRNA operons and other genes in order to detect the presence of specific organisms or groups of organisms in clinical specimens, foodstuffs, and environmental samples.

PCR plays an essential role in most molecular techniques, which are required high nucleic acid amounts to perform. Thus, minimizing biases in PCR amplifications increase the accuracy of these techniques.

PCR-associated problems are; differential amplification of different rRNA templates, sensitivity to profeeding activity of taq enzyme [113], RNA gene copy number, primer specificity, sensitivity to template concentration, amplification contaminant rRNA, formation of chimeric sequences, the secondary structure of the target, G+C content of the target [53]. However, several methods were developed to prevent these PCR biases [56]

Especially, for preferential amplification, it was suggested to

- Exclude the degenerated primers in the case of using domain level primers [56]
- Use high concentration of target DNA [56]
- Mix the separately amplified same PCR mixture and [56]
- Decrease the number of amplification cycles [54]
- For other important bias, the formation of chimeric sequence, it was suggested to keep the longer elongation time [55]
-

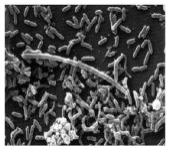
These factors also bring the question whether the PCR based molecular analysis precisely reflects the microbial diversity of different habitats and consequently the phylogenetic based taxonomy. For that reason, especially planning the experimental procedure and interpreting the results of PCR based molecular tools, and the abovementioned factors should be considered very carefully [56]

3.3.3 Cloning, sequencing and phylogenetic analysis

Cloning and sequencing of the gene codes of the 16S rRNA is the most widely used molecular technique in molecular ecology [114]. It has a large application range from ecology to medical researchers. Sequencing is a way to identify the biodiversity in environmental samples and dominant species. Woese et al.stated that the most appropriate way to determine the phylogenetic relationship from sequence data is to construct phylogenetic trees [32]. Giovannoni et al. (1990) is the first researcher who used cloning and sequencing technique to target 16S rRNA for marine ecology [170]. Cloning and rRNA gene library approaches are very effective and powerful when applied with other techniques such as DGGE, FISH and quantitive PCR. Cloning method allows obtaining full 16S sequence of the species in sample that have not been yet cultured or identified and covers most of the microorganisms inside the sample including minority communities, which are not easy to detect with other techniques. Although it has many advantages beside other techniques, in high diversified ecosystems, cloning-sequencing approach can be very laborious, time-consuming and expensive [150].

Molecular cloning is theoretically to isolate large quantities of specific genes or chromosomal fragments in pure form [116]. Cloning technique has consisted of several steps such as cloning, screening, sequencing and construction of the phylogenetic tree.(0)

Many cloning methods have been established for cloning. Thus, these methods are separated into two due to ligation properties such as, ligation dependent and ligation independent. In ligation dependent cloning firstly, extracted DNA’s are amplified with specific primers to attain targeted size and genes of interest. Then, amplified fragments in other word inserts are ligated into recombinant plasmid vectors. Different methods can be use according to DNA ends of the inserts such as blunt-end cloning, sticky-end cloning, and T-A cloning [171].

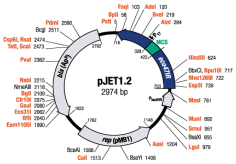


Environmental Sample →

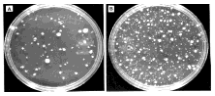
Homogenize sample & isolate DNA

← PCR amplification

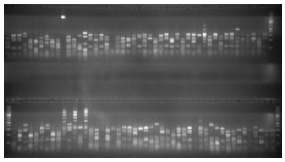
→ DNA Purification



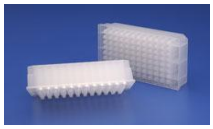
← Ligation of PCR products



→ Transformation into E.coli cells



← Selecting different clones



→ Plasmid Isolation

Figure 3.6 : Flow chart for the recovery, purification, and cloning of amplified DNA from environmental microorganisms.

Plasmids have some encoding functions that might not found in chromosome like antibiotic resistance genes so that they add important features to bacterial genetic diversity and plasticity [172]. Moreover, like chromosomes, they are replicated during bacterial division and each formed cell has at least one plasmid copy in their cytoplasm [173]. These features make plasmid vectors much preferable in cloning.

After then, ligated vectors are transformed into host organisms. E.coli widely used as a host organism, because of its rapid growth rate, ability to grow on chemically defined media and it is completely identified [174]. E.coli cells are grown on selective ager plates and screened in order to their plasmids abilities. After that, an additional screening is done for selecting different inserts, which can be done by different fingerprint methods such as Amplified Ribosomal DNA Restriction Analysis (ARDRA) [59]. and the Restricted Fragment Length Polyphormism (RFLP) [60].

These methods examined in following chapters. Selected clones were sequenced and compared with other sequences. A comparison was done with BLAST database provided by the computer programs, which are available on internet sites and determine the closest relatives. Blast approach is so widespread, approximately 108,431,692 sequences deposits in the NCBI database.(august 2009) [175].The comparison of the sequences with this database using computer programs is fast and reliable, which is very important in terms of finding the highest homology. The results of highest homology obtained do not clearly determine the relationships between the species when many sequences are compared [56]. The similarities and dissimilarities in phylogenetic trees can be visualized without difficulty [62]. Generation of phylogenetic trees can occur in two different ways, as distance matrix or as discrete character data [56]. Distance matrix calculations are based on evolutionary distances and the discrete character data calculations determine the nucleotide position independently [63].

3.3.4 The DGGE and the TGGE

DGGE technique based on the differing mobility on a gel of same size denatured DNA fragments but at least by one nucleotide can be separated by electrophoresis through a linear gradient of increasing chemical denaturants of urea and formamide. Thus, created band patterns directly reflected the specific genetic biodiversity of the samples [114, 115]. Electrophoretic mobility of partially melted DNA molecules in polyacrelamid gels cause separation in DGGE. Differentiation in completely helical form of the molecule of 16S rDNA genes from genomic DNA differ electrophoretic mobility of DNA molecules in polyacrelamid gels [64]. Polyacrylamide gels form after polymerization of monomeric acrylamide into polymeric polyacrylamide chains and cross-linking of the chains by N,N′-methylenebisacrylamide.

The polymerization reaction is commened by the addition of ammonium persulfate, and the reaction is accelerated by TEMED, which catalyzes the formation of free radicals from ammonium per sulfate [109]. DNA amplicons stop migrating at different levels of the gel, thus get separated. This separation, if it is based on linear gradient of denaturants at constant temperature is called DGGE [64], if based on linear temperature gradient at constant denaturants is called TGGE [65].DNA bands in the DGGE and the TGGE profiles can be visualized by ethidium bromide, SYBR Green I or silver staining [66].

Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) were used to obtain qualitative and semi-quantitative estimations of biodiversity [64]. Adding GC-rich sequences of approx.40 nucleotides to the 5'-end of the forward primer or the 3'-end of the reverse primer modify the melting behavior of the target fragment and close to % 100 of all possible sequence variations can be detected [64, 67]. The amplicon size is also limited with the 500 base pairs. Larger fragments are typically not used as the DGGE technique cannot resolve these into distinct bands [64]. Muyzer et al is the major pioneer of using PCR-DGGE technique in microbial ecology researches [64]. DGGE method became popular in other community ecosystem researches for monitoring the diversity changes and population shifts after his studies [68].

The ability of monitoring population shifts in DGGE is very important in environmental ecology researches for its time dependent community changes linked with changes in pH, feeding, HRT, temperature, mixing, acclimation and biomass concentration in engineered reactors. Further more, this method has a large usage to analyze the enrichment in cultures and the isolation of bacteria; the comparison of DNA extraction methods, the screening of the clone libraries and determination of PCR and cloning biases [69]. PCR approach can multiply and specify the gene which is allow to specify the species of interest. Using PCR specifity on genes, DGGE can monitor changes in target species or activity of interest. Moreover, when PCR is applied with universal primers and products are separated by DGGE, predominant bands can be cut out from the profile in polyacrelamide gel and subsequently subjected to re-amplification and sequence analysis. Beside cutting bands from gel, hybridization with oligonucleotide probes after blotting the DGGE profiles onto nylon membranes is an another way for understand the diversity or activity [64].

Although DGGE technique has a limitation in fragment size, it is possible to obtain complete sequence of species by constructing 16S library, which is obtained from combining DGGE with cloning, and sequencing technique [70]. If all bands in one profile are needed to be identified, it would be very laborious in terms of cloning each band fraction [56]. DGGE with DNA based PCR is analyze the diversity and change in diversity. To identify the active communities, RNA copies of the 16S rRNA were used in some studies [71].

3.3.5 Terminal restriction fragment length polymorphism(T-RFLP)

Terminal restriction fragment length polymorphism is a comparative rRNA fingerprint technique based on differences in restriction endonuclease (RE) cut sites in different same sized fragments that successfully applied environmental samples, such as agricultural soils[135,136], grassland [137], biological soil cuts [137, 138], activated sludge communities [139]. and wastewater communities [140]. T-RFLP in other name (ARDRA) is a rapid and semi automated fingerprinting technique [141,142]. Like other PCR-based techniques, a PCR is amplified to samples with fluorescence-labeled primers for determining specifity, length size and labeling of the fragments.

Each amplified fragment is digested with restriction enzymes with 4 base pairs recognition sites for gaining one-end labeled terminal restriction fragment (T-RF). According to Engebretson, by selecting the appropriate number and types of the restriction enzymes, accuracy of T-RF arrays which reflect the natural diversity of microbial communities in sample, can increase[143]. After digestion, T-RF's are separate on agarose gel and visualized as a peak.

The profile of T-RF's give an approximate number of phylotypes and, fluorescence intensity of peaks give the theorical amount of each phylotypes [144]. Profile dates analyzed by statistical methods include; similarity indices, hierarchical clustering algorithms, principal-component analyses, and self-organizing maps [143]. When these information combined with sequence information. T-RFLP became a powerful tool for environmental studies [145]. T-RFLP has a ability to detect less abundant taxa in communities. This make T-RFLP more sensitive over other fingerprint techniques such as DGGE and SSCP [146]. Although T-RFLP have very large application areas in environmental researches, it have some limitations including;

- If normalization procedures not applied, variations in the DNA loads can affect the profiles [148]
- Loss of small DNA fragments due to purification of samples [143].
- Difficulty in assigning accurate identity to each T-RF in complex profiles of 16S rRNA genes [147]
- RFLP can use only sequence information from restriction sites. This gives a limited resolution in identifying a specific phylogenetic group within a complex community [149].

3.3.6 Single-strand conformation polymorphism analysis (SSCP)

Single-strand conformation polymorphism analysis (SSCP) was first used in samples from natural habitat for evaluating the microbial diversity [149]. In SSCP, small same-sized PCR fragments with 100-300 bp., but different sequences are separated into different bands in a non-denaturing polyacrelamide gel due to its different mobility in gel, because of its altered folded structure [149]. Separation of fragments can detected by auto radiograms (radioactive detection), silver staining, ethidium bromide staining [150]. or amplifying DNA fragments with fluorescently labeled primers in PCR for automated DNA sequencer (non-radioactive detection) [150]. PCR-SSCP can detect very short changes (a few to 20-base) in fragment sequences in contrast to some other fingerprint techniques. But, the detection sensivity get decrease, when the lengths of the fragments are increase [151].

There are a few studies that SSCP was applied such as constructed wetlands [152], activated sludge's [153], and anaerobic reactors [154]. In these studies, SSCP was used for investigating the effect of substrate changes on the bacterial populations [154].

3.3.7 Stable Isotope Probing (SIP)

Stable isotope probing (SIP) is a culture –independent technique that allows identification of specific functional and active microbial groups using particular substrates [130]. Boschker and his group first used stable isotopes as a cellular biomarkers for identify microbial communities which are responsible for acetate oxidation in aquatic sediments [131]. C^{13}, N^{15}, H^{2} labeled compounds used by active groups of interest in anabolic process such as generating nucleic acids, and turn them into cellular biomarkers. SIP is a density separation based technique that separating labeled nucleic acids (DNA or RNA) from unlabelled by density gradient centrifugation, for obtain phylogenetic and functional information of active groups [130, 132].

Separated biomarkers are fingerprinted by DGGE or T-RFLP and characterized by sequence analysis of the SSU rRNA genes [133]. Either DNA or RNA can be used in SIP experiment. For DNA-SIP, isotopic enrichment is very important.

DNA synthesis rates in other name, replication of labeled cells are a limiting step for DNA based SIP [133]. When compared with DNA-SIP, RNA based SIP technique assimilates C^{13}-labelled substrate more efficient. RNA's have faster production according to DNA's in cell [133,134]. The first RNA-based SIP was applied on aerobic phenol-degrading community in an industrial wastewater treatment plant [133]. There are three important limitations to the application of the SIP method.

- dilution of a labelled substrate before incorporation in the cells [56]
- utilization of the labelled substrates by different trophic groups of microorganisms [56]
- the inability of reflecting the exact in situ conditions[56]

3.3.8 Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a hybridization-based technique that using 16S rRNA probes for determine microbial community composition and mRNA probes for determining specific metabolic activity, without PCR amplification [155,156]. This technique is directly applied to intact cells and gives information about their localization, morphology and abundance without the limitation of culture-dependent methods [56]. FISH technique allows supplementing the total cell count [157]. However, variations in absolute RNA content of each cell between the different community members allows a approximate quantification [47]

FISH technique consists of five main steps (0): (i) fixation, to permit visualization and permeabilization of the cells; (ii) preparation of the sample; (iii) hybridization with the respective probes for detecting the respective target sequences; (iv) washing steps to remove unbound probes; (v) mounting, visualization and documentation of results [156]

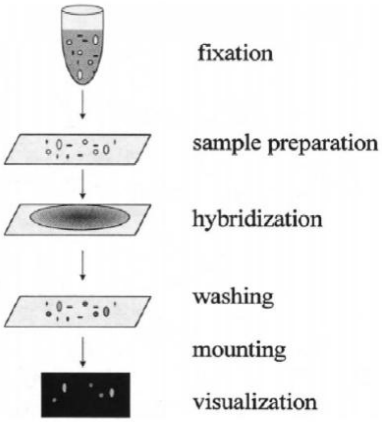


Figure 3.6 : Flow chart of a typical FISH procedure [156].

3.3.9 Microarrays

For understanding the microbial diversity with environmental factors and ecosystem complexity, it is important to characterize the functional group of interest and analyze the activity of individual members of the community under different environmental conditions [117]. Researchers used quantitive methods like a reverse transcriptase quantitive polymerase chain reaction (RT-PCR) and Northern blots for evaluating gene expression levels more than 20 years [118,119]. Microarray technology has great potential for characterizing microbial community, evaluating their function in the environment [120]. and gene expressions over other methods. It also enables to study of the interaction between different molecular pathways, identification and genotyping of bacteria based on genomic DNA-DNA similarities [117], population genetics [117], and detection thousands of genes (about 20.000-40.000) [121]. with probes in a single experiment. Patrick Brown's group was the first researchers to print arrays of PCR fragments amplified from cDNA on a glass surface the size of a standard microscope slide using a robotic printing tool [122]. Microarrays have a great sample throughput and parallel usage of hundreds to thousands of carrier-bound DNA probes in the basis of hybridization process [123]. Microarray is the combination of powerful amplification methods with hybridization oligonucleotide probes that is specific for mutable target sequences [124]. Oligonucleotide microarrays (DNA-chips) are produced by a light directed chemistry and each chip need a large number of photolithographic masks [125]. The chips surface contains thousands probes and targets (DNA, RNA samples) that labeled with fluorescent dyes for hybridization.

After hybridization, fluorescent dye activated by laser light and emission amount from fluorescent dyes indicated the hybridization intensity that gives the estimate of the relative amounts of the differentiation in gene composition with high sequence identity [123, 117].Although microarray has great potential in environmental ecology, it have some limitations in use. These are;

- obtaining a sufficient amount of mRNAs from environmental samples for analysis[126],
- homologous genes from many different organisms that may share a high degree of sequence similarity.

- limited number of probes and are mainly used for method development and optimization[127],
- expense of microarray printing and imaging equipment [128],
- the time and labour required for manual sample handling, nucleic acid purification, and associated volume reduction [128],
- inefficient purification or concentration of nucleic acids at low target concentrations [128],
- co extraction of inhibitory compounds that interfere with subsequent molecular manipulations, especially PCR[129]

3.3.10 Quantitative real time PCR

Conventional PCR techniques are not quantitative, but modifications to primers and measuring devices make real-time PCR a quantitive technique. Real-time PCR was first develop in the early 1990s [158]. In principle, Real-time PCR is combined with a fluorescence labeled probe or a specific, intercalating dye to measure the fluorescence changes after each cycle or continuously [159, 56]. DNA standards and test samples are amplified with samples at the same time and their Ct values are obtained and compared for constructing standard curve. Starting amount of template DNAs in samples are calculate using standard curve [160]. Real-time PCR target the 16S rRNA genes due to specific probes. This technique allows rapid quantification of minimum 24 samples in 1 to 2 hour in 96 –well format [160].

Several quantitive PCR methods were applied to detect populations in environmental samples. In complex environmental samples that studying population dynamics the DNA extraction method in real-time PCR requires a uniform DNA recovery and high output [161].

3.3.11 Comparisons of molecular techniques

Advantage and disadvantages of molecular techniques in microbial ecology are summarized in table 3.2.

Table 3.2: Summary of molecular techniques for microbial ecology

Technology /Approach	DNA/ RNA Extr.	PCR	Pattern Analysis Fingerprint	Advantages	Disadvantages	Ref.
Fluorescence in situ hybridization (FISH)	No	No	NA	▪ Direct analysis and quantification ▪ No heterogeneity problem ▪ Suitable for targeting specific group/species	▪ Requires genes/RNA with high number of copies ▪ Limit for total diversity mapping	[162]
Microarrays	Yes	No/Yes	NA	▪ Suitable for mapping total diversity/patter analysis ▪ Further sequencing not required to identify group/species ▪ High sensivity ▪ Fast and high throughput ▪ Not laborious	▪ Do not reveal unexpected species, except using more probes	[163]. ,[164], [165]
Denaturing gradient gel electrophoresis (DGGE)	Yes	Yes	Melting	▪ Suitable for pattern analysis or microbial community diversity ▪ Further sequencing not required, except using a non-heterogeneous gene	▪ Can produce multiple bands from one template with bias PCR of heterogeneous genes(e.g. 16S) ▪ Lacks resolution ▪ Needs sequencing for species ▪ May have PCR bias problems ▪ Cannot actually indicate species richness	[164] [166] [167] [162]
Tempreture gradient gel electrophoresis (TGGE)	Yes	Yes	Melting	▪ Suitable for pattern analysis	▪ Lacks resolution ▪ Needs sequencing ▪ May have PCR bias problems ▪ Cannot actually indicate species richness	[164]
Terminal restriction fragment length polymorphism (T-RFLP)	Yes	Yes	Size	▪ Suitable for pattern analysis ▪ One band per species ▪ High resolution ▪ Sensitive detection	▪ Restriction enzyme selection with unknown sequences ▪ May have PCR bias problems ▪ Cannot actually indicate species richness ▪ Some species may show the same length of fragments	[164]
Clonnig and sequencing	Yes	Yes	NA	▪ Contains larger sequences ▪ Provide more positive ID	▪ A large number of clones must be sequenced for positive diversity ▪ Bias from PCR ▪ Sequences need to compare with each other and libraries ▪ Laborious and time consuming	[164] [168] [162]
Amplified rDNA restriction analyses (ARDRA)	Yes	Yes	Size	▪ Suitable for screening clone libraries or isolates before sequencing	▪ Not suitable for pattern analysis ▪ Produces multiple bands for one species ▪ Cannot actually indicate species richness	[164]
Restriction fragment length polymorphism (RFLP)	Yes	Yes	Size	▪ Suitable for screening clone libraries or isolates before sequencing	▪ Not suitable for pattern analysis ▪ Produces multiple bands for one species	[164]
Single-stranded conformation polymorphism (SSCP)	Yes	Yes	Size	▪ Suitable for screening clone libraries or isolates before sequencing	▪ Not suitable for pattern analysis ▪ Produces multiple bands for one species	[164]
Real-Time PCR	Yes	No	NA	▪ Rapid	▪ Laborious	[169]
Dot-Blot hybridization	No	No	NA	▪ Direct analysis	▪ Laborious at species level ▪ Requires sequence data	[169]

4. MATERIALS AND METHODS

4.1 Operation of the reactors

4.1.1 Inoculums

As seed microorganisms, inoculum were collected from the anaerobic sludge digesters of Tuzla Wastewater Biological Treatment plant and inoculated into fermentation and methanization reactors of this study. The characterization of the collected flocculant sludge which was acclimated to the biological sludge was given in the Table 4.1.

Table 4.1: The characterization of the Tuzla WWTP digester sludge

Parameter	Inoculum
pH	7.0±5
Temperature(^{o}C)	36.0 ±0.5
TVFA(mg/l)	142.0±0.5
TS(mg/l)	30.0±0.2
TVS(mg/l)	14
COD(mg/l)	23.290

4.1.2 Reactors

Biomethanization pilot plant is a two-stage wet system and consists of mainly five physical and biological processes such as grinding, pulping, fermentation, digestion and settling.

Grinder: Dinner hall and vegetable hall wastes generally consist of large particles that have to be grind into smaller particles to increase substrate surface for easily hydrolyze and degrade. Substrates surface areas regulate the hydrolysis speed by increasing the active sites that enzymes easily connect to substrate and form substrate-enzyme formation. Grinder was used mainly for increasing the surface area of the substrate in this research. The capacity of the grinder is 500 kg per hour.

Figure 4.1: View of the Grinder

Pulper: Pulper reactor was used to mix, homogenize and dilute the grinded wastes. In wet systems (<%10 TS), solid wastes that have %20-%50 TS content have to be diluted before entering the process flow. The solid content in this study was diluted with clean water. For making grinded substrate homogeneous, increasing its solubility and adjust the solid matter content, 750L volume pulper is used with a active volume of 580L and with mechanical mixer in pilot plant.

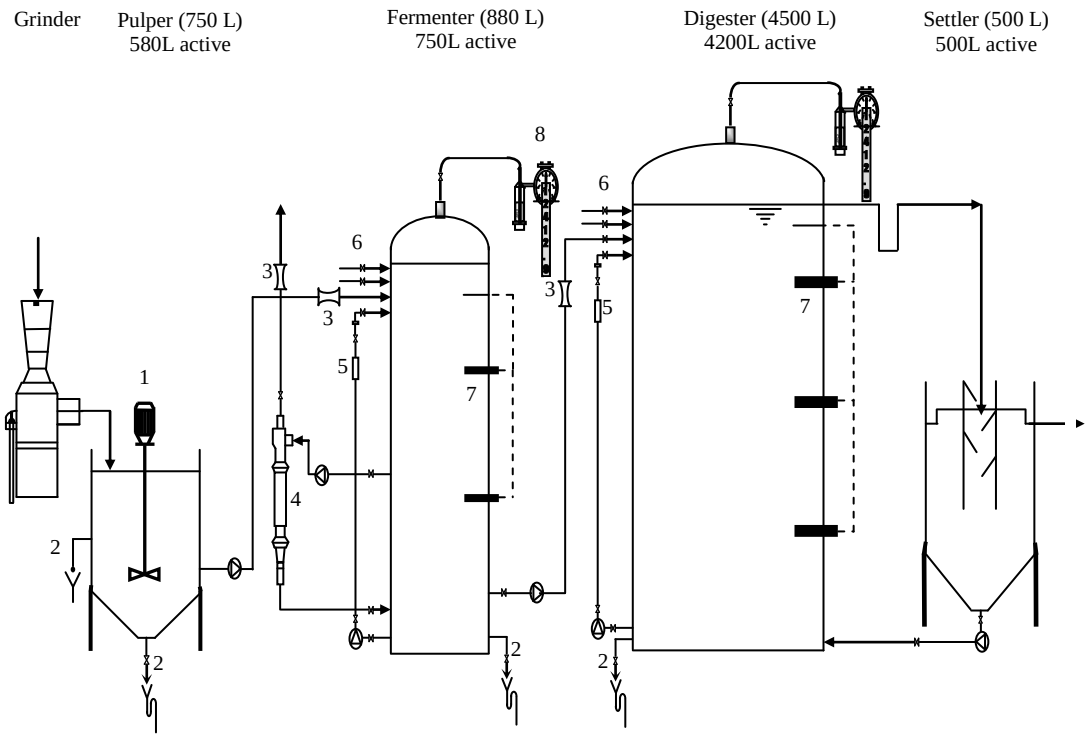
Figure 4.2: View of the pulper

Fermentation Reactor: First biological reactor (fermenter) inside the pilot plant is used as a hydrolysis and fermentation reactor. Thus, phase separation and optimum conditions for methane production achieved in the second biologic reactor. Fermentation reactor is a CSTR reactor, operated with mesophilic conditions and has 750 L volume. Active volume of the reactor is 480L. Mixing achieved by circulation of bottom water to the upper part of the reactor with 350L/hour capacity monopumps. Continuous feeding was done with 400 l/hour capacity monopumps.

Figure 4.3: View of the fermenter

Anaerobic Digester: Main advantages of the two-stage systems are having a stable treatment performance for some wastes, which cause problems in single-stage digesters. Some short fluctuations of organic loading rates can cause high loadings in single-stage digesters but fluctuations in organic loadings in two–stage systems stabilized in fermentation reactor for providing a uniform feeding to second stage. The stabilization effect of the first stage is suitable for wastes that have low cellulosic content like vegetable hall wastes [73]. Thus, methanization reactions are occur in second stage and protected against shock organic loadings and inhibition from slowly degradable substrates [74]. Pilot plant's digester has a volume of 4.5 m^3 with active volume 4.2 m^3 and mixing achieved by circulation of bottom water to the upper part of the reactor with 350L/hour capacity monopumps.

Figure 4.4: View of the anaerobic digester



1-Mixer, 2-Discharge, 3-Flow meter, 4-Seperator, 5-pH meter, 6-acid/base line, 7-heater, 8-Wet gas meter, 9-Discharge

Figure 4.5: Schematic view of the Biomethanization Pilot Plant

4.2 Molecular Characterization of the Reactor Sludge

4.2.1 Feeding and Sampling Schedule

Dinner Hall Wastes

Food waste collected from a dining hall (I.T.U dining hall, Istanbul) was used as the substrate of this study. Food waste composition changed each day according to the menu of the day. Wastes were sealed in plastic bags and directly taken to the pilot plant in an hour and grinded with water before fed into pulper. Since the food waste as collected from dinner hall of the university, it contained considerable impurities such as metal (spoon, fork) and plastics (cups, packages). Operational personnel of the pilot plant did screening of these inorganic materials.

To gain the compositional variability of the food waste and performance of the reactors daily sampling was perform. Samples were initially taken on three days (Monday, Wednesday, and Friday) of one week. Since the food waste was only collected on weekdays, no sampling was performed on the weekend. Sampling schedule for molecular experiments are shown in table 4.2. Samples were directly taken from circulation lines of the reactors.

Table 4.2 : Sampling schedule of molecular samples for dinner hall waste

Fermenter		Co-Digester	
Date	Operation Time(Day)	Date	Operation Time(Day)
03.04.2007	Inoculum	03.04.2007	Inoculum
09.11.2007	37	09.11.2007	37
12.12.2007	68	12.12.2007	68
07.01.2008	94	07.01.2008	94
30.01.2008	117	30.01.2008	117
08.02.2008	126	08.02.2008	126
13.02.2008	131	13.02.2008	131
01.04.2008	179		

Vegetable Hall Wastes

Vegetable Hall waste collected from a vegetable hall (Istanbul Fruit and Vegetable Hall, Istanbul) was used as the substrate of this study. Food waste composition changed monthly according to incoming seasonal fruit and vegetables to the hall. Wastes were stored in open air and near pilot plant. Hall waste contained considerable impurities such as (wood, metals, rope, plastics, and aluminium packages). Operational personnel of the pilot plant did screening of these inorganic materials.

Figure 4.6 . Unscreened hall waste

To gain the compositional variability of the food waste and performance of the reactors daily sampling was perform. Samples were initially taken on three days (Monday, Wednesday, and Friday) of one week. Since the food waste was only collected on weekdays, no sampling was performed on the weekend. Sampling schedule for molecular experiments is shown in table 4.3. Samples were directly taken from circulation lines of the reactors.

Table 4.3 : Sampling schedule of molecular samples for vegetable hall waste

Pulper		Fermenter		Co-Digester	
Date	Operation Time(Day)	Date	Operation Time(Day)	Date	Operation Time(Day)
07.05.2008	9	07.05.2008	9	07.05.2008	9
04.06.2008	37	04.06.2008	37	04.06.2008	37
06.08.2008	100	23.06.2008	56	23.06.2008	56
08.09.2008	133	06.08.2008	100	06.08.2008	100
24.09.2008	149	08.09.2008	133	08.09.2008	133
		24.09.2008	149		

4.2.2 Extraction of DNA

Samples were taken every month directly from circulation lines of digesters. Then, directly transferred to 15 ml sterilized tubes and centrifuged 15 minutes with 14000 rpm, +4^{o}C. After the removal of the supernatant, samples were washed with PBS (phosphate-buffered saline) buffer and stored for DNA extraction at -20^{o}C degrees. Total DNA was extracted from 1 ml of concentrate biomass by using a ZR Soil Microbe DNA Kit™ (Zymo Research Corp., Orange, USA) protocol. First of all, Zymo-Spin™ IV-HRC Spin Filters prepared before the extraction by centrifugation (8000 × g, 3 min, 22°C) (Rotina 35R; Hettich, Tuttlingen, Germany). The samples were washed with phosphate-buffered saline (PBS) and 500µl of sample was transferred into a DNase-free 1.5ml ZR BashingBead™ Lysis Tube consisted of 300 mg of zirconium beads with 750 µl Lysis Solution. The homogenization of the cells was carried out with MP FastPrep®-24 system (MP Biomedicals LLC, USA) by bead beating of 3min at a speed of 4.5m/s. After homogenization, the insoluble material from homogenate was removed by centrifugation at 12,000xg for 1 minute. The supernatant was transferred into a new Zymo-Spin™ IV Spin Filter in a collection Tube and centrifuge at 7,000 rpm (~7,000 x g) for 1 minute.

Then, 1200.µl of Soil DNA Binding Buffer added to the filtrate in the collection tube. 800 µl of the mixture transferred into Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10,000 x g for 1 minute. Flow through was discarded from the collection tube and filtrate was centrifuged at 10,000 x g for 1 minute again. After centrifugation, 200 µl DNA Pre-Wash Buffer added to the Zymo-Spin™ IIC Column in a new collection tube and centrifuged at 10,000 x g for 1 minute. Zymo-Spin™ IIC Column was transferred into a clean 1,5 ml microcentrifuge tube and 100 µl DNA Elution Buffer added directly to the column matrix and centrifuged at 10,000 x g for 30 seconds to elute DNA. Eluted DNA was transferred into prepared Zymo-Spin™ IV-HRC Spin Filter in a clean 1.5 ml microcentrifuge tube and centrifuged at exactly 8000 × g for 1 minute.

4.2.3 PCR amplification

Aliquots (1 µl) of the DNA solution were used for subsequent bacterial and archaeal PCR amplifications. In this study, different polymerase enzymes were used for different methods. PCR was performed in 50 µl reaction mixtures using DreamTaq DNA polymerase (Fermentas, Vilnius, Lithuania), high fidelity enzyme mix (Fermentas, Vilnius, Lithuania), DreamTaq™ Green DNA Polymerase (Fermentas, Vilnius, Lithuania) and Taq DNA recombinant polymerase (Fermentas, Vilnius, Lithuania) were used for DGGE, cloning (before selection), cloning (after selection) and sequencing respectively. For the PCR mixture contained 5 µl of 10× PCR buffer, 3 µl of 50mM $MgCl_2$, 1 µl of deoxynucleoside triphosphate (dNTP)s containing 10 mM each dNTP, 1 µl of 10 mM forward and reverse primers, 0.25 µl of the Taq DNA polymerase (5 U/µl), and nuclease free water for a final volume of 49 µl. Later, 1 µl target DNA was added. Before the digestion of clones, 25 µl mixtures were prepared with same ratios for the clones beside 50 µl. The samples were amplified using the BioRAD Mycycler (Hertfordshire, U.K). Complete bacterial 16S rRNA was selectively amplified for DGGE, RFLP, cloning and sequencing using specific primers. All primers were collected from IDT (Ebersberg, Germany) and shown in Table 4.4.

Tablo 4.4 : Primers used in PCR amplifications

Primer	Target	Technique	Region	Sequence (‘5 →‘3)	Ref.
Bact-968F-GC	Bacterial 16S rRNA	DGGE	V6	(CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG)AACGCGAAGAACCTTAC	[75]
Bact-1401R			V8	CGG TGT GTA CAA GAC CC	[75]
Bact-27F		Cloning	V1	AGAGTTTGAT(C/T)(A/C)TGG CTCAG	[75]
Bact-1492R			V8	CGGCTACCTT GTTACGAC	[75]
Arc-109(T)-F	Archaeal 16S rRNA	DGGE	V2	ACT GCT CAG TAA CAC GT	[76]
Arc-515R-GC			V3	(CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG)ATCGTA TTACCGCGGCTGCTGGCA	[75]
Arc-109F		Cloning	V2	ACK GCT CAG TAA CAC GT	[76]
Arc-515R			V3	ATCGTATTACCGCGGCTGCT GGCA	[75]
P1.2F	Cloning vector pJET1.2 Eco47IR	Clone screening	Eco32I	CGACTCACTATAGGGAGAGC GGC	Fermentas
P1.2R			Eco32I	AAGAACATCGATTTTCCATG GCAG	Fermentas

The reaction mixture was subjected to PCR for DGGE (550bp) under the following conditions: denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s; primer annealing for 1 min (annealing temperatures are for bacteria and archaea, 56 and 52, respectively); and DNA extension at 72°C for 1 min. After the last amplification cycle, samples kept for final extension at 72°C for 5 min and were immediately cooled to 4°C. The size and the yield of the amplicons (5 µl) were checked by electrophoresis on a 1% (wt/vol) agarose gel.

The reaction mixture was subjected to PCR for cloning and sequencing (1500bp) under the following conditions: denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s; primer annealing for 1 min (annealing temperatures are for bacteria and archaea, 52 and 52, respectively); and DNA extension at 72°C for 1 min. After the last amplification cycle, samples were kept for final extension at 72°C for 5 min and were immediately cooled to 4°C. The size and the yield of the amplicons (5 µl) were checked by electrophoresis (BioRad) on a 1% (wt/vol) agarose gel in 1X TAE buffer and run 20 minutes with 100V. The 100bp DNA marker controlled the products quality and amount. (GeneRuler^{TM} 100bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania) Agarose jel was stained with ethidium bromide and visualised by a BioRad illuminator. (GelDoc, BioRad, Hercules, CA)

4.2.4 Cloning and sequencing

Extracted DNA's are amplified with primers in table 2.16 and high fidelity PCR enzyme mix. The amplified V2-V8 regions for bacteria and V2-V3 regions for archaea of the 16S rRNA (~1500, ~400 bp) were purified with a Roche PCR purification kit (Roche High Pure PCR Product Kit, USA) following the manufacturer's instructions. The quality and the amount of purified PCR products were determined by electrophoresis on a 1% (wt/vol) agarose gel with a DNA marker (GeneRuler^{TM} 100bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania). These sticky-end amplicons were cut into blunt-end products with blunt-end enzyme. After that products were ligated into 50 ng/µl pJET 1.2/blunt vectors (MBI Fermentas) with 3:1 vector/targetDNA ratio by CloneJET^{TM} (MBI Fermentas) and were sequentially cloned into JM107 Competent cells (MBI Fermentas) with $CaCL_2$ transformation protocol [108]. Clones that were determined with ampicilin (100 µg/ml) and lethal restriction enzyme selection and were transferred into 20 µl TE buffer and were then incubated at 95°C for 10 min in order to lyse the cells. The

lysed cells were amplified with pET1.2F (5′-CGACTCACTATAGGGAGAGCGGC-3′) (CloneJET^{TM}, Fermentas) and pET1.2R (5′-AAGAACATCGATTTTCCATGGCAG -3′) (CloneJET^{TM}, Fermentas) primers, and these amplicons were digested with *Alu* I, *Cfo* I, and *Msp* I (MBI Fermentas, Vilnius, Lithuania) restriction enzymes mixture at 37°C for 90 min. To differentiate these digested clones, ARDAR analysis was conducted using OWL 03-14 Submerged Gel Electrophoresis System (Thermo Fisher Scientific, Waltham). Digested PCR products were run in the 3% (wt/vol) agarose gels (Prona) with a DNA marker (GeneRuler^{TM} 50 bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania) at 100V, 30 °C and for 45 min, and were visualized with ethidium bromide staining. Some clones, which have different profiles in ARDRA, were selected for further DGGE and sequencing analysis. Selected isolates amplified with DGGE primers and matched with DGGE bands of total community profiles. Plasmids of selected clones were purified using the High Pure Plasmid Isolation Kit (Roche High Pure Plasmid Isolation Kit, USA). The sequencing analysis was sent to REFGEN Biotechnology (Ankara). The molecular work was carried out in the new laboratory in I.T.U where no culture of anaerobic community was ever present and studied.

The assembly of DNA sequence data DNA sequences were edited manually to correct falsely called bases and trimmed at both the 5’ and 3’ends using. GeneDoc Multiple Sequence Alignment Editor& Shading Utility Version 2.6.002 (http://www.psc.edu/biomed/genedoc). The homology searches of the databases were conducted using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) [61]. The alignment studies were performed on ARB program (Editor 4.1) manually [77].and checked using GeneDoc Multiple Sequence Alignment Editor& Shading Utility Version 2.6.002 (http://www.psc.edu/biomed/genedoc). A phylogenetic tree was constructed in the ARB program (http://www.arb-home.de/) using the neighbor-joining algorithm [78]. All sequences were checked for chimeric artifacts using the CHIMERA_CHECK program version 2.7 in the Ribosomal Database Project II (RDP II) [79]. Operational taxonomic units (OTUs) were defined as clones with >97% sequence identity using Mothur v.1.9.0 program (http://www.mothur.org)

4.2.5 DGGE

GC clamped amplicons were run in the 8% polyacrylamide (37.5:1 acrylamide-bisacrylamide) gels containing urea and formamide using DCode TM System apparatus (BioRad, Hercules, CA). For this purpose, two aliquots of the 8% polyacrylamide gel (PAG) stock solutions, one with a 0% denaturing property, and the other with a 100% denaturing property, thus containing 7 M urea (AppliChem Cat.No: A5470,5000) and 40% (v:v) formamide (Merck Cat.No: 1.09684.1000), were prepared from the commercially available 40% acrylamide/bis solution (37.5:1 acrylamide/bis, BioRad, Cat. No: 161-0148). Glycerol (Merck Cat.No: 1.04093.2500) was also added to the solutions. From these stock solutions, working aliquots with 35% and 60% for bacteria and from 30% to 55% for archaea denaturing capacity, the optimum for the resolution of the desired DGGE bands were prepared. Gelbond® PAG film (Amersham Pharmacia Biotech, 80-1129) was placed on the rear glass plate of the casting sandwich assembly prior to casting to physically support the polymerized gel and to allow an ease in handling the gel after electrophoresis. Right before pouring the gel, the 10% ammonium persulfate (Merck Cat.No: 1.01200.1000) and TEMED (AppliChem Cat.No: A1148, 0250) were added to the working solutions to initiate and accelerate polymerization. The PAG was casted with the help of the DCode System, (DCode System, BioRad, Hercules, CA) from which the working solutions were steadily pumped into the casting sandwich assembly providing a denaturing gradient. After loading the 10 μL of PCR products with 3 μL loading dye to the polymerized PAG, electrophoresis was performed first at 200 Volts for 5 min, then at 85 Volts for 16 hours in the 1xTAE buffer. The electrophoresis temperature was 60^{o}C. By the completion of the electrophoresis, the gel supported by Gelbond® PAG film was removed from the casting assembly and was silver-stained [80]. Images of the DGGE bands were captured in the ".tif" format by scanning the gels with a scanner. Selected and sequenced clones from cloning and sequencing matched with amplified bands in DGGE profiles.

DGGE gel, resulting band profiles digitally normalized by comparison with Tuzla sludge samples as a reference profile and dendograms was constructed, using the BioNumerics v4.0 software package (Applied Maths).

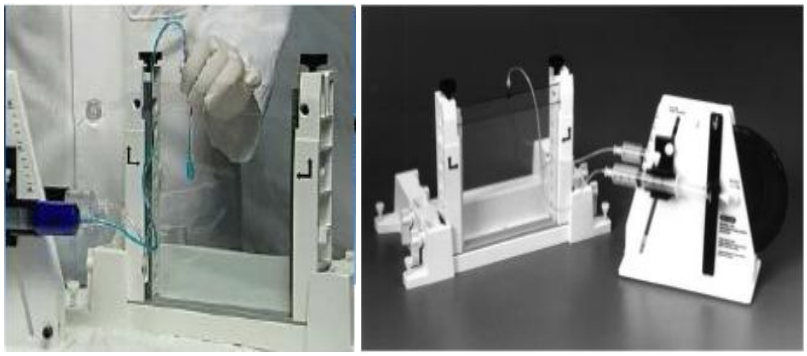


Figure 4.7 : Casting of the polyacrelamide gel with gradient delivery system (DCode System, BioRad, Hercules, CA)

4.2.6 Statistical Analysis

A principal component analysis (PCA) was used to ordinate sites according to physico-chemical analysis, after standardization. Canonical correspondence analysis (CCA) was used to determine the relationships between environmental variables and DNA fingerprints of microbial communities (197). Monte Carlo tests based on 499 permutations were used to test the null hypothesis that microbial communities were unrelated to environmental variables (198). The resulting ordination biplots approximated the weighted average of each samples (existence of each OTU) with respect to each environmental variable, represented by an arrow. Multivariate analyses were done in CANOCO, version 4.5, for Windows (Microcomputer Power, Ithaca, NY).

4.2.7 Analytical Techniques

The following parameters were monitored and measured: temperature, total solids, total volatile solids, pH, alkalinity, total and soluble COD, VFAs, the Total Solids (TS), Total Volatile Solids (TVS), pH, alkalinity, COD, were analyzed according to "Standart Methods" [99]. The pH in the fermenter and digester was monitored using a continuous in-line pH probe that located in circulation lines of the reactors.

Samples, which were taken for soluble COD and VFA, were centrifuged at 9000g for 15 min and the resulting supernatant filtrated through a Millipore PVDF filter (0,22mm). COD samples were preserved with H_2SO_4, VFA samples with 10M H_3PO_4. Volatile fatty acids (VFA) concentration was determined by a gas chromatograph (Agilent 6890N) equipped with a flame-ionisation detector and capillary column filled with Innowax (silicon based polymers, polysiloxanes, polyethylene glycols and solid adsorbents). The temperature of the injection port and detector were 230 and 250°C, respectively. The oven temperature reached 100°C in first 5 minutes and then 160◦C in following 5minutes and fixed at 230°Cin 3 min. Helium was carrier gas at 25ml/min. In addition, hydrogen gas was used at 40 ml/min flow rate. The sample (1.6mL) was transferred into a gas chromatography vial to which 0,2mL of 10% phosphoric acid was added.

5. THE ANAEROBIC DEGRADATION OF DINNER HALL WASTES IN TWO–STAGE DIGESTER

5.1 Introduction

This chapter remark physiological traits of the two-stage digestion in chemostat reactors at 37°C as a main objective. A better understanding of the fermenter and digester reactors and possible operating parameters for anaerobic digestion with dinner hall wastes was investigated with this study. In this concept, performance of the reactors was determined with COD, sCOD, TS parameters and in terms of total for all two reactors. Moreover, the research is also examining the VFA production and utilization inside the reactors.

5.2 Operation of the Reactors

Biomethanization system was operated totally 158 days, divided into three periods according to different loading rates. Operating periods and parameters are shown on table 5.1

Table 5.1: Operating Periods and Parameters

Parameters	1th period (0-52 days)		2nd period (52-83 days)		3th Period (83-158 days)	
	Fermenter	Digester	Fermenter	Digester	Fermenter	Digester
Flow, (L/day)	120	120	120	120	120	120
HRT(day)	4	35	4	35	4	35
VLR (kg COD/m^3 day)	7.5	0.7	13.8	1.2	24.3	2.6
VLR(kg TVS/m3.day)	5.1	0.3	10.0	0.8	12.3	1.6
Feeding TS(%)	2.2±0.7	1.3±0.7	4.1±1.4	3.1±1.6	6.0±2.2	6.3±2.7
Temperature (^oC)	35±3	35±3	34±3	35±3	35±3	35±3

5.3 Performance of the Reactors

Over 158 day's operation of the reactors, complete VFA and soluble COD utilization was observed. The last day's weeks of the third period, due to mechanical problems about mixing and acid-base adjustment, digester's performance was dropped seriously. Biomethanization system's performance for these three phases summarized on the table 5.2, 5.3 and 5.4. Every reactor was analyzed according to each phase under different sections.

Table 5.2: Biomethanization systems 1st period performance

Parameter	1st Phase (0-52 days)		
	Pulper	Fermenter	Digester
pH	3.6±0.2	4.72±0.17	7.3±0.3
Alkalinity, mg $CaCO_3$/l	-	-	3121±408
TS, %	2.2±0.7	1.3±0.7	2.0±1.1
TVS, %	2.0±0.6	0.8±0.5	1.2±0.8
Tot. COD, mg/l	30000±11600	24000±7440	20800±5000
sCOD, mg/l	13200±5550	10800±2000	2200±970

Table 5.3: Biomethanization systems 2nd period performance

Parameter	2nd Phase (52-83 days)		
	Pulper	Fermenter	Digester
pH	3.8±0.2	4.17±0.2	7.8±0.2
Alkalinity, mg $CaCO_3$/l	-	-	3275±179
TS, %	4.1±1.4	3.1±1.6	1.6±0.4
TVS, %	3.8±1.3	2.7±1.6	0.8±0.3
Tot. COD, mg/l	55000±12600	43000±11900	18400±5300
sCOD, mg/l	26150±7300	17000±8400	1600±840

Table 5.4: Biomethanization systems 3rd period performance

Parameter	3th Phase (83-158 days)		
	Pulper	Fermenter	Digester
TS, %	6.0±2.2	6.3±2.7	1.8±0.8
TVS, %	5.0±2.1	5.6±2.4	1.0±0.4
Tot. COD, mg/l	97000±35000	92250±28500	33400±21800
sCOD, mg/l	34460±15630	31910±13000	17160±13600

5.3.1 Fermenter

Influent dinner hall wastes were grinded with water to achieve approximately 2.1%, 4.1% and 6.0% total solid content and fed to pulper. In the first period, fermentation reactor was fed with averagely 2% TS content waste. It was increased to %4 in second period and %6 in the third period (Figure 5.1, 5.2). The average TVS/TS ratio in third period is 0.89. An organic loading rate of fermenter in first period was 4.1 kg TVS/m^3 due to the increasing TS loading rate in 2^{nd} and 3^{th} periods. It was increased to 10 and 12.3 kg TVS/m3 respectively.

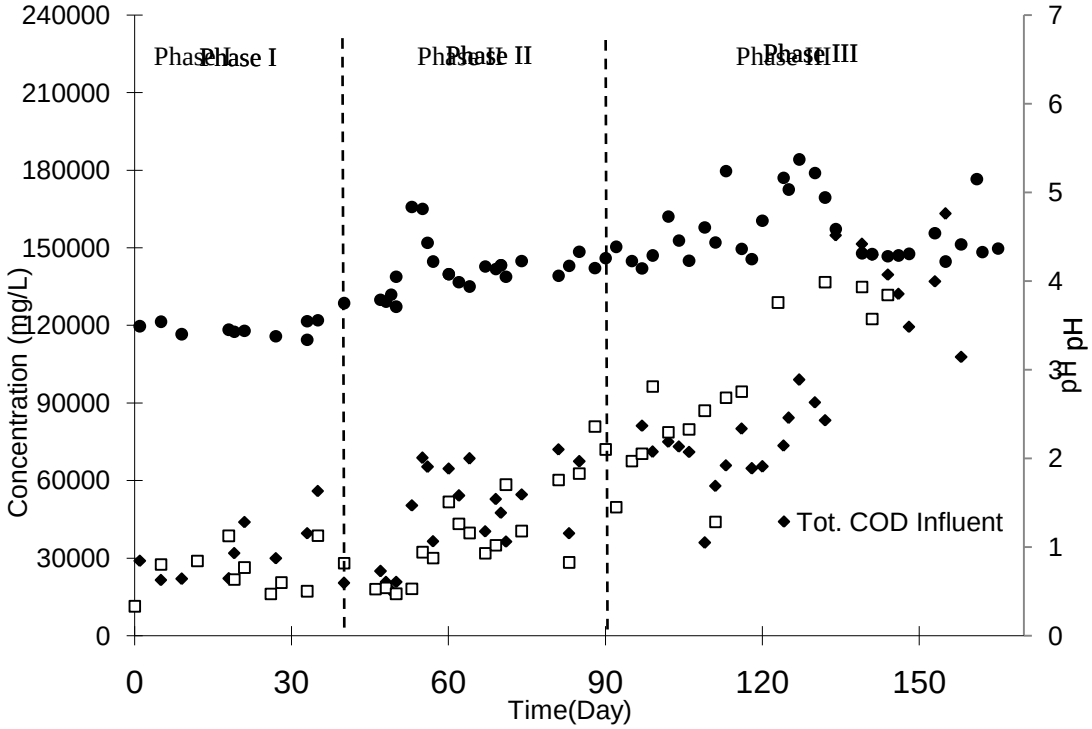


Figure 5.1 . Influent/Effluent TVS (%) values in fermenter

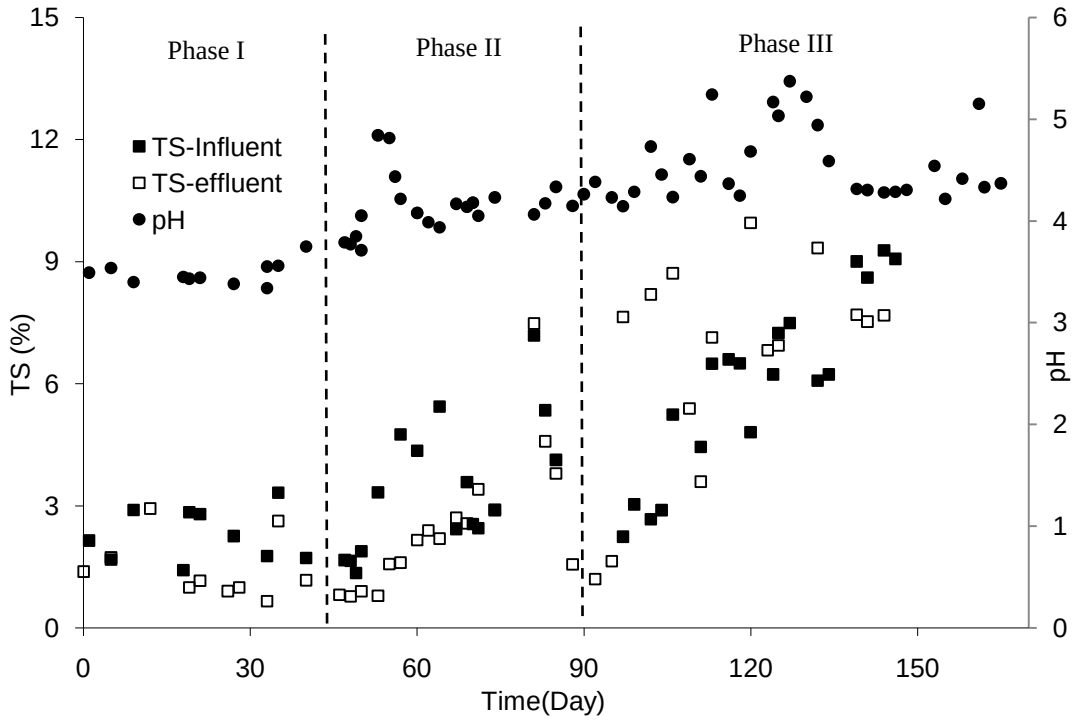


Figure 5.2 : Influent/Effluent TS values(%) in fermenter

Evaluating all periods with soluble COD and total COD parameters, fermenter's influent soluble COD and total COD values are respectively 56216±38500 and 26400±14600 mg/l (Figure 5.3, 5.4). sCOD/totCOD ratio in influent flow of fermenter with all periods is 0,41. Soluble COD values in influent flow are close to effluent values. With microbiologic analysis, it is observed that influent substrate concentration contain high soluble and readily COD content. In fermenter due to low pH and substrate conditions, it can be said that hydrolysis process is not occurred. Moreover, high soluble COD and VFA's are converted to each other especially lactic acid.

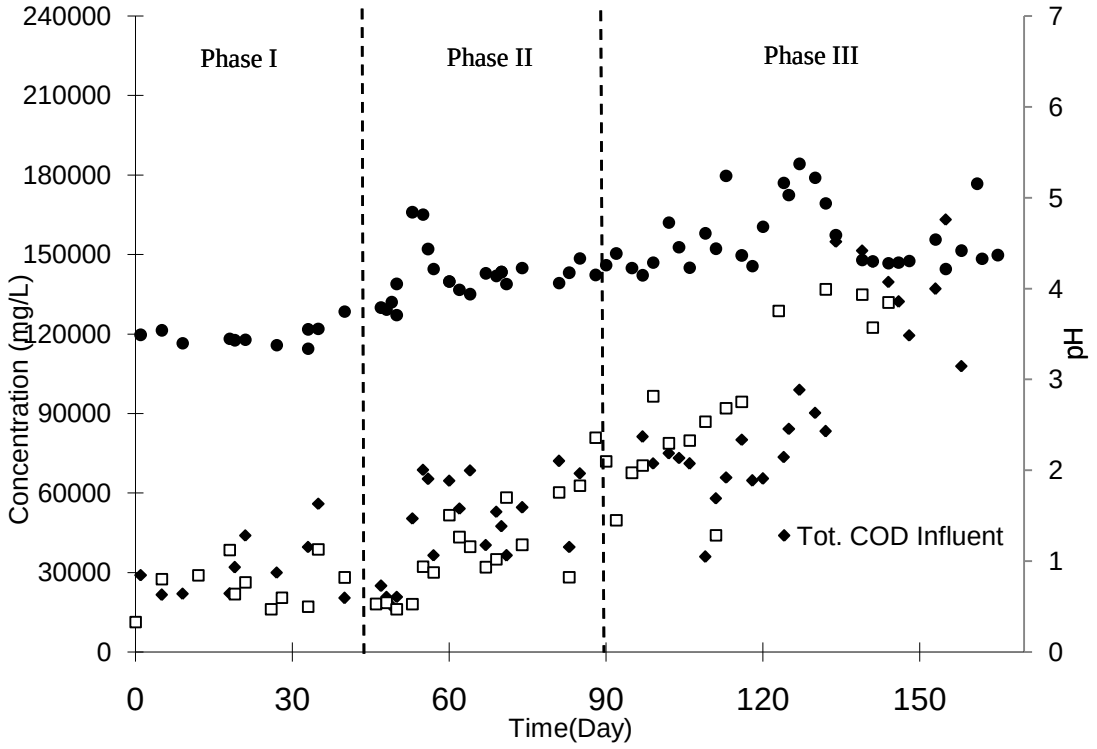


Figure 5.3 : Influent/Effluent total COD, pH values in fermenter

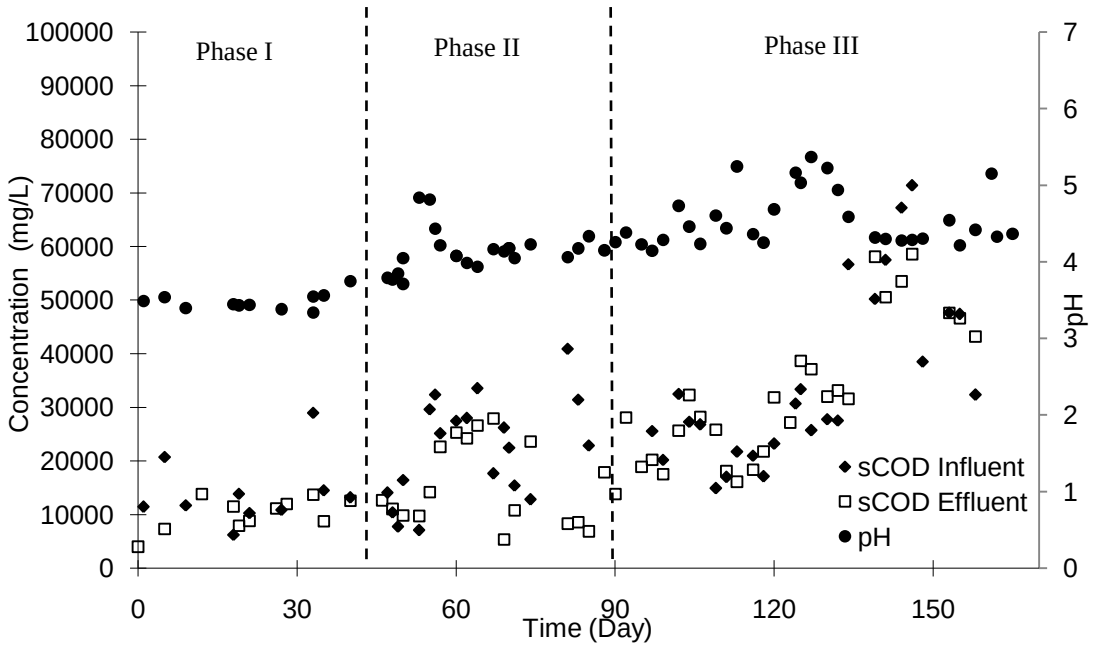


Figure 5.4 : Influent/Effluent soluble COD and pH values in fermenter

Figure 5.5 shows the volatile fatty acid values and their COD equivalent measured inside all reactors. Figure 5.5 also shows that VFA concentrations inside the fermentation reactor are increase according to pH conditions. Microorganism composition inside the reactor was effected and changed by pH. Operation period between the 32 and 96 days, VFA amount inside the soluble COD of influent flow is %4 (773/19450) and this ratio is increase to %28 (3668/13350) inside the reactor.

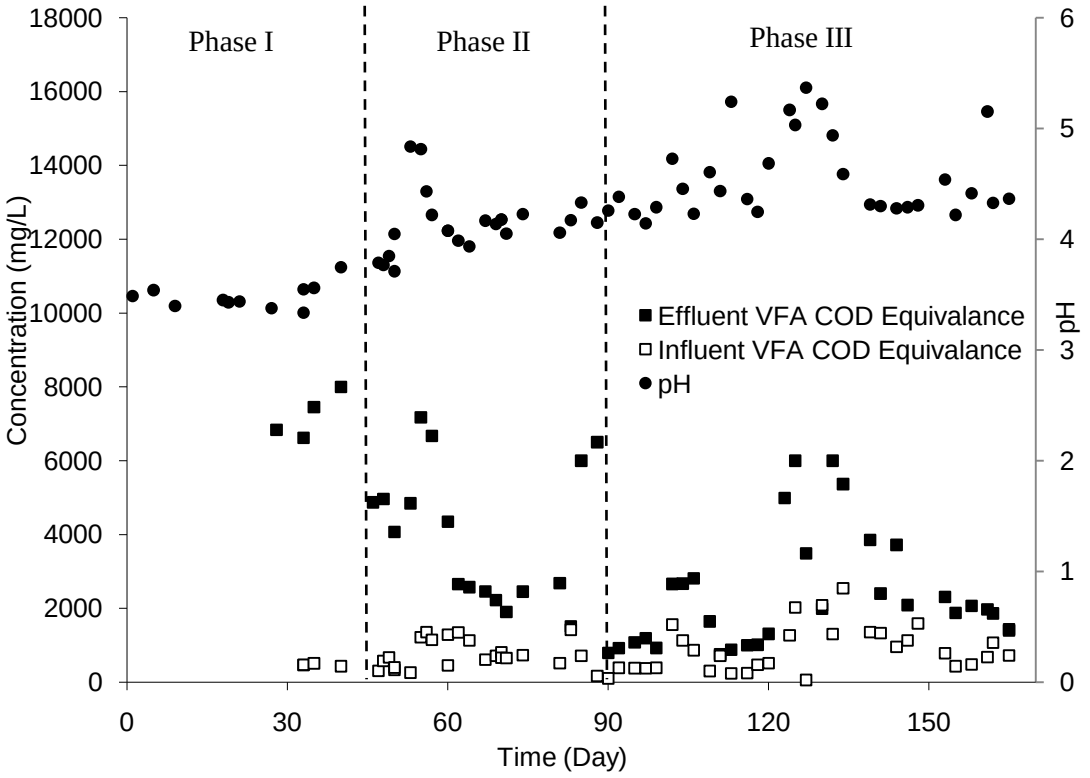


Figure 5.5 : Influent/Effluent VFA concentrations in fermenter

Acetic acid was the dominant VFA inside raw substrate. The VFAs produced were mainly lactic acid with minor concentrations of acetic acid and propionic acid. Difference between acetic acid and propionic acid values in influent flow and effluent flow of fermenter are higher than other VFAs. Average, propionic and acetic acid values in influent were, 595 mg/l and 31, 35 mg/l and in effluent 1350 mg/l and 820 mg/l respectively. Average lactic acid concentration in fermenter was 6523, 87 mg/l. Lee also stated that lactic acid ratio in acidogenic fermenter among total acids was about %73 under the conditions of different HRT's [181]. Average ethanol concentration in fermenter was 1074 mg COD/l. Dinner hall garbage's contain high concentrations of nutrition which makes the waste ideal raw materials for lactic acid, ethanol and hydrogen [182-183]. According to Ma's study, 50 g/l of ethanol production was maintained in anaerobic system feeding with kitchen garbage wastes, without pH adjustment and under non-sterilized conditions [184]

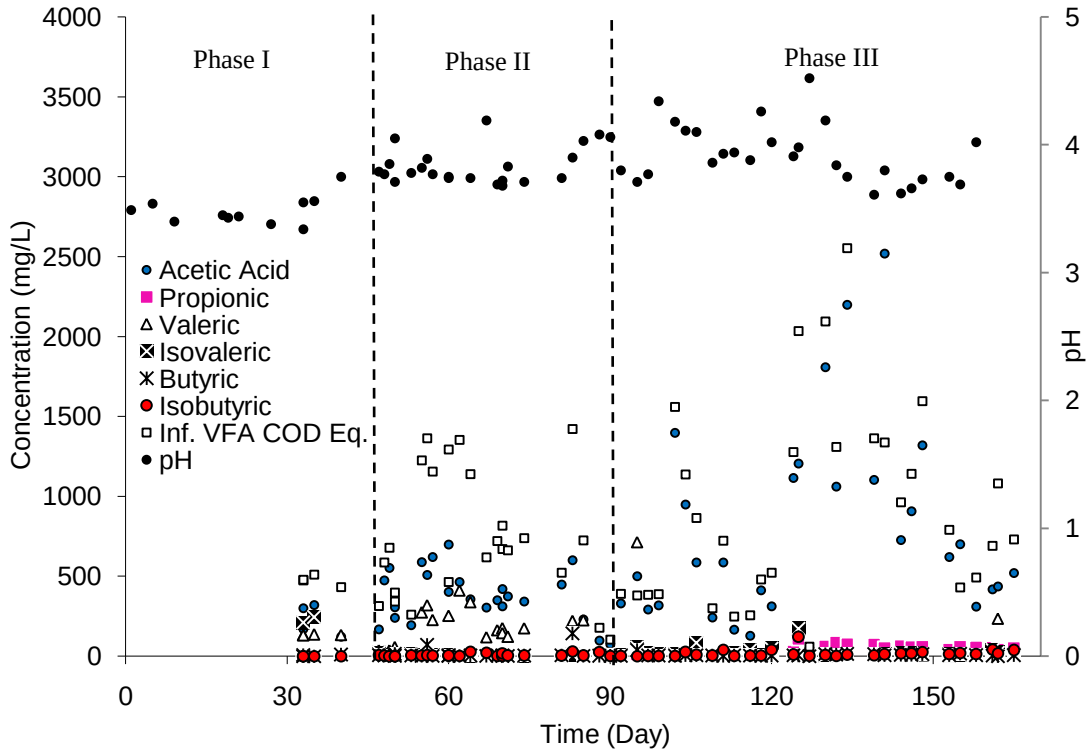


Figure 5.6 : Influent VFA components to fermenter

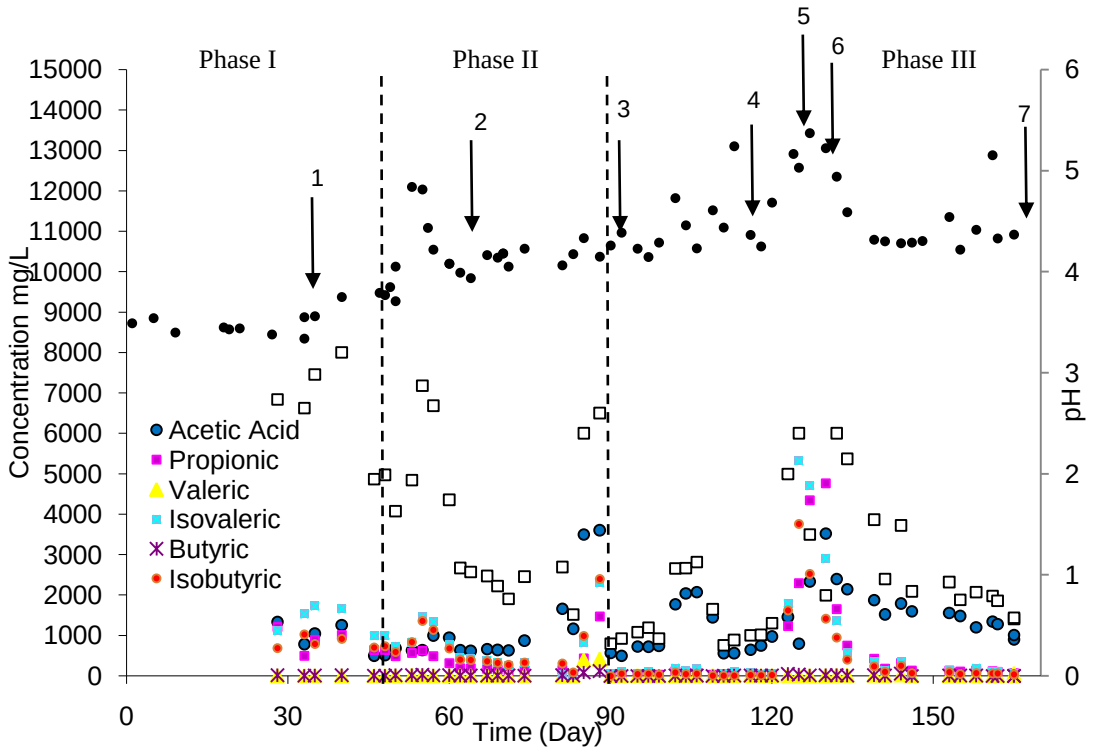


Figure 5.7 : VFA components in fermenter. Arrows with numbers are samples that were taken for microbiologic experiments.

Ethanol and lactic acid were the dominant products inside the fermenter. Average nitrate, phosphate and sulphate concentrations inside the fermenter were 110.37 mg/l, 407.41 mg/l and 235.91 mg/l respectively.

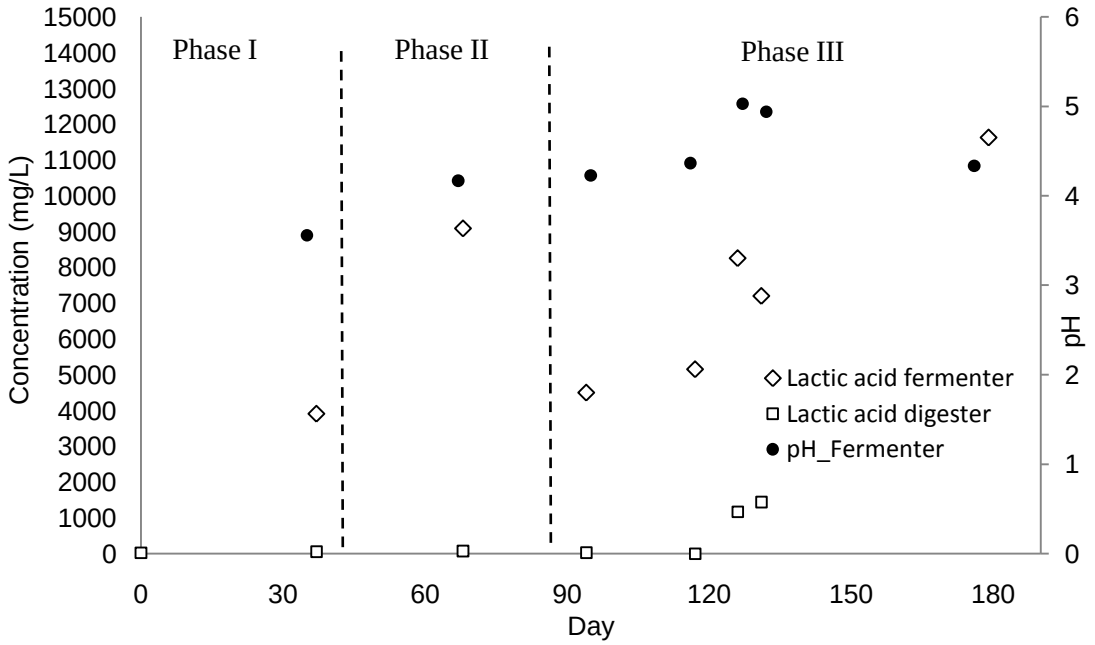


Figure 5.8 : Lactic acid concentrations in fermenter and digester.

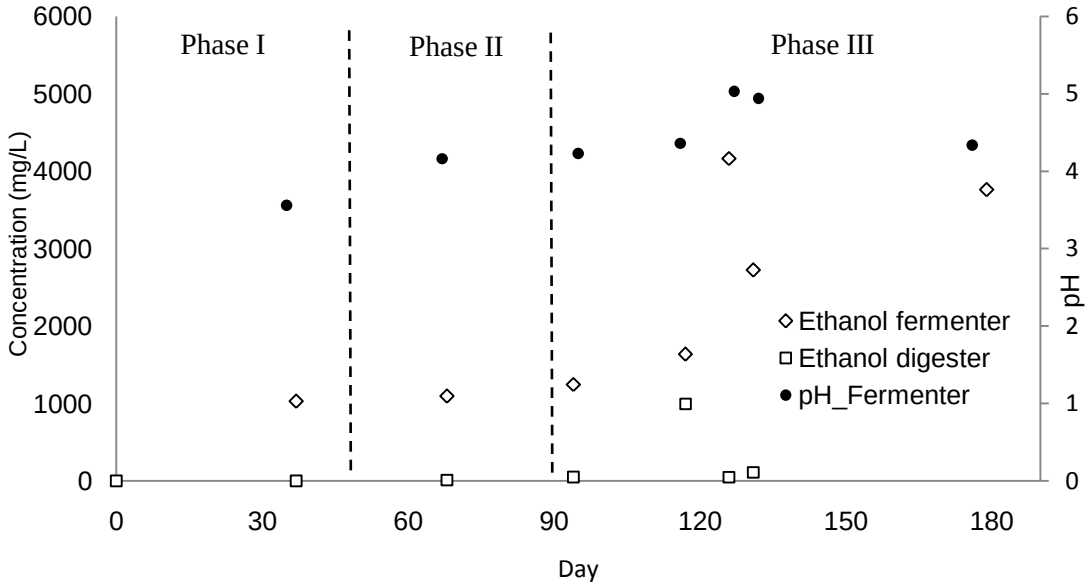


Figure 5.9 : Ethanol concentrations in fermenter and digester.

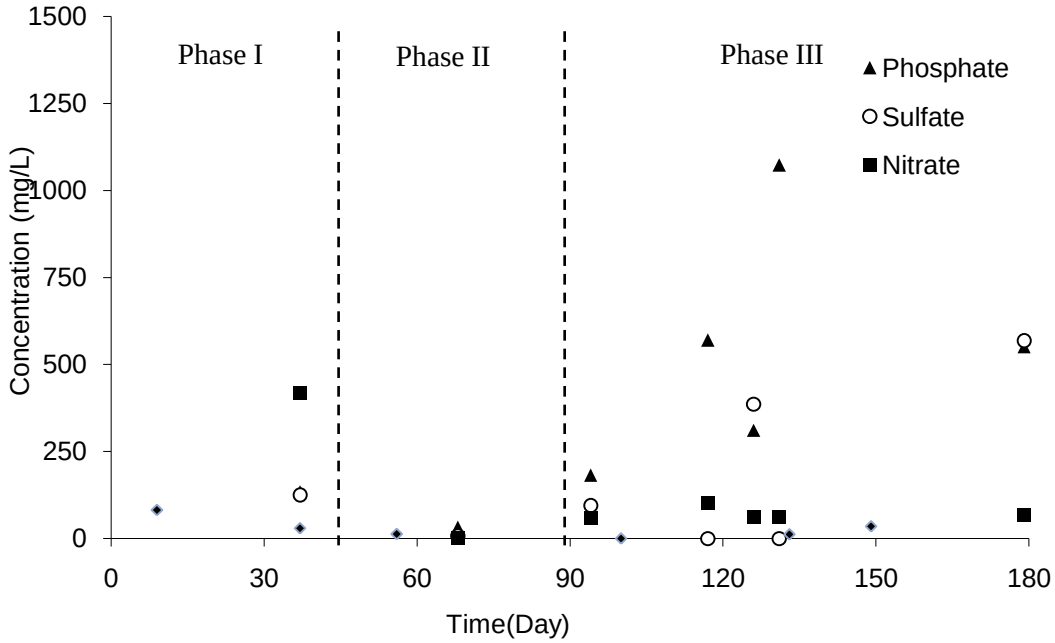


Figure 5.10 : Sulfate, Nitrate, Phosphate concentrations in fermenter.

5.3.2 Digester

Digester is the second stage of the biomethanization system. Hydraulic retention time of the reactor is 35 days. As it shown before on table 5.1, digester was fed with approximately %1.3 TS content waste in first period. In second and third period respectively TS content of the feeding is increased to approximately %3.1 and %6.3.(table 5.8). The average TVS/TS ratio in third period is 0.89. Organic loading rates of digester in first period was 0.3 kg TVS/m3, due to the increasing TS loading rate in 2nd and 3th period, it was increased respectively to 0,8 and 1,6 kg TVS/m3.

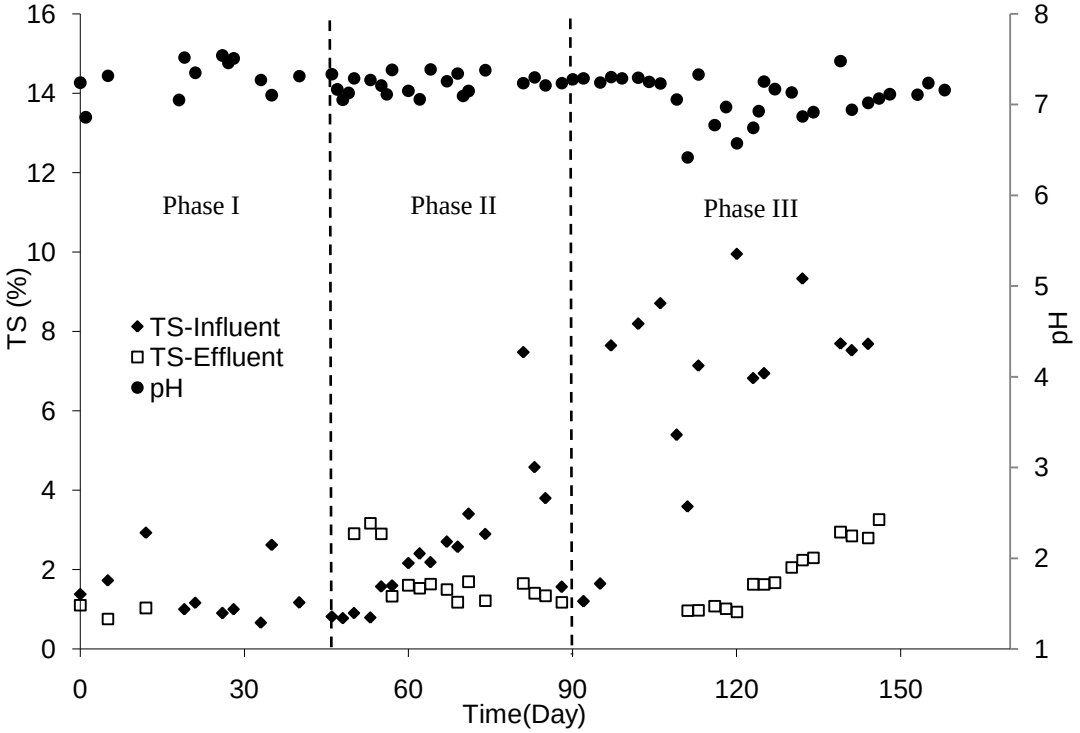


Figure 5.11 . Influent and Effluent TS and pH values in digester

Evaluating all periods with soluble COD and total COD parameters, digester influent soluble COD and total COD values are respectively 56200±35800 and 23150±13860 mg/l (Fig. 5.9, 5.10). sCOD/totCOD ratio in influent flow of digester with all periods is 0,41.High methanization rates observed in digester.

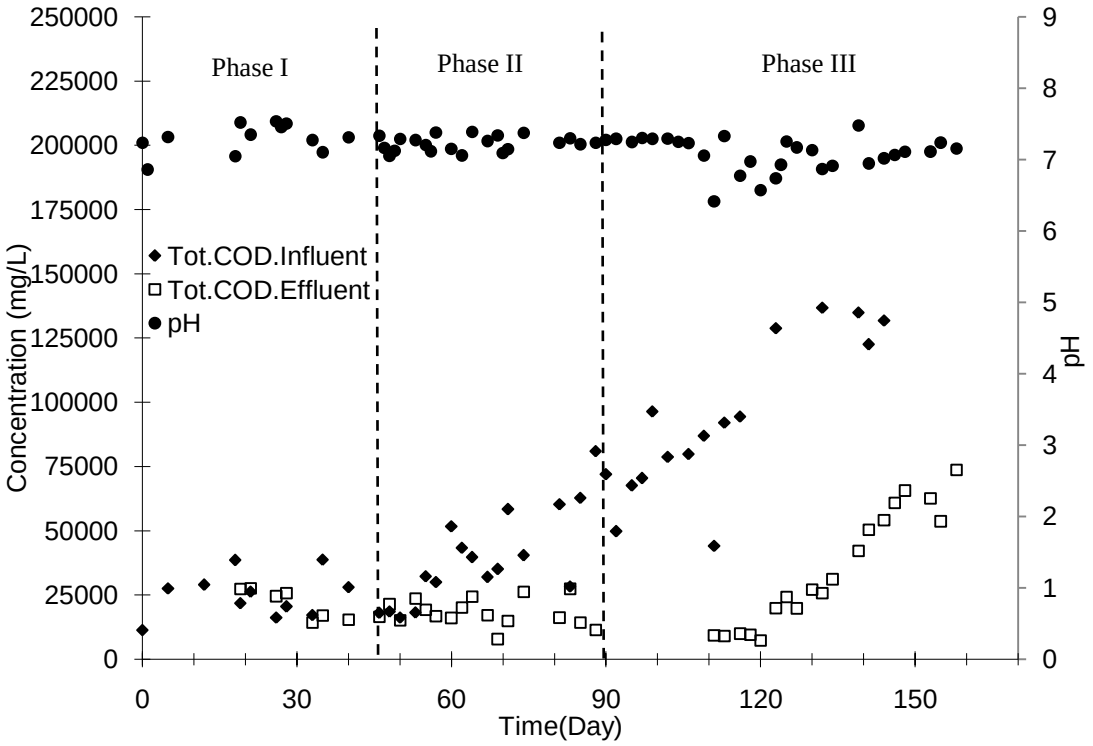


Figure 5.12 : Influent and Effluent total COD and pH values in digester

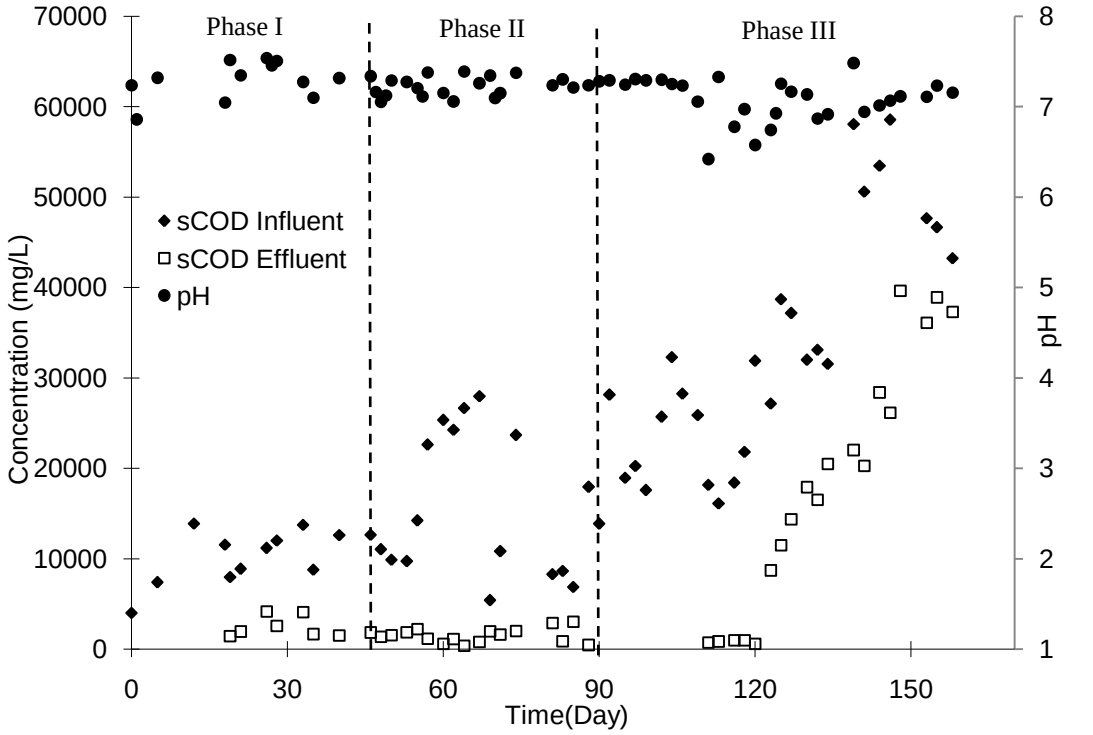


Figure 5.13 : Influent and effluent soluble COD, pH values in digester

In the first period, soluble COD and total VFA concentrations are respectively 1877 mg COD/l and 70 mg COD/l. However, high organic loadings in period three after 120 operational days caused some mechanical mixing problems and a rapid decrease in pH to 6 caused an inhibition in methanogenic activity. All sCOD and VFA values were rised rapidly in digester. This result showed that methanogens were very sensitive to pH changes.

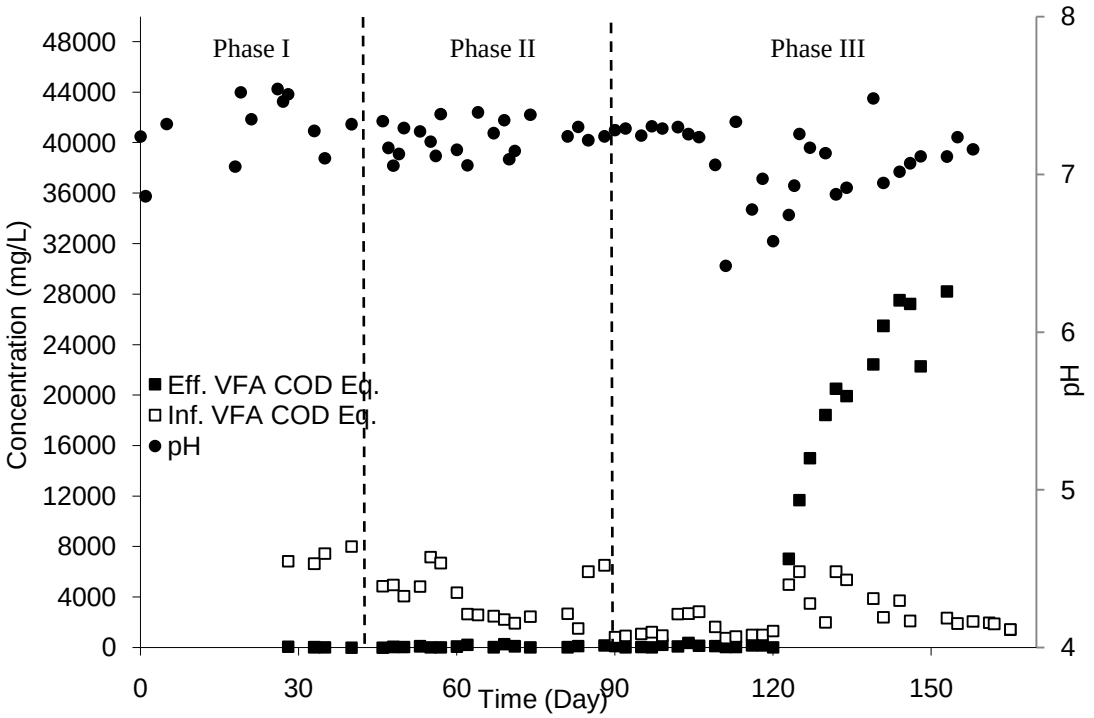


Figure 5.14: Influent and effluent VFA and pH values in digester

VFA concentrations are very low in effluent of the digester (average 70 mg COD/l). After 120 days operation time, pH inhibition caused VFA values to increase rapidly. After inhibition occurred, digester micro flora was not able to use VFA's for growth and maintenance. High lactic acid, ethanol concentrations in influent flow of the digester was not observed in effluent concentration. High anaerobic activity until inhibition time converts lactic acid and ethanol concentration into last products such as CO_2, CH_4.

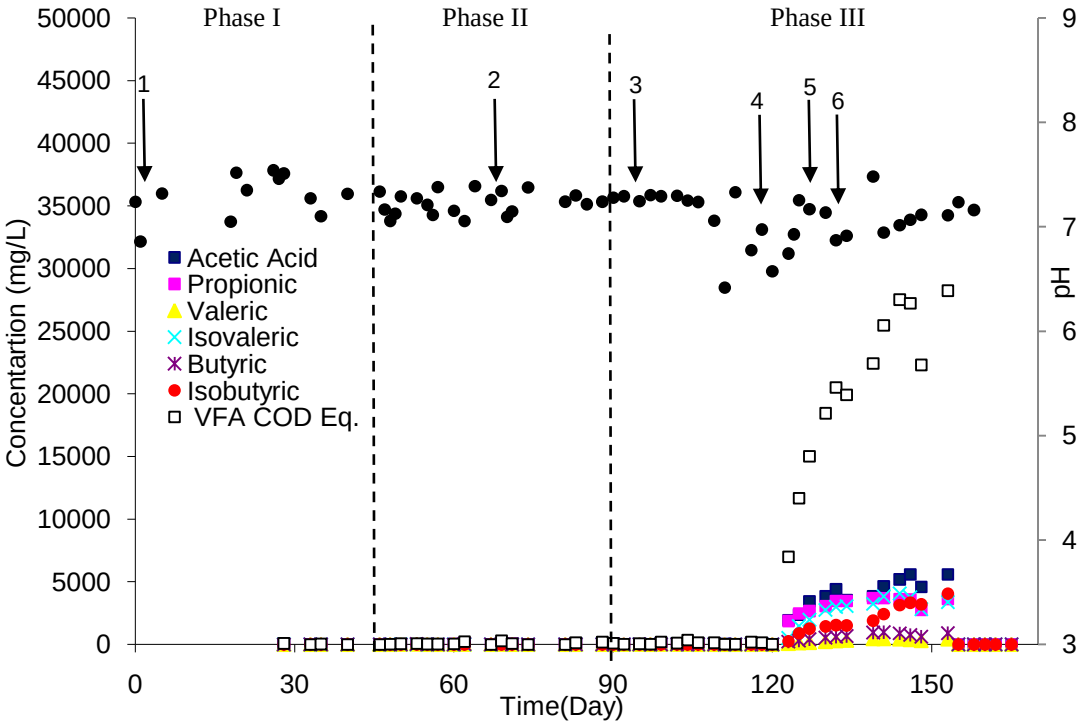


Figure 5.15 : Influent and effluent VFA and pH values in digester. Numbers with arrows are sampling days.

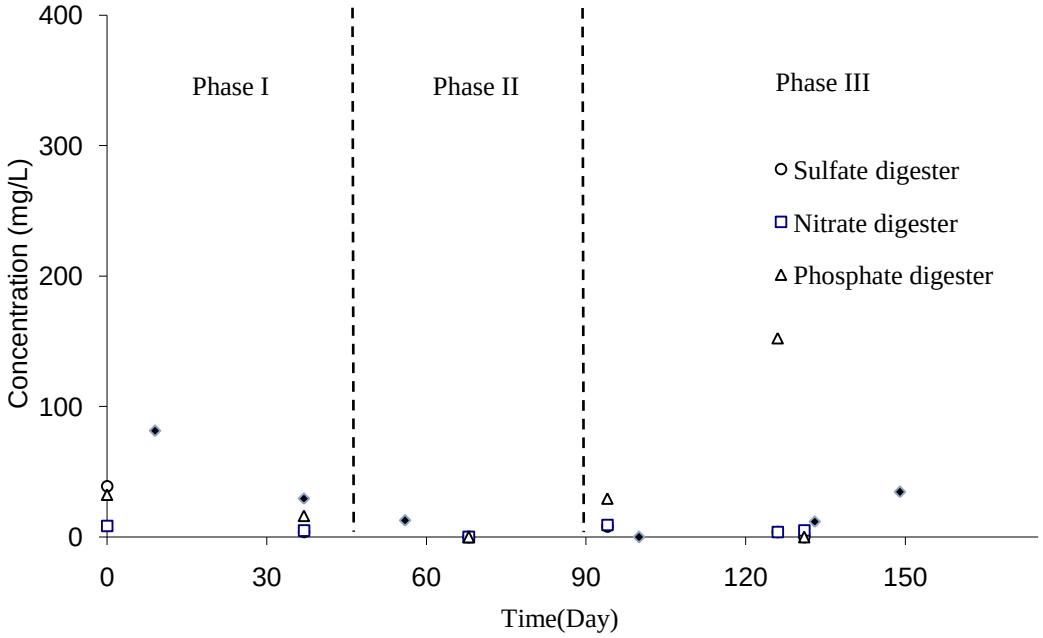


Figure 5.16 : Sulfate, Nitrate, Phosphate concentrations in digester.

6. POPULATION DYNAMICS OF TWO-STAGE ANAEROBIC DIGESTER TREATING DINNER HALL WASTES

By conducting a molecular culture-independent methodology, the polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE); and cloning-sequencing was applied in order to characterize the reactors' sludge fed with dinner hall wastes. The operational conditions of the reactors were described as in Chapter 4. The PCR amplified V6-V8 and V2- V3 regions of 16S rRNA for bacteria and archaea respectively were analyzed by DGGE to visualize the predominant bacteria in the reactors' sludge. DGGE analysis was also used to observe the population dynamics together with diversity along the operational period of the reactors. Moreover, 16S rRNA clone libraries were assessed to gain more insight to the identification of the microorganisms.

6.1 DGGE analysis of 16S rRNA gene fragments.

6.1.1 PCR amplification

Extracted DNA's were amplified with Bac968F-GC [75]. and Bac1401R [75]. primers for bacterial population, Arc109(t)F [76]. and Arc515-GC[75]. primers for archaeal population to generate 433bp and 406bp DNA fragments respectively.

Archaeal PCR amplifications were repeated third times for understand the reason of low-light samples which may result of PCR biases. 68th, 179th day of fermenter sludge sample and have very low archaeal population and in 37th day of fermenter sludge sample no products were observed when checked for on agarose gels, indicating that the Archaea comprise a small fraction of the fermenter microbial community. Thus, in 68th of digester sludge sample has low archeal population.

6.1.2 DGGE Result

The DGGE profiles derived from DNA amplicons of the reactors' sludge were given in Figure 6.1 and Figure 6.2. Population shifts of bacterial communities were assessed by comparing the DGGE banding patterns of 13 samples taken during the operation of all reactors (1-180 days). (Figure 6.1-6.2). In this figures, numbers and Tuzla indicated the representative sampling days, and the marker for the DGGE analysis.DGGE profiles of the bacterial PCR amplification products obtained from the fermenter and digester reactors' sludge at different time intervals were shown in Table 4.2. The prominent bands in the DGGE pattern should give the dominant members of the microbial community. So, the dominant isolations, such as D11, B2, B4, A12 and A1 bands in fermenter, E22, E12, E4, F17 bands in digester profiles were represented by the dark bands in the corresponding DGGE patterns. However, other important players could have been underestimated for complex environments. Such a statement could be derived from the PCR biases and and might cause the weakening or absence of the bands in the patterns. Suchlike situation was observed for the A8 pattern for the fermenter sludge. Since it covered the clone library with 12,5%, it showed a weak band in the DGGE profile representing the sludge sample taken at the day of 37.These differences between the DGGE profiles and the 16S clone library could be due to the bias of the PCR amplification.Another important observation was the detection of multiple bands corresponding to one species. For example, *Leuconostoc mesenteroides* in 94^{th} day sample was represented by two differently located bands on the same DGGE profile such as B2 and B3(Figure 6.1). Marked bands were identified in table 6.1 for bacteria and table 6.2. For biodiversity analysis, theorically, it can be said that each band represents a unique species, with band density according to species amount insample. But, the limitiations in the DNA extraction, PCR ampification and chance of chimeric product formation from mixed populations,create a failure on this assumption [79]. Thus,commigration of different sequences to same band, could give band two identifications.

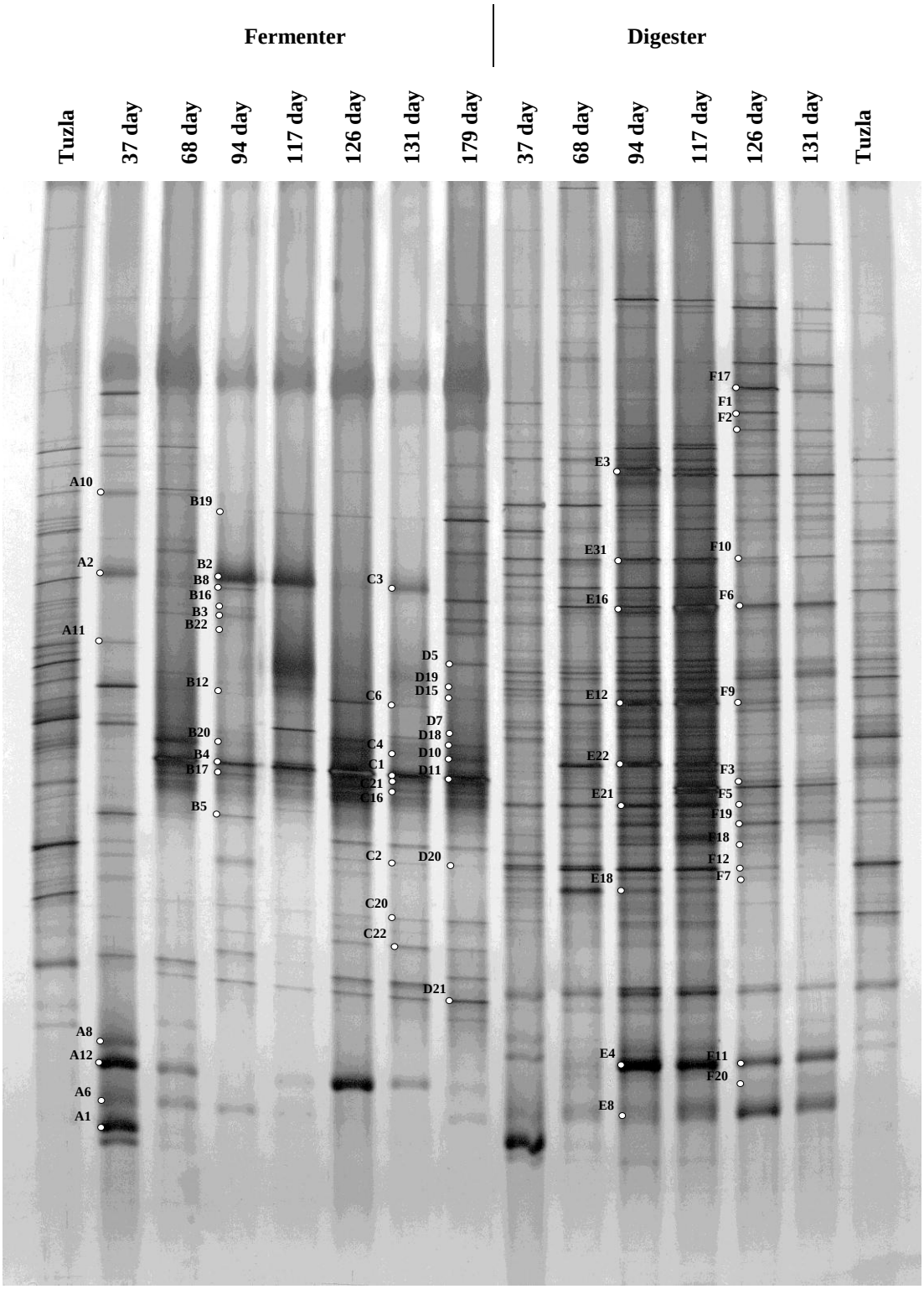


Figure 6.1 : DGGE analysis of the bacterial community during dinner hall waste fermentation and digestion. 16S rRNA gene amplified with primers 968F-GC and 1401R from DNA samples extracted at different times, as indicated. The formamide-urea denaturing gradient ranged from 35% to 60%. Marked patterns were determined by cloning technique and successfully identified by sequencing (Table 6.1) were reported as band numbers and sequentially numbered from top to bottom.

Table 6.1 :Sequence similarities to closest relatives and phylogenetic affiliations of DNA, matched with bacterial DGGE bands of DHW wastes

Band no.[a]	Identified Species	Number of clones	% of Total	Similarity(%)[b]	NCBI accession no	Order	Family
A1	Bacterium LB01	14	29,2	99	EF053126	Veillonellaceae	Firmicutes
A2	Leuconostoc pseudomesenteroides strain L7	3	6,3	99	DQ523483	Leuconostocaceae	Firmicutes
A6	Bacterium LB01	4	8,3	99	EF053126	Veillonellaceae	Firmicutes
A8	Uncultured Prevotella sp. clone 9B	6	12,5	99	EU887831	Prevotellaceae	Bacteroidetes
A10	Lactococcus lactis subsp. lactis KF147	2	4,2	99	NC_013656	Streptococcaceae	Firmicutes
A11	Lactobacillus sp.	1	2,1	87	AB016864	Lactobacillaceae	Firmicutes
A12	Megasphaera elsdenii S2	1	2,1	97	U95028	Veillonellaceae	Firmicutes
B1	Lactobacillus brevis ATCC 367	7	7,3	98	CP000416	Lactobacillaceae	Firmicutes
B2	Leuconostoc mesenteroides ATCC 8293	8	8,3	99	CP000414	Leuconostocaceae	Firmicutes
B3	Leuconostoc mesenteroides ATCC 8293	2	2,1	95	CP000414	Lactobacillaceae	Firmicutes
B4	Lactobacillus amylovorus strain LAB31	30	31,3	94	EF120373	Leuconostocaceae	Firmicutes
B5	Uncultured bacterium clone SM6	2	2,1	94	DQ318873	Lactobacillaceae	Firmicutes
B6	Paenibacillus sp. PRE17	2	2,1	91	EU880530	Paenibacillaceae	Firmicutes
B7	Lactobacillus brevis strain 14G	2	2,1	96	FJ532360	Lactobacillaceae	Firmicutes
B8	Lactobacillus amylovorus strain LAB52	1	1,0	98	EU000489	Leuconostocaceae	Firmicutes
B12	Uncultured bacterium clone TKW-HPB-19	2	2,1	97	GQ505035	Lactobacillaceae	Firmicutes
B16	Uncultured Leuconostoc sp. clone T0233	4	4,2	98	GU458929	Leuconostocaceae	Firmicutes
B17	Lactobacillus amylovorus strain LAB2	1	1,0	98	EF120368	Lactobacillaceae	Firmicutes
B19	Lactobacillus plantarum strain AF1	4	4,2	98	FJ386491	Lactobacillaceae	Firmicutes
B20	Lactobacillus sp. oral taxon 052 strain DoxG2	8	8,3	95	GQ422710	Lactobacillaceae	Firmicutes
B22	Leuconostoc mesenteroides ATCC 8293	4	4,2	93	CP000414	Lactobacillaceae	Firmicutes
C1	Lactobacillus amylovorus strain LAB31	29	30,2	99	EF120373	Lactobacillaceae	Firmicutes
C2	Lactobacillus coryniformis subsp. torquens strain 30	1	1,0	98	NR_029018	Lactobacillaceae	Firmicutes
C3	Leuconostoc gelidum gene strain: NJ 319	5	5,2	97	AB485958	Leuconostocaceae	Firmicutes
C4	Lactobacillus amylovorus strain LAB31	1	1,0	98	EF120373	Lactobacillaceae	Firmicutes
C6	Lactobacillus kitasatonis	1	1,0	95	AB107637	Lactobacillaceae	Firmicutes
C16	Uncultured bacterium clone TKW-HPB-19	1	1,0	99	GQ505035	Lactobacillaceae	Firmicutes
C20	Uncultured bacterium clone 130-1E18	1	1,0	93	FJ673040	Lactobacillaceae	Firmicutes
C21	Lactobacillus amylovorus strain LAB52	1	1,0	95	EF120375	Lactobacillaceae	Firmicutes
C22	Lactobacillus mucosae strain CCUG 43179	1	1,0	96	NR_024994	Lactobacillaceae	Firmicutes
D5	Lactobacillus parabuchneri strain: YIT 0272	1	1,0	98	AB429372	Lactobacillaceae	Firmicutes
D7	Lactobacillus brevis strain: NRIC 0138	3	3,1	99	AB362619	Lactobacillaceae	Firmicutes
D10	Lactobacillus amylovorus strain LAB31	23	24,0	98	EF120373	Lactobacillaceae	Firmicutes
D11	Lactobacillus amylovorus strain LAB31	2	2,1	99	EF120373	Lactobacillaceae	Firmicutes
D15	Lactobacillus casei BL23 strain BL23	2	2,1	99	FM177140	Lactobacillaceae	Firmicutes
D18	Lactobacillus parabuchneri strain: YIT 0272	1	1,0	99	AB429372	Lactobacillaceae	Firmicutes
D19	Uncultured bacterium clone RRH_aaa01a08	1	1,0	95	EU474872	Lactobacillaceae	Firmicutes
D21	Propionibacterium acnes strain: JCM 6425	1	1,0	95	AB538431	Propionibacteriaceae	Actinobacteria

a Bands are numbered according to Figure 6.1

b Identity represents the % identity shared with sequences in the Genbank database

Table 6.1 (Continued)

Band no.[a]	Identified Species	Number of clones	% of Total	Similarity(%)[b]	NCBI accession no	Order	Family
E3	*Uncultured bacterium clone BUD12*	5	5,2	99	DQ447172	Thermotogaceae	Thermotogae
E4	*Uncultured bacterium clone BS7_62*	5	5,2	91	GQ458234	Synergistaceae	Synergistetes
E7	*Uncultured spirochete clone AUSPI67*	4	4,2	98	AY648566	Spirochaetaceae	Spirochaetes
E8	Eubacterium callanderi strain DSM 3662	1	1,0	92	NR026330	Eubacteriaceae	Firmicutes
E12	Clostridium sp. ID5	1	1,0	88	AY960574	Ruminococcaceae	Firmicutes
E16	Uncultured Aminobacterium sp. clone 11IISN	1	1,0	99	EU887808	Synergistaceae	Synergistetes
E18	Uncultured spirochete clone AUSPI67	3	3,1	91	AY648566	Thermotogaceae	Thermotogae
E21	Eubacterium callanderi strain DSM 3662	1	1,0	92	NR026330	Eubacteriaceae	Firmicutes
E22	Uncultured Aminobacterium sp. clone 11IISN	6	6,3	99	EU887808	Synergistaceae	Synergistetes
E31	Uncultured bacterium clone 2F4_cons	1	1,0	99	EF688168	Porphyromonadaceae	Bacteroidetes
F1	Uncultured Bacteroidetes clone D004022F04	1	2,1	95	EU721793	Ruminococcaceae	Firmicutes
F2	Uncultured bacterium clone SJTU_G_05_90	3	6,3	97	EF405016	Ruminococcaceae	Firmicutes
F3	Lactobacillus amylovorus strain LAB31	2	4,2	98	EF120373	Lactobacillaceae	Firmicutes
F6	Uncultured clone HAW-RM37-2-B-1600d-Y	3	6,3	92	FN563296	Porphyromonadaceae	Bacteroidetes
F7	*Uncultured rumen clone L406RC-1-E12*	2	4,2	88	GU303569	Lachnospiraceae	Firmicutes
F9	*Uncultured bacterium clone 2F4_cons*	1	2,1	96	EF688168	Porphyromonadaceae	Bacteroidetes
F11	*Uncultured Bacteroidetes clone QEDV2BF11*	3	6,3	91	CU919602	Rikenellaceae	Bacteroidetes
F12	*Uncultured bacterium clone BUD12*	3	6,3	99	DQ447172	Thermotogaceae	Thermotogae
F17	*Uncultured rumen bacterium clone L3A_B06*	1	2,1	95	EU381538	Ruminococcaceae	Firmicutes
F18	*Desulfovibrio magneticus RS-1*	1	2,1	87	AP010904	Incertae Sedis XI	Firmicutes
F19	*Lactobacillus amylovorus strain LAB31*	1	2,1	93	EF120373	Lactobacillaceae	Firmicutes
F20	*Uncultured bacterium clone 21e07*	1	2,1	97	EF515222	incertae_sedis	Bacteroidetes

a Bands are numbered according to Figure 6.1

b Identity represents the % identity shared with sequences in the Genbank database

Bacterial population shifts seen in the DGGE profile were mostly linked to changes in the pH. Obviously, bacterial patterns and biodiversity of fermenter samples differ clearly from digester samples. Existence of smaller bacterial variety of microorganisms in fermenter is that the pH, feed composition and hydraulic retention time difference of the reactors. Amplification of the V2-V3 region of the archaeal 16S rRNA genes, followed by DGGE analysis of the DNA isolated from each of the samples, were shown in Figure 6.2. Although, a high diversity was seen in fermenter, sequence results showed that universal archaeal 109(T)F and 515-GC primers could amplified bacterial targets. These sequences were selected and taken in bacterial clone library. According to cloning and sequencing results, archaeal diversity in fermenter samples was very low due to low pH and high organic loadings. Most of the sequenced clones that amplified with archaeal primers in fermenter, identified as a bacterial gene. Moreover, for fermenter profiles diversity, which were not named with a band number, may not represent archaeal diversity. Table 6.2 showed identifications of marked bands. Digester methanogenic community significantly changed after 120 day operation. Inhibition that caused from pH adjustment was stated on previous chapters. This showed the instability of methanogen population due to pH changes in anaerobic conditions.

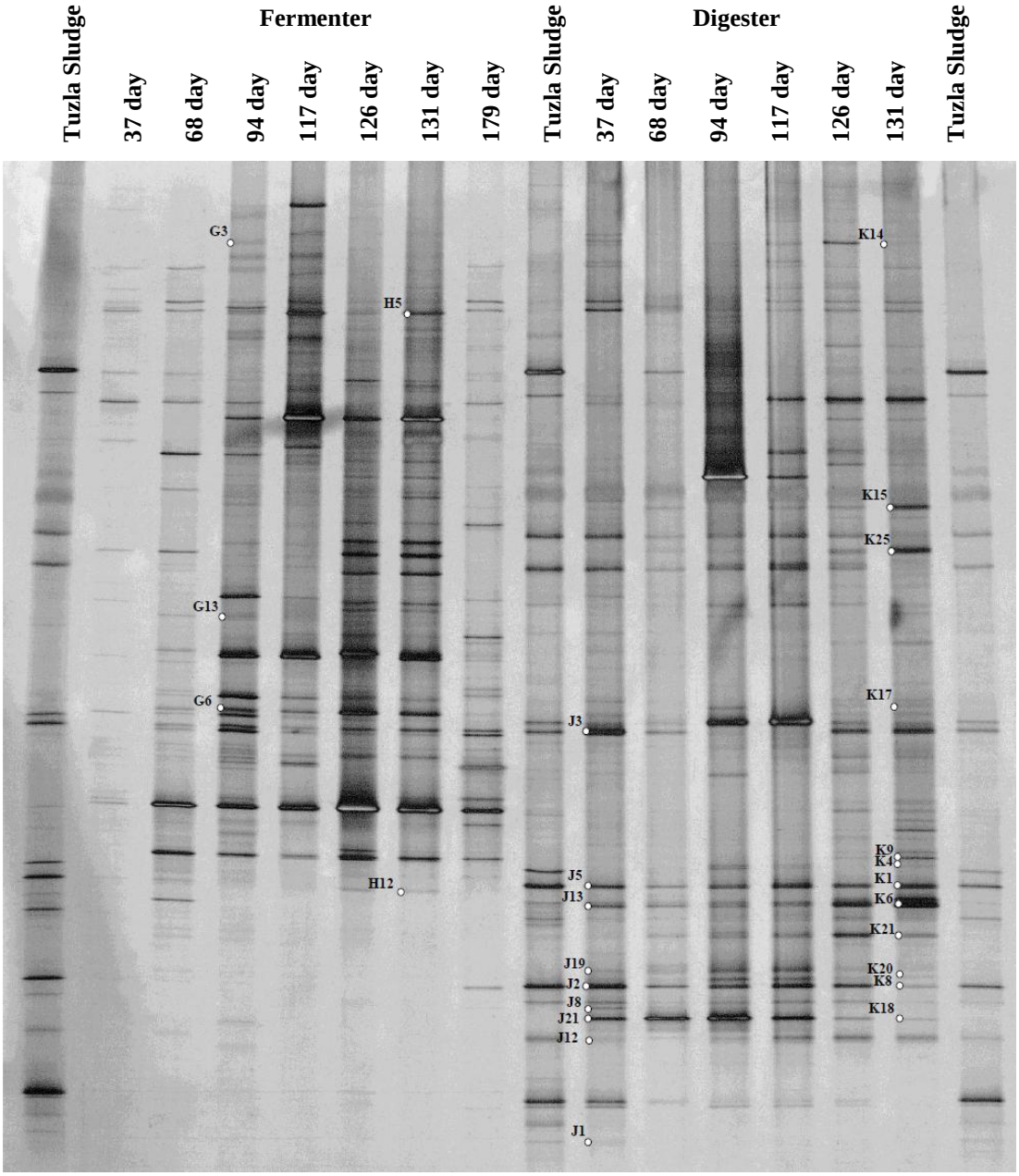


Figure 6.2 : DGGE analysis of the archaeal community during dinner hall waste fermentation and digestion. 16S rRNA gene amplified with primers 109(t)F and 515R-GC from DNA samples extracted at different times, as indicated. The formamide-urea denaturing gradient ranged from 30% to 55%. Marked patterns were determined by cloning technique and successfully identified by sequencing (Table 6.2) were reported as band numbers and sequentially numbered from top to bottom.

Table 6.2: Sequence similarities to closest relatives and phylogenetic affiliations of DNA, matched with archaeal DGGE bands of DHW wastes

Band no.[a]	*Identified Species*	Number of clones	% of Total	Similarity(%)[b]	(NCBI accession no.)	Order	Family
G3	*Methanobrevibacter acididurans strain ATM*	11	11,5	99%	NR_028779	Methanobacteriaceae	Methanobacteriales
G6	*Methanofollis liminatans*	2	2,1	98%	AY196677.1	Methanomicrobiaceae	Methanomicrobiales
G13	*Uncultured archaeon clone 822_6_pH7_2*	1	1,0	99%	GQ453660	Methanobacteriaceae	Methanobacteriales
H5	*Uncultured rumen archaeon clone T2PL03_D12*	3	3,1	95%	FJ586827	Methanobacteriaceae	Methanobacteriales
H12	*Methanosarcina barkeri str. CM1*	1	1,0	98%	AJ002476	Methanosarcinaceae	Methanosarcinales
J2	*Uncultured archaeon clone TRA-10*	23	24,0	100%	EF512457	Methanosaetaceae	Methanosarcinales
J3	*Uncultured archaeon clone AS15*	13	13,5	97%	AY464836	Methanobacteriaceae	Methanobacteriales
J5	*Uncultured archaeon clone QEED1BH111*	1	1,0	99%	CU917195	Methanosaetaceae	Methanosarcinales
J7	*Uncultured Methanosaeta sp. clone KA1*	3	3,1	99%	DQ085318	Methanosaetaceae	Methanosarcinales
J8	*Uncultured Methanosarcinalesclone LiM 3B-2H*	1	1,0	95%	FN646491	Methanosaetaceae	Methanosarcinales
J12	*Uncultured archaeon clone B16-A48*	1	1,0	98%	GQ458183	Methanosarcinaceae	Methanosarcinales
J13	*Uncultured archaeon clone:HNA-02*	17	17,7	99%	AB509221.1	Methanosarcinaceae	Methanosarcinales
J19	*Uncultured archaeon clone TRA-10*	1	1,0	100%	EF512457	Methanosaetaceae	Methanosarcinales
J21	*Uncultured methanogenic archaeon clone WD7_2*	1	1,0	87%	GQ453601	Methanosaetaceae	Methanosarcinales

[a] Bands are numbered according to Figure 6.1
[b] Identity represents the % identity shared with sequences in the Genbank database

Table 6.2 (Continued)

Band no.[a]	*Identified Species*	Number of clones	% of Total	Similarity(%)[b]	(NCBI accession no.)	Order	Family
K1	*Uncultured methanogenic archaeon clone WD7_18*	4	4,2	95%	GQ453597	Methanosarcinaceae	Methanosarcinales
K4	*Uncultured archaeon clone 8-23*	2	2,1	92%	FJ479787	Methanosarcinaceae	Methanosarcinales
K6	*Uncultured archaeon gene clone:HNA-02*	1	1,0	99%	AB509221	Methanosaetaceae	Methanosarcinales
K8	*Uncultured archaeon clone AS22*	6	6,3	96%	EU358672	Methanosaetaceae	Methanosarcinales
K9	*Methanospirillum hungatei*	7	7,3	94%	AB517987	Methanospirillaceae	Methanomicrobiales
K14	*Methanobrevibacter acididurans strain ATM*	10	10,4	98%	NR_028779	Methanobacteriales	Methanobacteriales
K15	*Uncultured methanogenic clone decelerated_CH4_C7*	11	11,5	96%	GQ453581	Methanosarcinaceae	Methanosarcinales
K17	*Uncultured euryarchaeote clone:BLA05*	12	12,5	96%	AB248608	Methanomicrobiaceae	Methanomicrobiales
K18	*Uncultured Methanospirillum sp. clone A07*	13	13,5	96%	EU888810	Methanospirillaceae	Methanomicrobiales
K20	*Uncultured archaeon clone TRA-10*	15	15,6	98%	EF512457	Methanosaetaceae	Methanosarcinales
K21	*Uncultured archaeon clone C89T102a*	16	16,7	94%	FJ941348	Methanomicrobiaceae	Methanomicrobiales
K25	*Uncultured Methanosarcina sp. clone X4Ar32*	17	17,7	92%	AY607257	Methanosarcinaceae	Methanosarcinales

[a] Bands are numbered according to Figure 6.1
[b] Identity represents the % identity shared with sequences in the Genbank database

6.1.2.1 Fermenter analysis

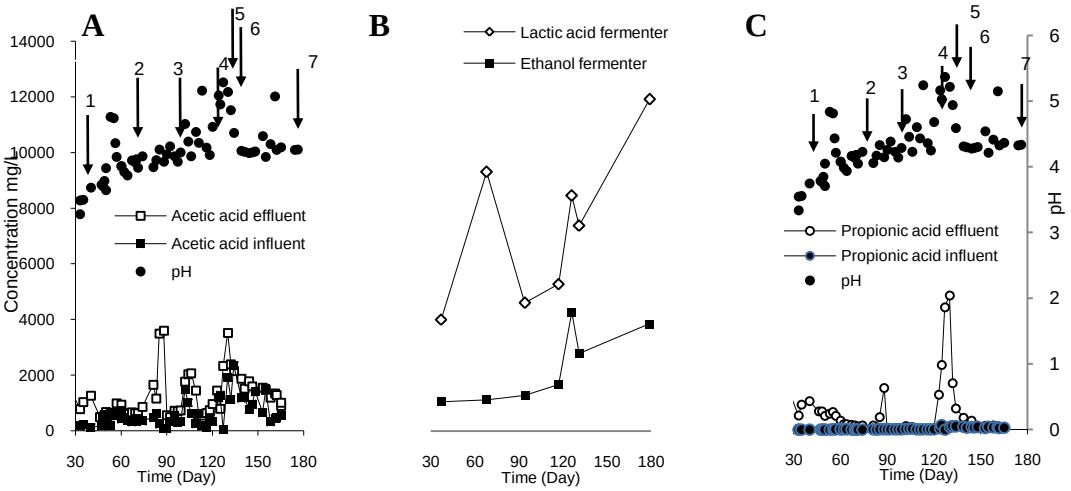


Figure 6.3 : VFA and pH values in fermenter. (A: acetate, B: lactic acid and ethanol, C: propionic acid)

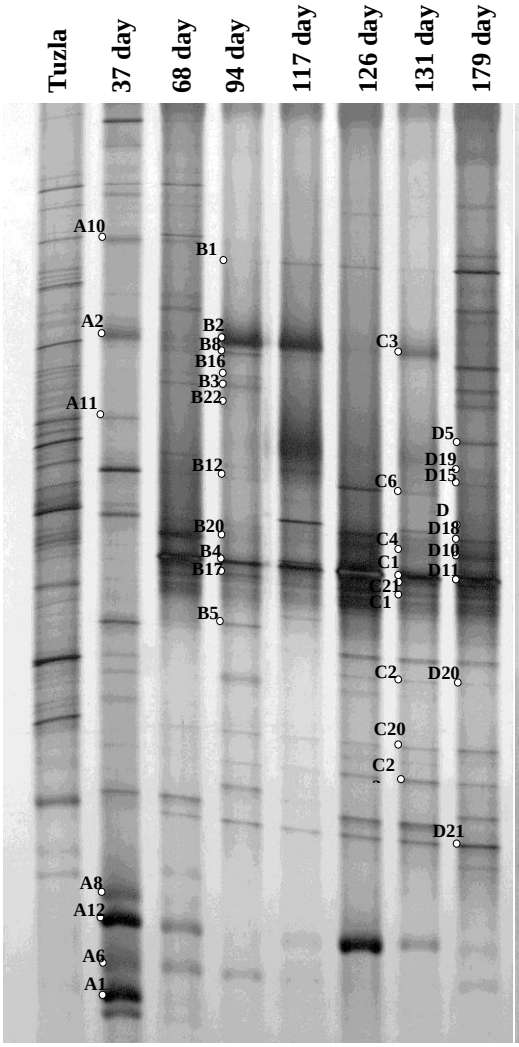


Figure 6.4 : DGGE profile of bacterial population in fermenter

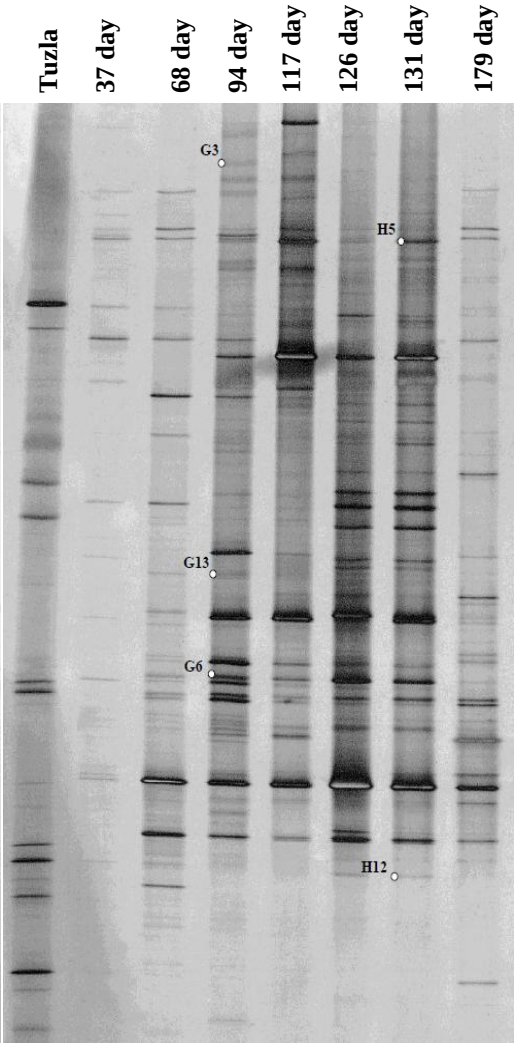


Figure 6.5 : DGGE profile of archaeal population in fermenter

Due to the increasing organic loading rate and substrate composition, fermenter's microflora selectivity was increased in first 131 days of the fermentation. Solubilization rates were stated in previous chapters, dinner hall wastes were made up of mostly carbohydrate with readily biodegreadable COD that didn't need to hydrolyze. Thus, this nature of the substrate caused acidic conditions in reactor and with mesophlic conditions promote growth of Lactobacillales group of microorganisms which are supposed to rapidly metabolize reducing sugars and available volatile fatty acids. Organic acids are important constituents of fermentation because they directly influence the pH during fermentation. B4, C1, D11 dominant bands were identified as *Lactobacillus amylovorus*. In early stages of the fermentation (1 month) process, diversity and species richness is high in the sludge. *Megasphaera elsdenii* and *Bacterium LB01* that belonged *Veillonellaceae* order were dominant inside reactor. This was the reason of first organic loadings to inoculum sludge that was taken from anaerobic wastewater sludge treatment plant. When the lactic acid concentration began to increase, *Megasphaera elsdenii* utilized lactate in preference to glucose when the two substrates were present and did not utilize glucose until lactate decreased to a low concentration. And LB01 strains of Veillonellaceae order efficiently converts toxic lactate and excessive acetate to butyrate can prevent lactate and acetate accumulation. This dominance was show the system recovery from inhibition of high VFA esspecially lactic acid [185]. The increased loading rates changed fermenter ecology to *Leuconostocaceae* and *Lactobacillaceae* dominance with high selectivity of acid tolerant organisms. It was interesting that *Methanobrevibacter acididurans strain ATM* from Methanobacteriales phylum and *Methanofollis liminatans* from Methanomicrobiales phylum was found inside the reactor. Although, the growing conditions of *Methanobrevibacter* species was in pH range 5.0--7.5, with maximum growth at pH 6.0 [186], and *Methanofollis* species was in pH 7.0, [187]. were found in the acidic (pH 4-4,5) fermenter ecology. After 120 operation days, fermenter diversity richness bagan to increase with increased pH. Dinner hall wastes consist of high amounts of carbonhydrates, lipids and proteins. Production of ammonia with proteolysis and utilization of the organic acids by bacteria might be responsible for the pH shift from 3,5 to 5 [188]. But, according to McInerney et al, carbohydrates were tend to suppress the synthesis of exopeptidases, which is a group of enzymes facilitating protein hydrolysis[189]. In 179th day of fermenter *Lactobacillaceae* species were

still dominant and lactic acid values still high. But, as Figure 6.4 showed a significant band formation in the last period of the reactor with increased pH.

6.1.2.2 Digester analysis

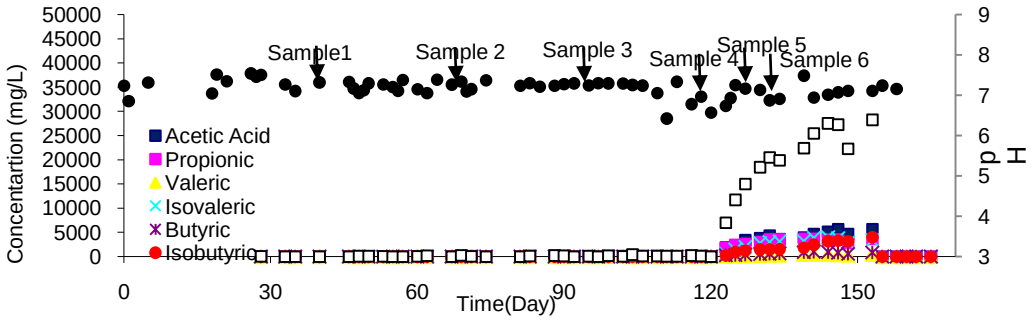


Figure 6.6 : VFA and pH values in digester

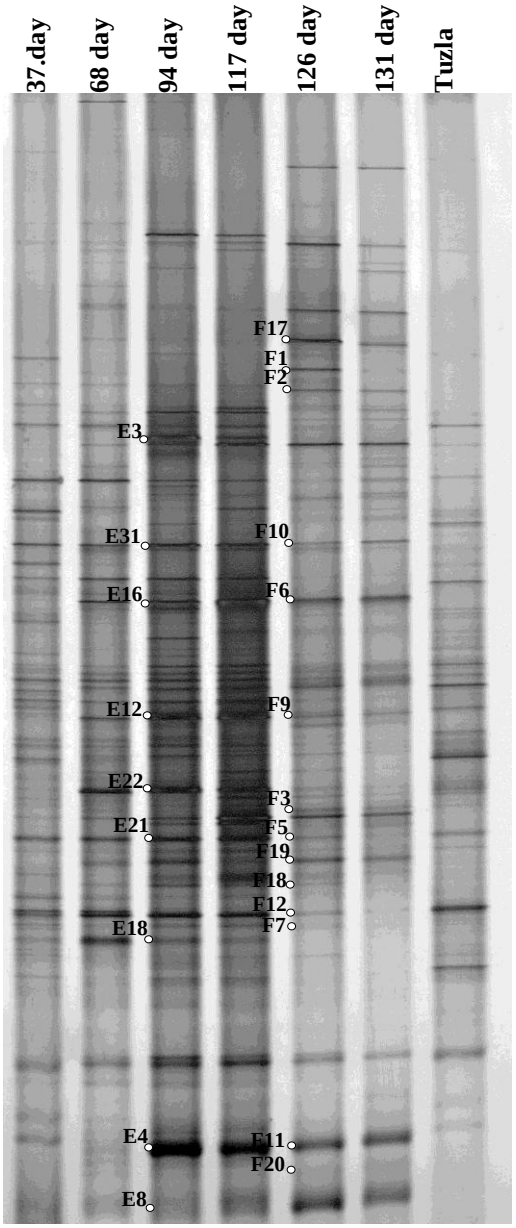


Figure 6.7 : DGGE profile of bacterial population in digester

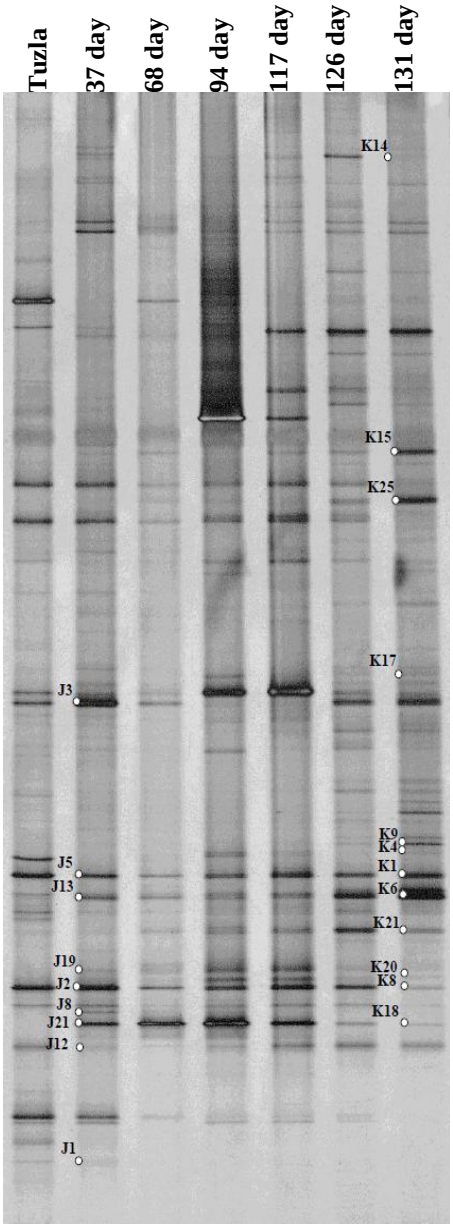


Figure6.8 : DGGE profile of archaeal population in digester

Long HRT values inside the reactor made the digester to worked with high performance. As stated before, all VFA's and soluble COD's were degraded into last step of methanization process. Ecology of the digester was very similar to Tuzla. Dominant bands such as E3, E12, E22, E4 F6 were belong to Thermotogae, Firmicutes, Synergistetes, Synergistetes, Bacteroidete phylum's respectively. In 120th day of digester, pH of the reactor was rapidly decreased to 6,3. Rapid decrease was directly effected bacterial communities as seen in Figure 6.7. After inhibition, *Ruminococcaceae* species occurred and 3 bands such as F7, F12, and F18 were disappered. DNA based microbiological studies give information about existing and disappearing species inside samples. Activation of the species was not able to determine.

6.1.2.3 Dendrogram of Bacterial Profiles

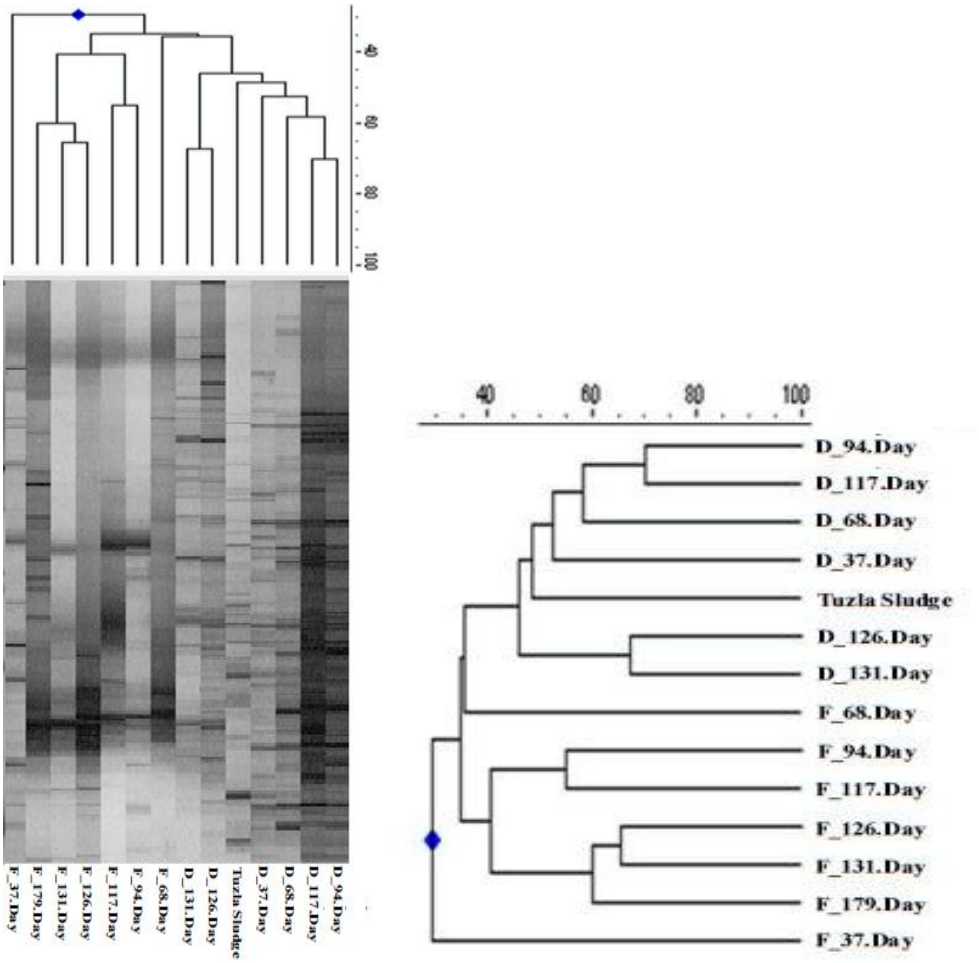


Figure 6.9: Illustrations of the cluster analysis of the bacterial PCR-DGGE profiles of dinner hall waste's fermentation and digestion. Dendrograms were based on the Dice coefficient of similarity (weighted) and were obtained with the UPGMA clustering algorithm. Samples were indicated by reactor name and operation day. (F_37.day means 37th day in fermenter)

Cluster analysis of the PCR-DGGE bacterial profiles of fermentation and digester was given in Figure 6.9. Fermenter and digester ecology was very different form each other with 35%similarity. Especially pH and HRT of the reactors made a great difference in biodiversity of the reactors. 37th and 68th day of fermenter was less similar to other operational days. High similarity between 126, 131 and 179 days of fermenter samples showed the stability of the communities and end of the acclimation period. Digester samples 50% similar to Tuzla inoculums. It was observed that stability increased in digester day by day. In 94th and 117th day's bio diversity was nearly 70% similar. As stated before, inhibition that occurred in 120th day of operation made a significant change in the microbial diversity. 65% differentiation was observed in diversity of digester after inhibition.

6.1.2.4 Dendrogram of Archaeal Profiles

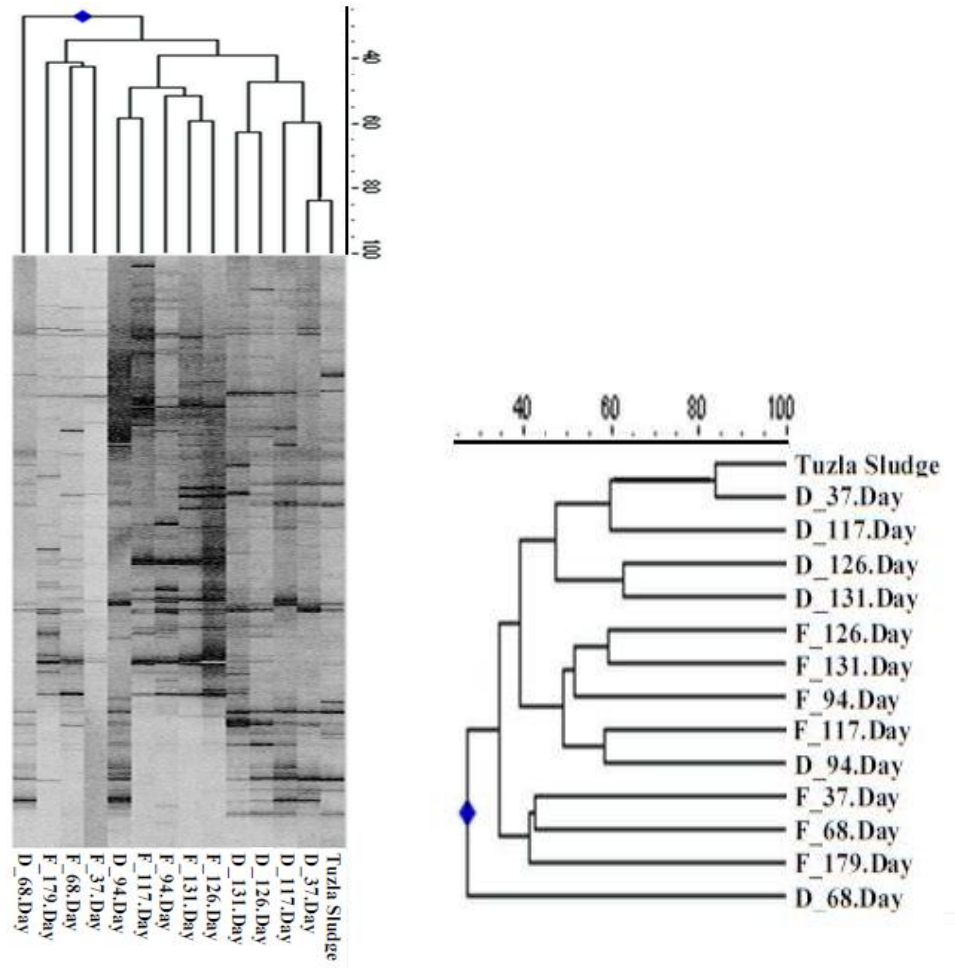


Figure 6.10: Illustrations of the cluster analysis of the archaeal PCR-DGGE profiles of dinner hall waste's fermentation and digestion. Dendrograms were based on the Dice coefficient of similarity (weighted) that obtained with the UPGMA clustering algorithm. Samples were indicated by reactor name and operation day. (D_37.day means; 37th day in digester)

Cluster analysis of the PCR-DGGE archaeal profiles of fermentation and digester was given in Figure 6.10. In previous section, the biases of PCR results were stated. Archaeal communities in fermenter were not reliable except marked bands. In first month of the operation, archaeal diversity was very close to inoculums with 85% similarity. Inhibition changes were also occurred in methanogenic population. 126th and 131th day samples have 45% similarity with other sampling days.

6.1.2.5 Canonocial Correspondence Analysis(CCA) of Bacterial Profiles

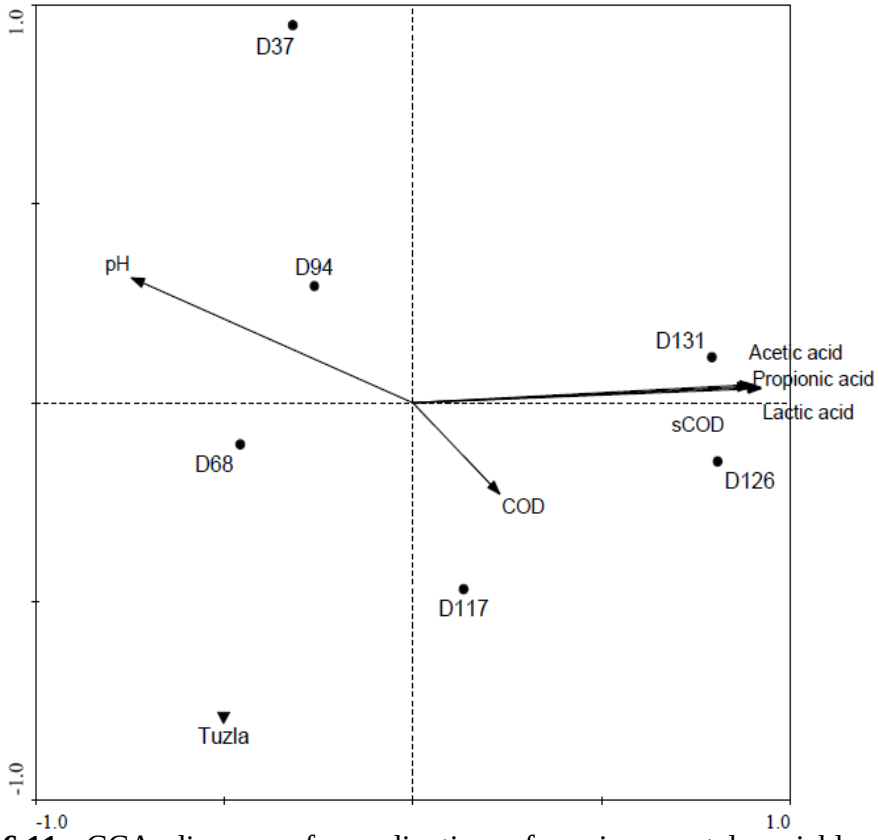


Figure 6.11: CCA diagrams for ordination of environmental variables such as volatile fatty acids (acetic acid, propionic acid, lactic acid), COD, pH for digester and of the 7 digester samples from bacterial DGGE fingerprints. D68 indicates digester sample on day 68.

CCA of DGGE fingerprints of digester bacterial communities showed that acetic acid, propionic acid, lactic acids in the digester sludge were highly correlated with changes in the structure of anaerobic bacteria communities especially D126 and D131, which were changed after inhibition of pH in 120 day of operation. The pH of the reactor was highly correlated with community structures. It has a positive correlation with D94, D68 and Tuzla sludge, negative correlation with D117, D131, and D126. COD variable has lower correlation with community changes. Acetic acid, propionic acid, lactic acid concentrations were the variables that best correlated with the structure of anaerobic bacteria communities in digester, followed by COD concentration. Community structures of the samples were far from Tuzla sludge. Increasing TS% loading rates and VFA concentrations depending on increased loading rates community structures were not stable and accumulate in a small area. After inhibition of digester, D126 and D131 samples accumulated in a small area. This was the significant effect of pH on selectivity of microbial communities in anaerobic digester ecology.

6.1.2.6 Canonocial Correspondence Analysis(CCA) of Archaeal Profiles

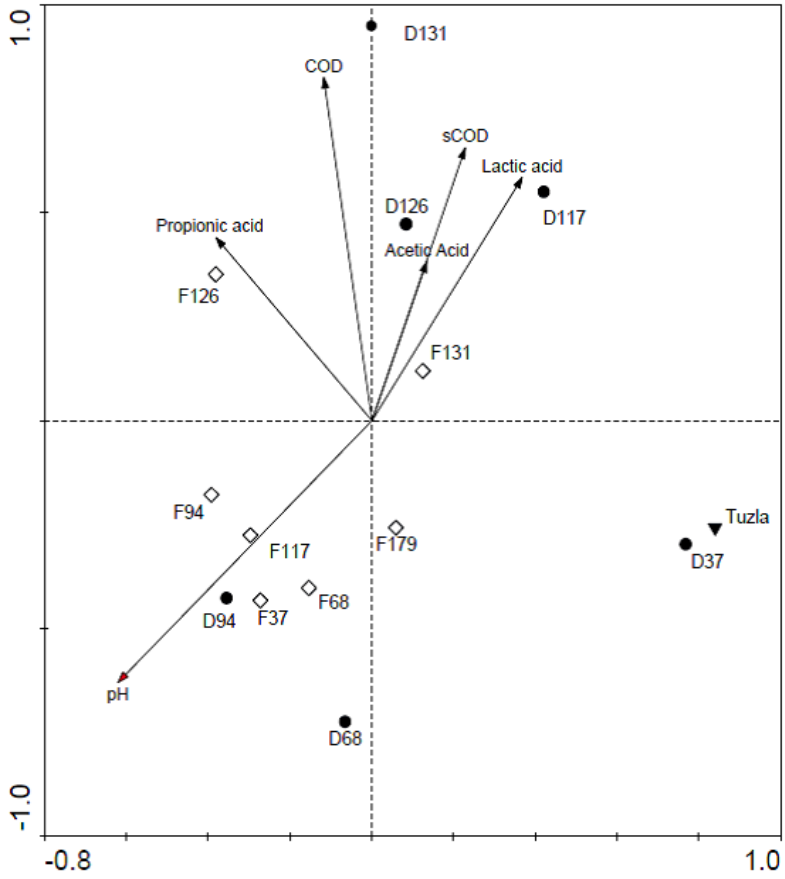


Figure 6.12: CCA diagrams for ordination of environmental variables such as volatile fatty acids (acetic acid, propionic acid, and lactic acid), COD, sCOD, pH and 14 fermenter and digester samples from archaeal DGGE fingerprints. D94 indicates digester sample on day 94, F94 indicates fermenter sample on day 94.

CCA of DGGE fingerprints of digester archaeal communities showed that VFA, soluble COD, COD values were positively correlated with digester samples. Similar to bacterial CCA profile, inhibition in digester effects D117, D126 and D131 samples, significantly changed community structure of these and made stable afterwards. The pH of the reactor highly and negatively correlated with digester community structures. Inoculums archaeal communities not changed in one-month acclimation as shown in the figure 6.12 as D37. After 60 days operation community diversity in digester was changed much move to negative gradient such as D68 and D94. Fermenter archaeal community diversity were very close each other. F37, F68, F94, F117 were accumulated in a small area with a negatively gradient. Samples were positively and highly correlated with pH and have lower correlation with VFA's. Increased TS% loading rates changed of the F126, F131, F179 sample's archaeal structures.

6.2 The clone library of the fermenter and digester sludge

There are potential experimental errors starting from extraction of the nucleic acids through the sequencing of the clones. To prevent those potential errors, many precautions were taken in the process of experimental works. For the clone libraries, PCR is the crucial step due to primer selectivity in the amplification and have the variable rRNA gene copy numbers.

Dinner hall waste study's biomass samples collected at the days of 37, 94, 131, and 179 from fermenter, 94, 117 and 133 from digester for bacterial analysis, subjected to cloning and sequencing analysis. Moreover, for archaeal analysis, biomass samples collected at the days of 94, 131 for fermenter and 37, 131 for digester, were subjected to cloning and sequencing analysis. In this respect, 96 bacterial and clones randomly picked for each sample and these clones were screened by RFLP. 22 out of 1056 clones were found as a shorter fragment compared to the 16S rRNA length and were eliminated for further analysis.

For bacterial cloning, 80 transformants from fermenter, 62 transformants from digester were selected for approximately 1500 bp sequencing using RFLP screening. For archaeal cloning 33 transformants from fermenter, 42 transformants from digester were selected for approximately 500 bp sequencing using RFLP screening Based on these partial 16S rRNA sequences with DGGE technique, 217 clones were compared with each other, and a total of 80 OTU were attained for bacteria, 65 OTU were attained for archaeal. Representative clones for each OTU was sequenced completely. The total OTU distributions for the fermenter and digester reactors were 84 and 61 respectively. Six of these sequences were detected as chimera and were taken aside for further analysis. .96 16S rRNA archaeal gene clones selected from each reactor were subjected to screening and subsequently to cloning and sequencing. It was found that all clones were closely related to the lineages of *Euryarchaeota*; including *Methanosarcinales*, *Methanomicrobiales*. Although, complete 16S rRNA nucleotide sequences showed a small similarity with the cultured bacteria, they showed great similarity with the cultured Archaea species.. Table 6.1-6.2 lists the percentage of similarities between transformants sequences of all three reactors and BLAST database after analysis using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi). A homology search showed that the main

groups could be classified into 2 major phylotypes in fermenter that belong to *Firmucutes*, *Bacteroidetes* phylum's. In addition, some minor OTUs were in *Synergistetes, Spirochaetes, Actinobacteria, Thermotogae* phylums (Tables 6.1-6.2). Thus, with the results of homology search, digester ecology could be consisting of 4 main groups such as *Firmicutes*, *Spirochaetes, Bacteroidetes* and *Synergistetes*. In addition, some minor OTUs were in *Thermotogae*phylum (Tables 6.1-6.2).

6.2.1 The clone library of the fermenter sludge

Bacterial Diversity

37th day

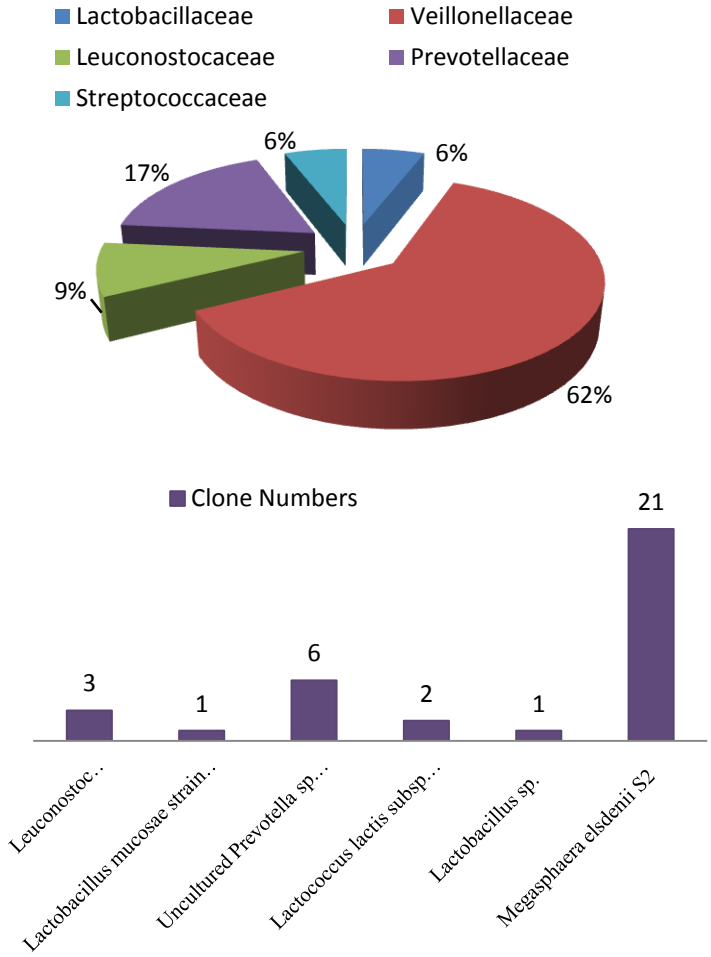


Figure 6.13: Phylogenetic distribution of bacterial 16S rRNA clones derived from 37th day of the fermenter reactor

Phylogenetic distribution of 16S rRNA clones derived from the 37th day of the fermenter reactor was given in Figure 6.11. Phylogenetic analysis of the 37th day clones revealed a community clearly dominated by diverse populations of Veillonellaceae (62% of all clones; Table 6.11);but *Leuconostocaceae* (9%), *Prevotellaceae* (17%), *Streptococcaceaes* (6%) and Lactobacillaceae (6%) were detected as well (Figure 6.11).The most abundant isolate 99% identical to *Bacterium strain LB01*. A lactate-utilizing, butyrate-producing bacterium, *strain LB01* had the high similarity with members of the genus *Megasphaera* and the metabolic characteristics that *strain LB01* efficiently converts toxic lactate and excessive acetate to butyrate can prevent lactate and acetate accumulation [185]. The second most abundant isolate 99% identical to *Prevotellaceae*, was a anaerobic gram-

negative bacilli and it was isolated from aerobic predigester [190]. The other abundant clone was the *Leuconostocaceae* and was closely related to the isolate *Leuconostoc pseudomesenteroides* strain L7 [191].

94th day

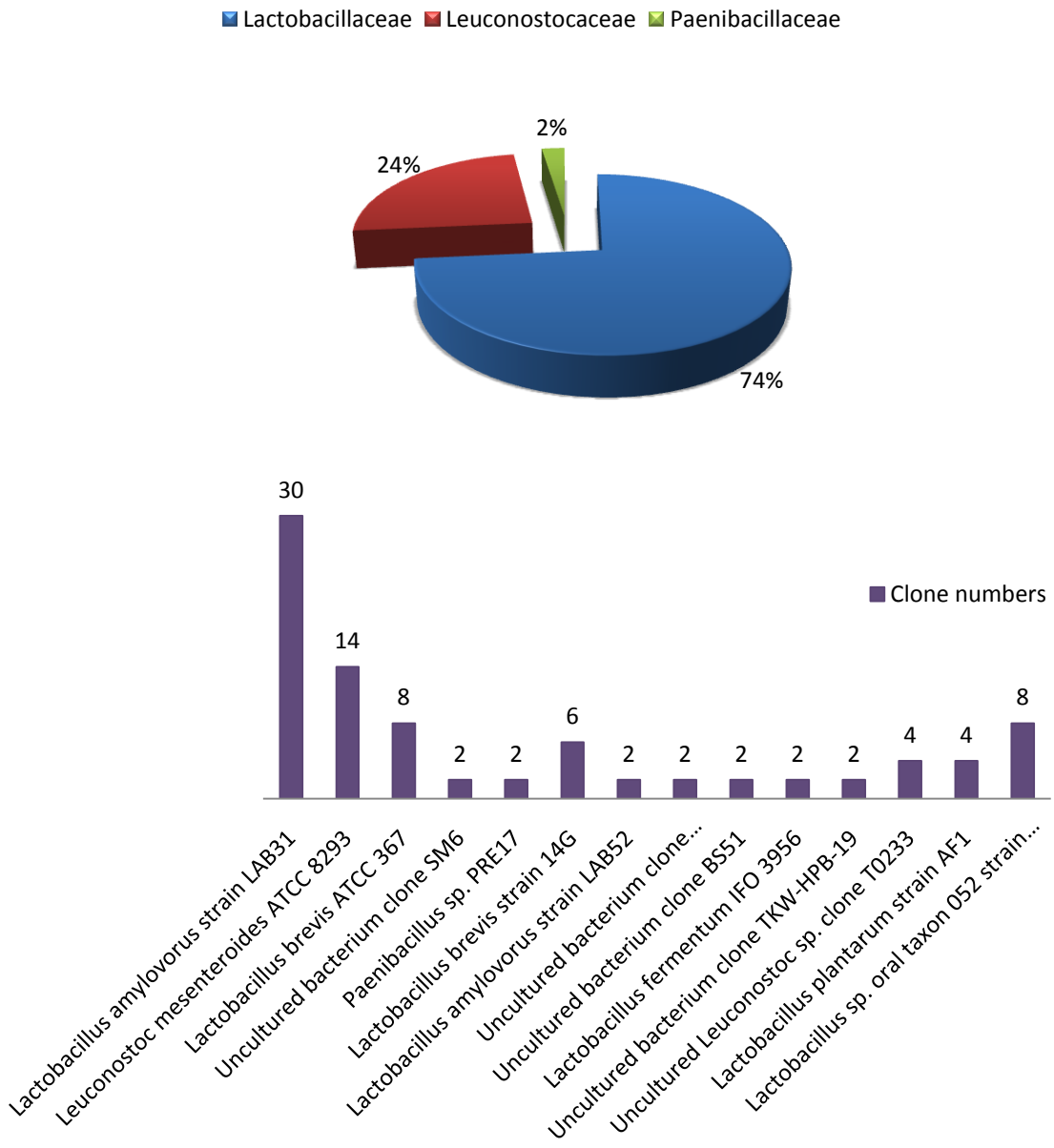


Figure 6.14: Phylogenetic distribution of bacterial 16S rRNA clones derived from 94th day of the fermenter reactor

Cloning of the 94th day of fermenter sludge clearly showed that Lactobacillaceae was the main dominant phylum in this clone library. The clones belonging to this phylum phylogenetically were scattered over several genus and were specialized on the guild of lactic acid producers.

The second most abundant isolate 99% identical to *Leuconostocaceae* and was closely related to the isolate *Leuconostoc mesenteroides ATCC 8293* [191]. *Leuconostoc* species are epiphylic bacteria that are widespread in the natural environment and play an important role in several industrial and food fermentations [192].Optimum temperature for growth are 20-30 ºC but growth can occur between 5ºC and 37ºC [193]. Beside this, clones belonging toPaenibacillaceae, esa detected a minor amount which accounts as 5% of the total library (Figure 6.12)

131thday

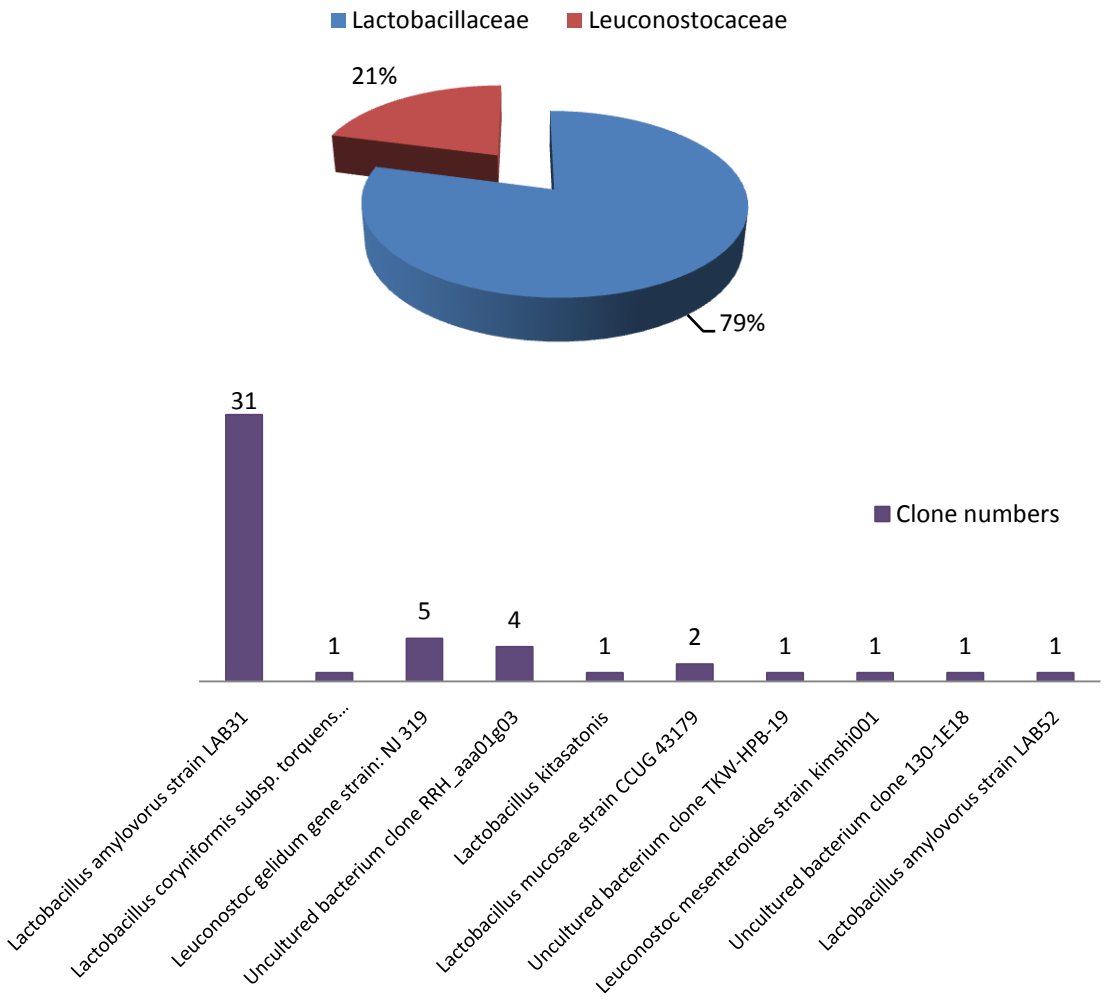


Figure 6.15: Phylogenetic distribution of bacterial 16S rRNA clones derived from 131th day of the fermenter reactor

Phylogenetic distribution of 16S rRNA clones derived from the 131th day of the fermenter reactor was given in Figure 6.13. Phylogenetic analysis of the 131th day clones revealed a community clearly dominated by diverse populations of Lactobacillaceae (79% of all clones; Table 6.13);but *Leuconostocaceae* (21%), was detected as well (Figure 6.13).The most abundant isolate 99% identical to

Lactobacillus amylovorus strain LAB52, a lactate-producing bacterium. The second most abundant isolate 99% identical to *Leuconostoc Gelidum. strain NJ319.*

179th Day

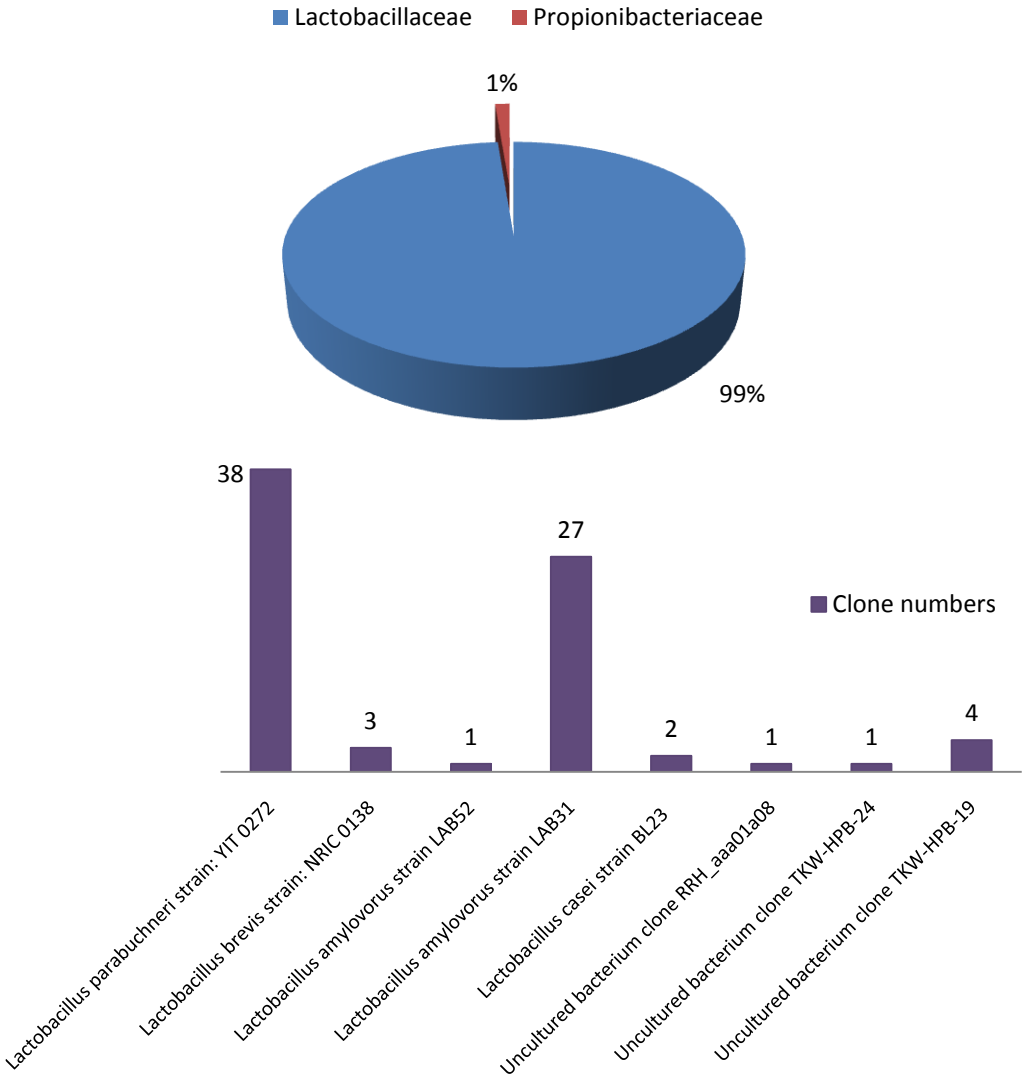


Figure 6.16: Phylogenetic distribution of bacterial 16S rRNA clones derived from 179th day of the fermenter reactor

Cloning of the 179th day of fermenter sludge clearly showed that Lactobacillaceae was the main dominant phylum in this clone library. The clones belonging to this phylum phylogenetically were scattered over several genus and were specialized on the guild of lactic acid producers.

The second most abundant isolate 95% identical to *Propionibacteriaceae* and was closely related to the isolate *Propionibacterium acnes strain: JCM 6425 ATCC 8293*

Propionibacterium species are slow-growing, nonsporulating, gram-positive anaerobic bacilli [194]

Archaeal Diversity

94th day

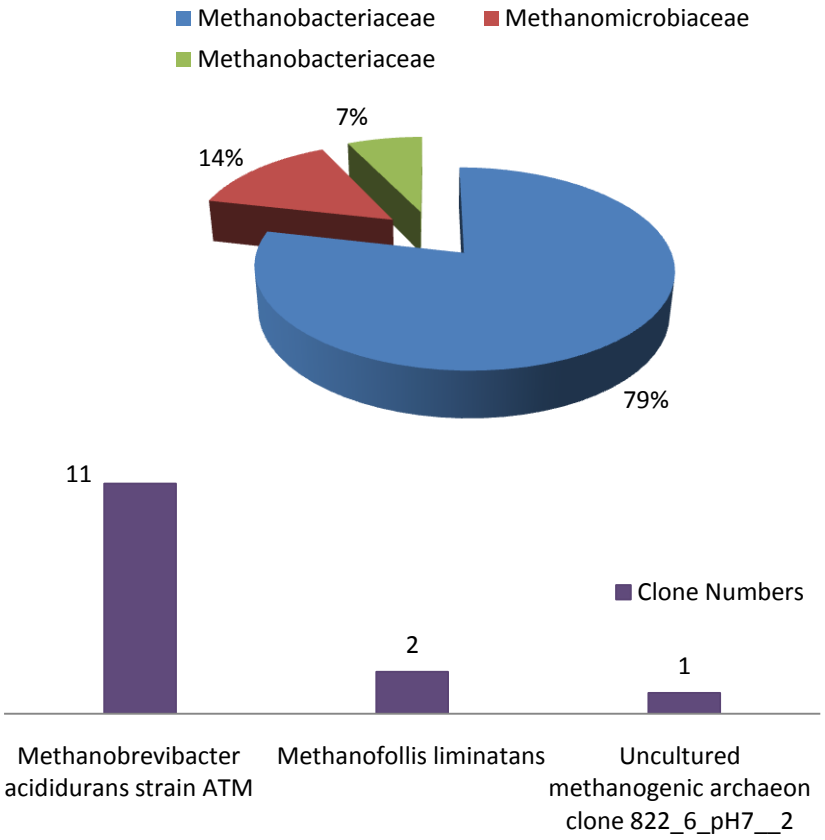


Figure 6.17: Phylogenetic distribution of archeal 16S rRNA clones derived from 94th day of the fermenter reactor

The archaeal composition of the 94th day of fermenter sludge was given in Table 6.2. Archaeal methanogenic community showed low diversity and did not change during all operational periods. The major member of the Archaea detected in the fermenter sludge was Methanobrevibacter acididurans strain ATM. The isolate could grow in the pH range 5.0--7.5, with maximum growth at pH 6.0. Rumen fluid and acetate were required for growth on H(2)/CO(2) [186]. The second dominant archaeal species in the 94th day of fermenter sludge were Methanofollis liminatans (14%). Methanofollis liminatans was a mesophilic, highly irregular cocci that use H_2/CO_2 and formate for growth and methanogenesis [187]. The other member of the archaeal community belongs to *Methanobacteriaceae* with an abundance of 7%.

131th day

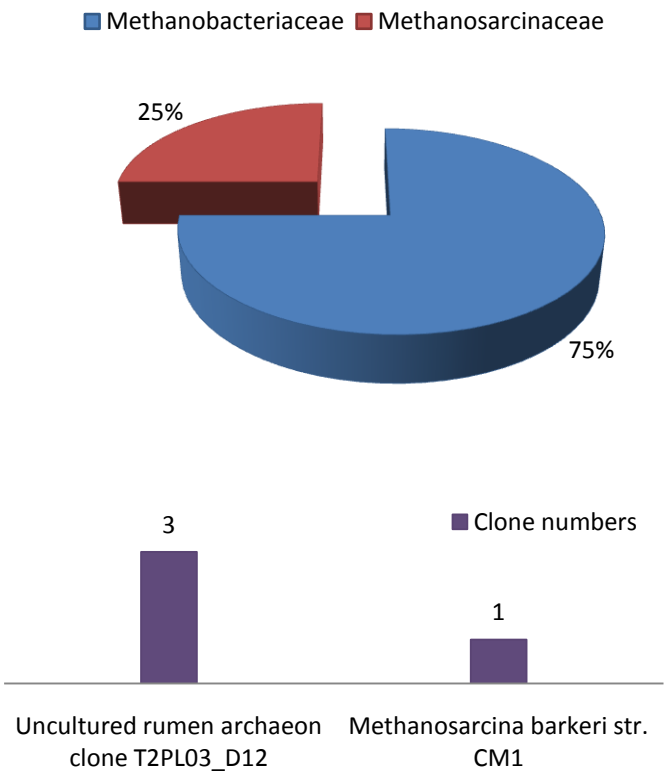


Figure 6.18: Phylogenetic distribution of archaeal 16S rRNA clones derived from 131th day of the fermenter reactor

The archaeal composition of the 131th day of fermenter sludge was given in Table 6.2. Archaeal methanogenic community showed low diversity and did not change during all operational periods. The major member of the Archaea detected in the fermenter sludge was Methanobacteriaceae. The second dominant archaeal species in the 131th day of fermenter sludge were Methanosarcina barkeri str. CM1 .(14%). with %98 abundance. Methanosarcina family are slow developers and are sensitive to sudden change in physical and chemical conditions, but they are capable of growth in a variety of substrates [195]

6.2.2 The clone library of digester sludge

Bacterial Diversity

68th day

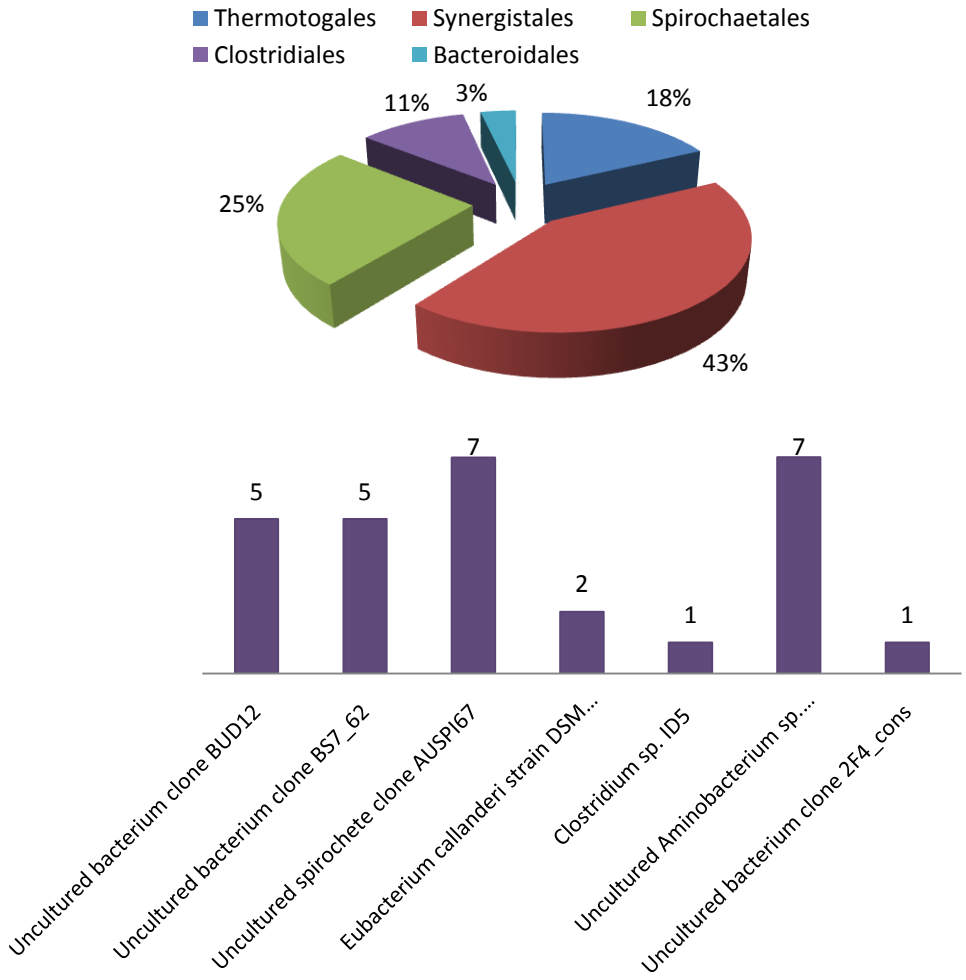


Figure 6.19: Phylogenetic distribution of bacterial 16S rRNA clones derived from 68th day of the digester reactor

Phylogenetic distribution of 16S rRNA clones derived from the 68th day of the fermenter reactor was given in Figure 6.17. Phylogenetic analysis of the 68th day clones revealed a community clearly dominated by diverse populations of Synergistales (43%) of all clones; but *Spirochaetales* (25%), *Thermotogales* (18%), *Clostridiales* (11%) and *Bacteroidales* (3%) were detected as well (Figure 6.15).

The most abundant isolate 98% identical to *Synergistaceae* the second most abundant isolate 99% identical to *Spirochaetales*, the other abundant clone was the *Thermotogales* and was closely related to the isolate *Uncultured bacterium clone BUD12* .

117th day

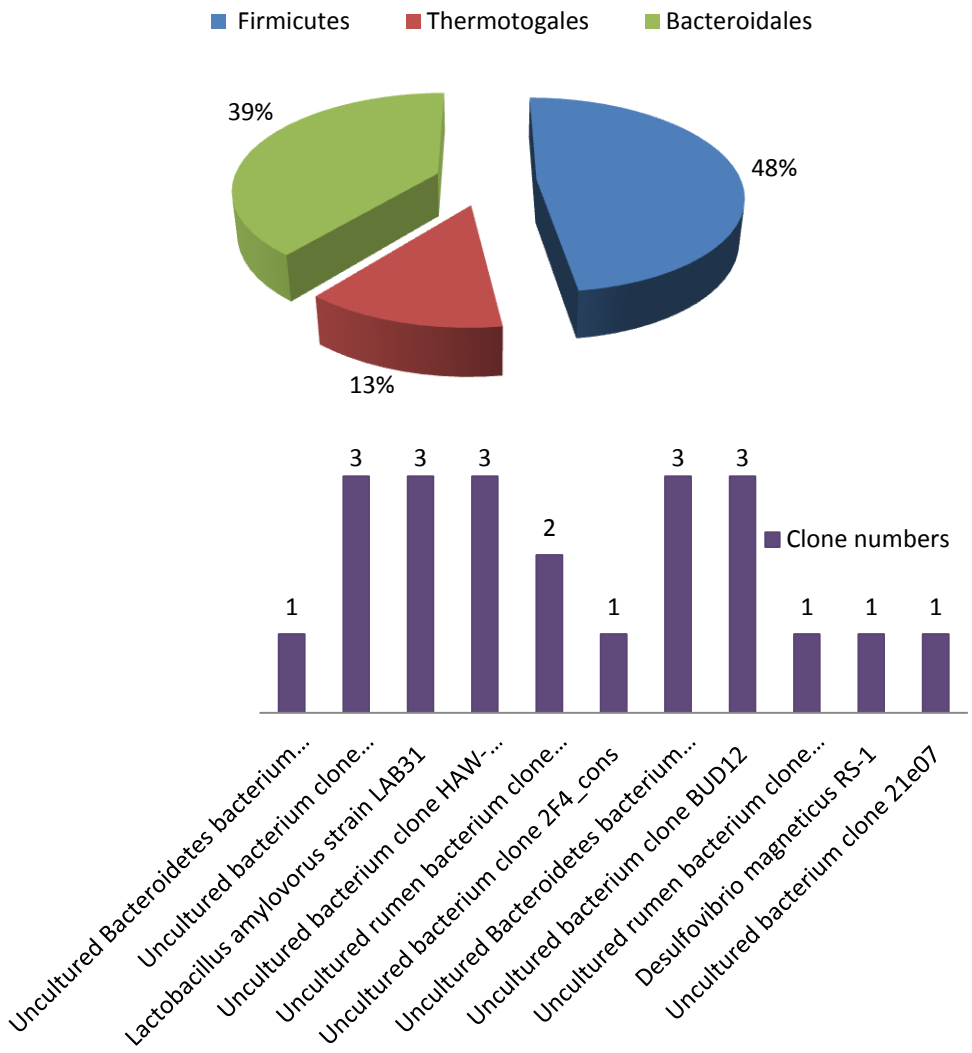


Figure 6.20: Phylogenetic distribution of archeal 16S rRNA clones derived from 117th day of the digester reactor

Cloning of the 117th day of fermenter sludge clearly showed that Bacteroidales was the main dominant phylum in this clone library. The clones belonging to this phylum phylogenetically were scattered over several genus and were specialized on the guild of *Porphyromonadaceae* and *Rikenellaceae.*

The second most abundant isolates identical to *Firmicutes* and were specialized on the guild of *Ruminococcaceae, Lactobacillaceae, Porphyromonadaceae* and *Lachnospiraceae.* Beside this, clones belonging *Thermotogales*,also detected a minor amount which accounts as 13% of the total library. (Figure 6.20)

Archaeal Diversity

37th day

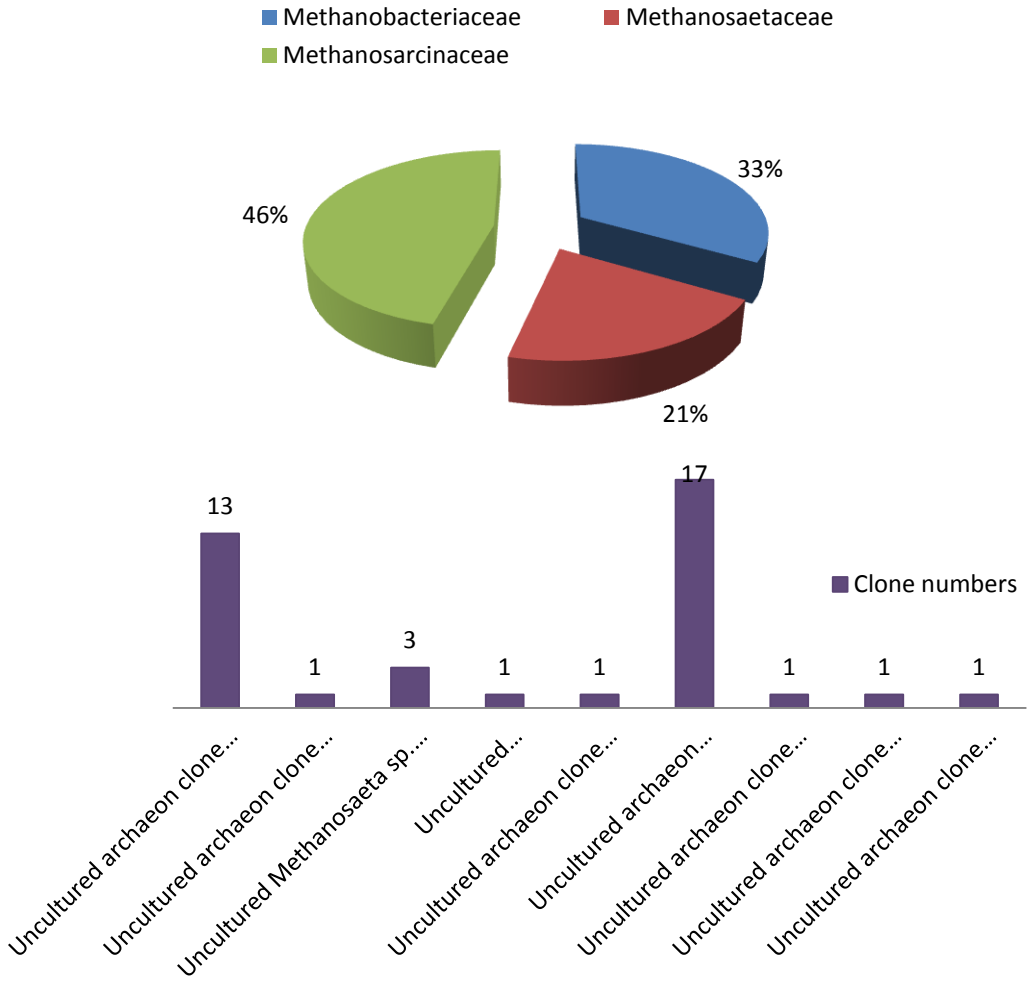


Figure 6.21: Phylogenetic distribution of archaeal 16S rRNA clones derived from 37th day of the digester reactor

The archaeal composition of the 37th day of digester sludge was given in Table 6.2. Archaeal methanogenic community showed high diversity and stable during all operational periods. The major member of the Archaea group detected in the digester sludge was *Methanosarcinaceae* (46%). and mainly *Uncultured archaeon clone:HNA-02*. The second dominant archaeal species in the 94th day of fermenter sludge were *Methanobacteriaceae* (33%). *Methanobacteriaceae* was known as hydrogenotropic group that use H_2/CO_2 for growth and methanogenesis. The other member of the archaeal community belongs to *Methanosaetaceae* with an abundance of 21%.

131th day

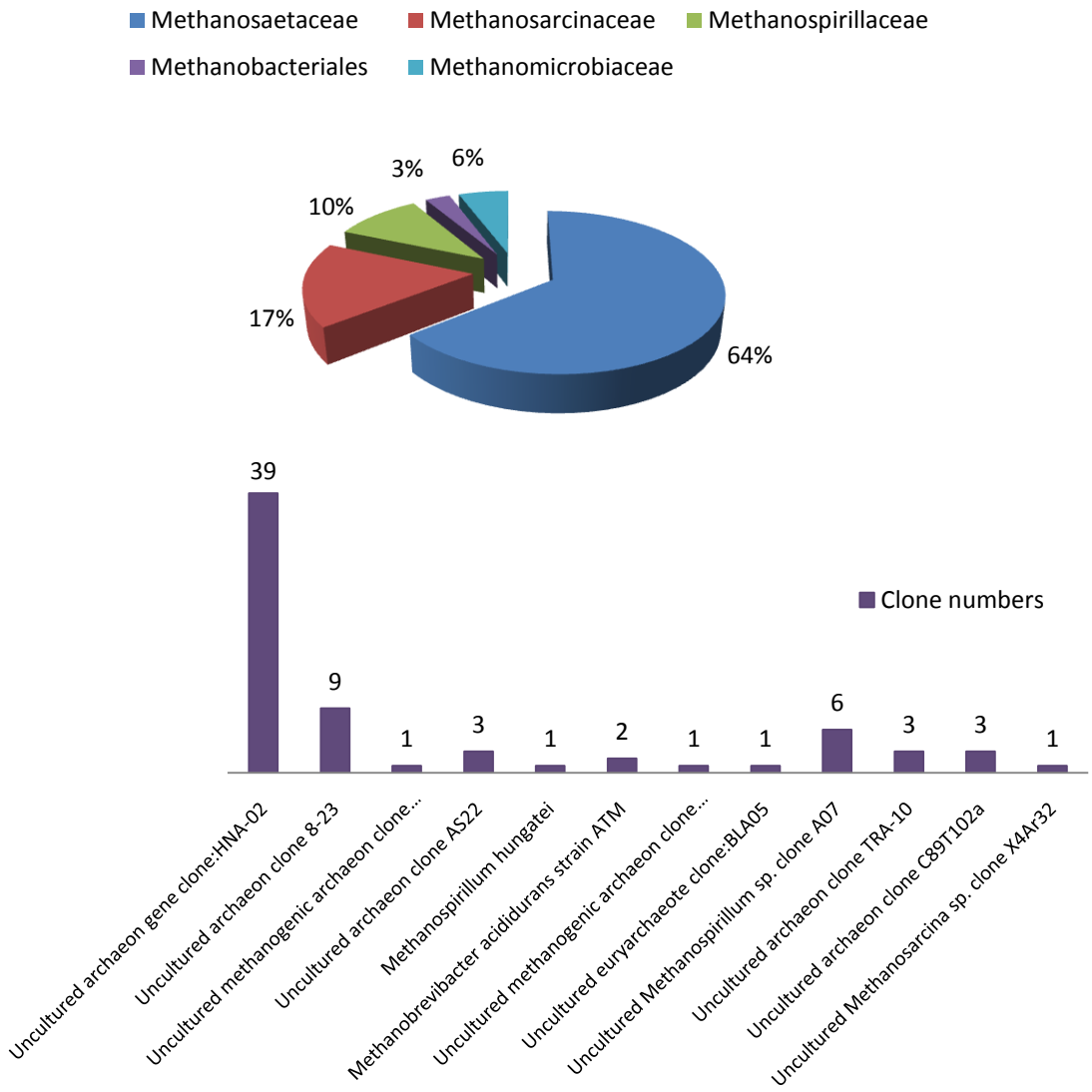


Figure 6.22: Phylogenetic distribution of archaeal 16S rRNA clones derived from 131th day of the digester reactor

The archaeal composition of the 131th day of digester sludge was given in Table 6.2. Archaeal methanogenic community showed significantly change in the ratios after 3 month period. *Methanosaetaceae* ratio was increased greatly to 64%. The major member of the *Methanosaetaceae* group detected in the digester sludge was *Uncultured archaeon gene clone:HNA-02*. The second dominant archaeal species in the 131th day of fermenter sludge were *Methanosarcinaceae* (17%). The other member of the archaeal community belongs to *Methanospirillaceae* with an abundance of 10%. Minor populations inside the sample were *Methanomicrobiaceae* and *Methanobacteriales* with the ratios %6,%3 respectively.

6.2.3 Phylogenetic Tree of bacterial clones

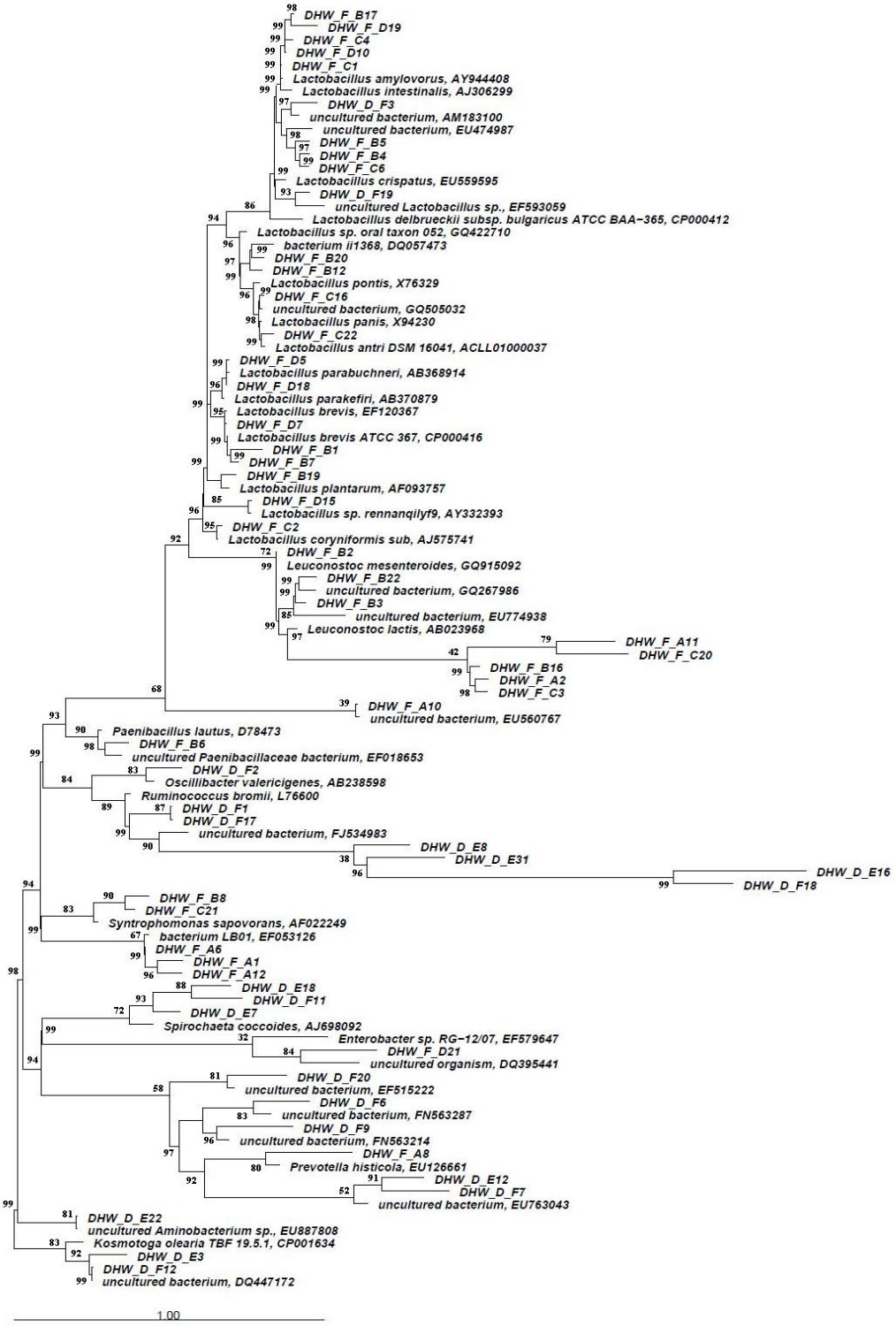


Figure 6.23: Phylogenetic relationships inferred from the alignment of partial bacterial 16S rRNA gene sequences of 38 isolated from fermenter and 22 from digester. GenBank accession numbers of reference sequences are reported. The DHW stands for dinner hall wastes, F and D represents fermenter and digester reactors, letters with numbers represents band

numbers in Figure 6.1. 16S rRNA genes sequences belong each isolation were aligned using CLUSTALX (editor 4.1) in ARB. The tree was constructed using the neighbor-joining method. The bar indicates 100% sequence divergence. Bootstrap values (expressed as percentages of 1,000 replications) are reported at each node.

6.2.4 Phylogeneric Tree of archaeal clones

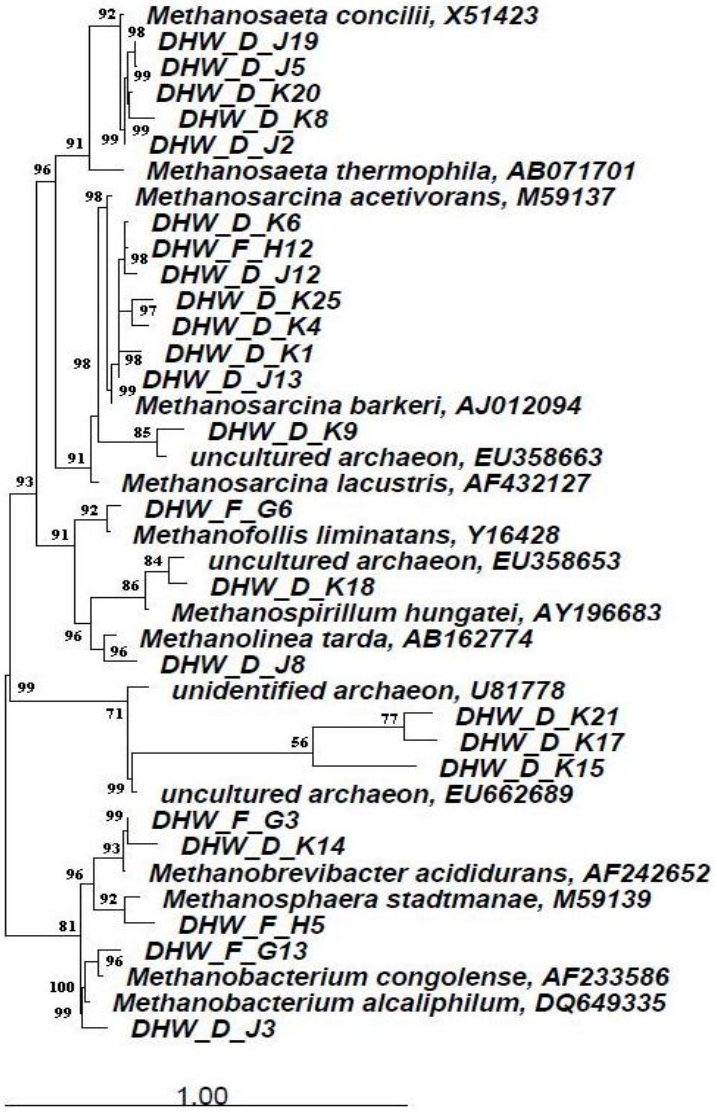


Figure 6.24 : Phylogenetic relationships inferred from the alignment of partial archaeal 16S rRNA gene sequences of 16 isolated from fermenter and 21 from digester. GenBank accession numbers of reference sequences are reported. The DHW stands for dinner hall wastes, F and D represents fermenter and digester reactors, letters with numbers represents band numbers in Figure 6.1. 16S rRNA genes sequences belong each isolation were aligned using CLUSTALX (editor 4.1) in ARB. The tree was constructed using the neighbor-joining method. The bar indicates 100% sequence divergence. Bootstrap values (expressed as percentages of 1,000 replications) are reported at each node.

7. THE ANAEROBIC DEGRADATION OF VEGETABLE HALL WASTES IN TWO–STAGE DIGESTER

7.1 Introduction

This chapter remark physiological traits of the two-stage digestion in chemostat reactors at 37°C as a main objective. A better understanding of the fermenter and digester reactors and possible operating parameters for anaerobic digestion with vegetable hall wastes was investigated with this study. In this concept, performance of the reactors was determined with COD, sCOD, TS parameters and in terms of total for all 2 reactors. The research is also examining the VFA production and utilization inside the reactors.

7.2 Operation of the reactors

The Biomethanization system was operated totally 133 days, Operating period and parameters are shown on table 7.1. Anaerobic digestion of solid wastes is rate limited by the hydrolysis step, and so physico-chemical treatments are often used to promote solubilization of organic matter [81]. Feeding composition in this study was not stable. Because of the inorganic or slowly degradable matters (wooden storage parts, plastics, metals) caused a decrease in the performance of the system. (Decrease in biogas production).Thus, a pre-treatment required for the pilot plant (Figure 7.1)

Table 7.1: Reactors treating vegetable hall wastes operating period and parameters

Parameter	Fermenter	Digester
Volume, m^3	0.65 (max:0.85)	4.29 (max: 4.50)
Flow, L/day	130	130
Hydraulic retention time, (day)	5	33
Volumetric Loading (kg COD/m3. day)	4.0	0.6
Volumetric Loading (kg TVS/m3. day)	4.1	0.5
Feeding TS (%)	2.1±0.8	1.6±0.8
Temperature,(^oC)	24±7	31±6

Figure 7.1 : Raw and waste, pre-treated feeding composition of vegetable hall wastes. (A: raw B. Pre-treated waste)

7.3 Performance of the reactors

Over 150 day's operation of the reactors, complete VFA and soluble COD degradation was observed. Very low VFA concentrations were detected at the effluent of the reactor. Every reactor is analyzed according to each period under different sections.

Table 7.2: Biomethanization system performance

Parametre	Period (0-133 days)		
	Pulper	Fermenter	Digester
pH	-	5.11±0.32	7.23±0.13
Alkalinity, mg $CaCO_3$/l	-	-	5600±610
TS, %	2.1±0.8	1.6±0.8	3.9±1.2
TVS, %	1.5±0.6	1.0±0.6	1.8±0.5
Tot. COD, mg/l	19800±6550	20100±7500	27200±8900
sCOD, mg/l	8600±4060	10500±3400	720±400

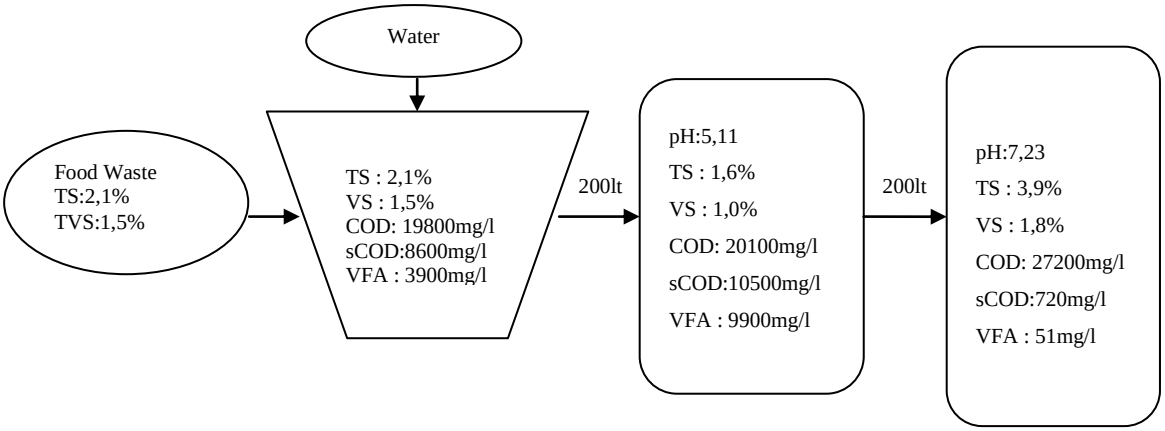


Figure 7.2 : Schematic view of the Biomethanization Pilot Plant with average performance values

7.3.1 Pulper

Influent vegetable hall wastes were grinded with water to achieve approximately 2.1% total solid content then, fed to pulper. Feed composition of the waste is shown on table 7.3.

Table 7.3 : Reactors treating vegetable hall wastes operating period and parameters

Parameter	Ave..	Median	Max	Min.	Std. Deviation
TS, %	2,1	1,9	3,8	0,8	0,8
TVS, %	1,5	1,4	3,1	0,6	0,6
Tot. COD, mg/l	19800	18700	37000	8600	6550
Soluble COD, mg/l	8600	8200	18800	1900	4060

7.3.2 Fermenter

Fermenter is the first biological process stage of the biomethanization system. Hydraulic retention time of the reactor is 5 days. Fermenter of pilot plant fed with approximately 3,0 kgTVS/m3.day organic loading and waste with %2,1 TS content. The average TVS/TS ratio in third period is 0.72. In this study OLR was not increased to reach high loading rates and stable between 1.5 and 5.0 kgTVS/m^3.day

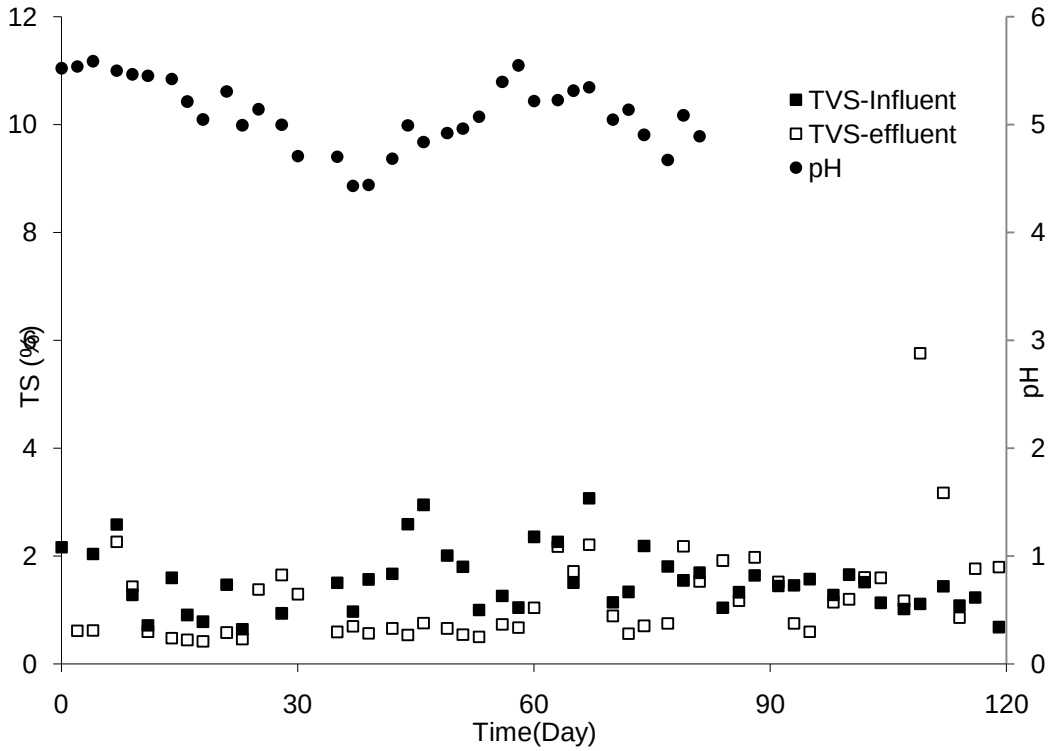


Figure 7.3 : Influent and Effluent TVS and pH values in fermenter

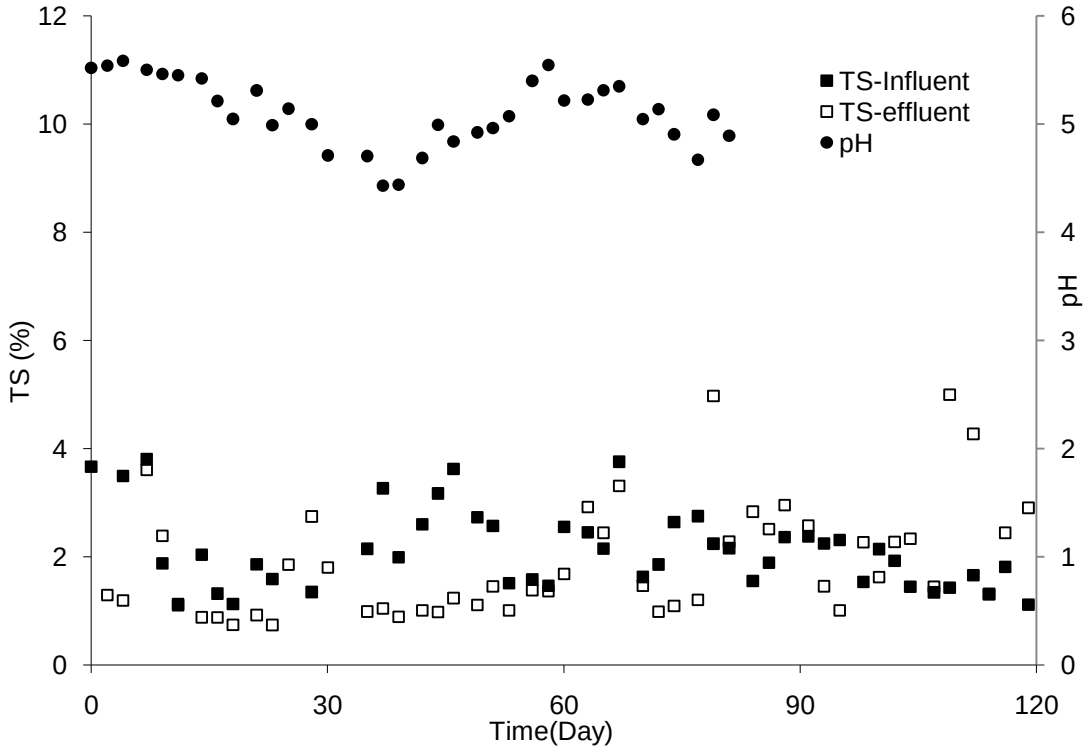


Figure 7.4 : Influent and Effluent TS and pH values in fermenter

During fermentation, pH and temperature are measured. The values are 5.11±0.32, 24±7 ^{0}C respectively. In operation period, fermenter had some heating problems.

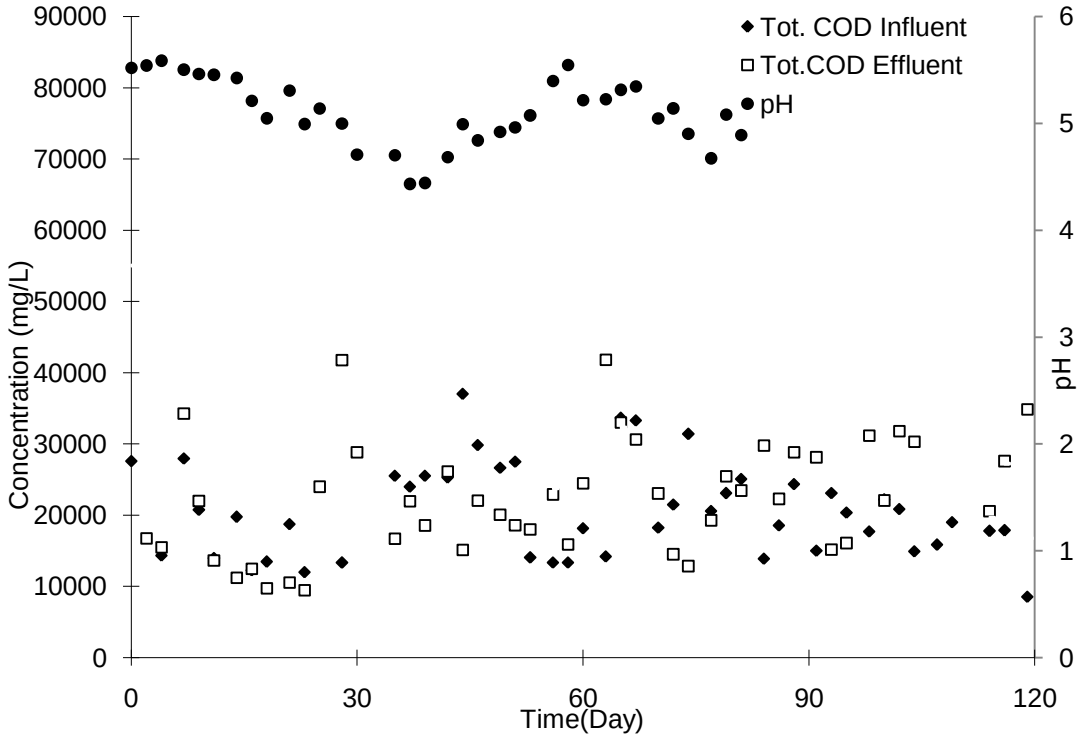


Figure 7.5 : Influent and Effluent tot.COD and pH values in fermenter

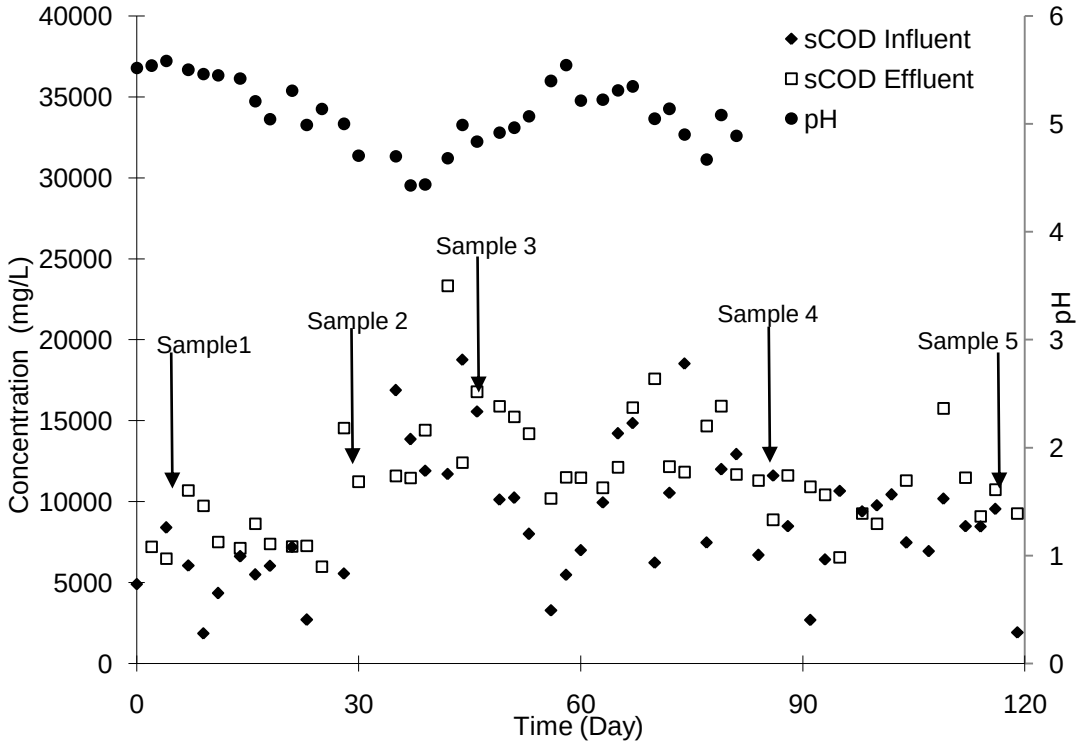


Figure 7.6: Influent and Effluent soluble COD and pH values in fermenter. Sample number shows samples times that were taken for microbiological analyses.

But, seasonal temperatures stabilized reactor temperature to 30°C.

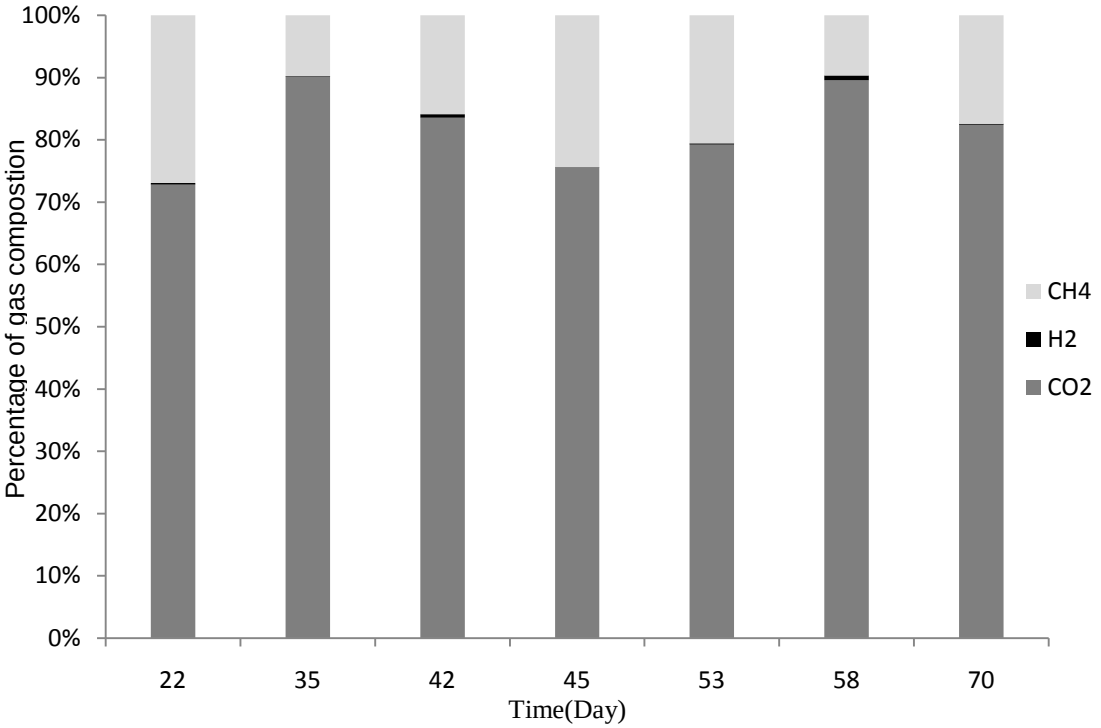


Figure 7.7: Gas composition in fermenter

The gas composition of the fermenter was measured as 17.8 %CH4, 81.9 %CO2 and 0.3 %H_2 (Figure 7.7).

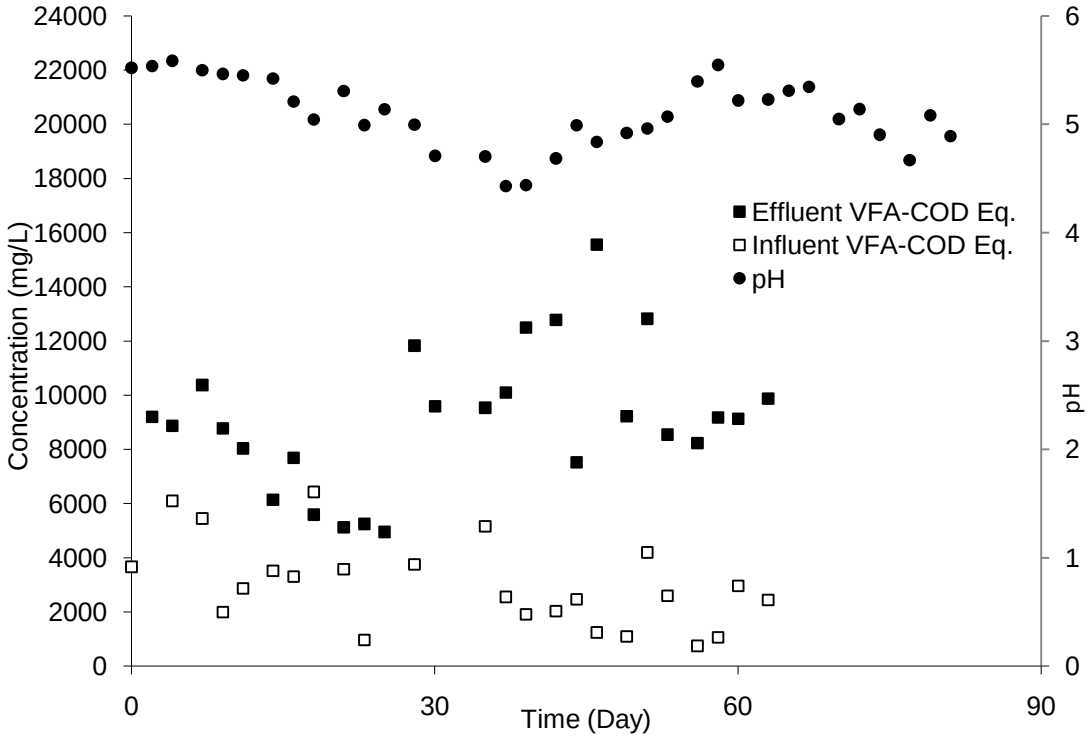


Figure 7.8 : Influent and effluent total VFA and pH values in fermenter. Sample numbers show samples that were taken for microbiological analyses.

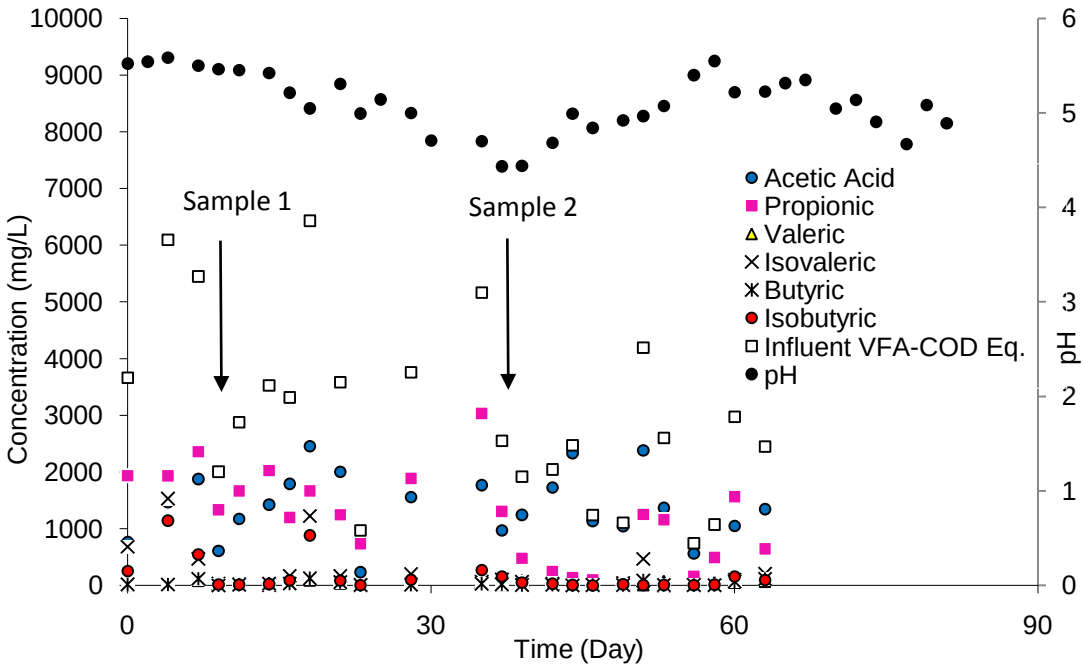


Figure 7.9 : Influent (Pulper) VFA components and pH values. Sample numbers show samples that were taken for microbiological analyses.

Soluble COD and VFA figures show (Figure 7.7, 7.9) that hydrolysis and fermentation processes were occurred. However, gas compositions and DGGE profiles show the methanogenic activity in the reactor.

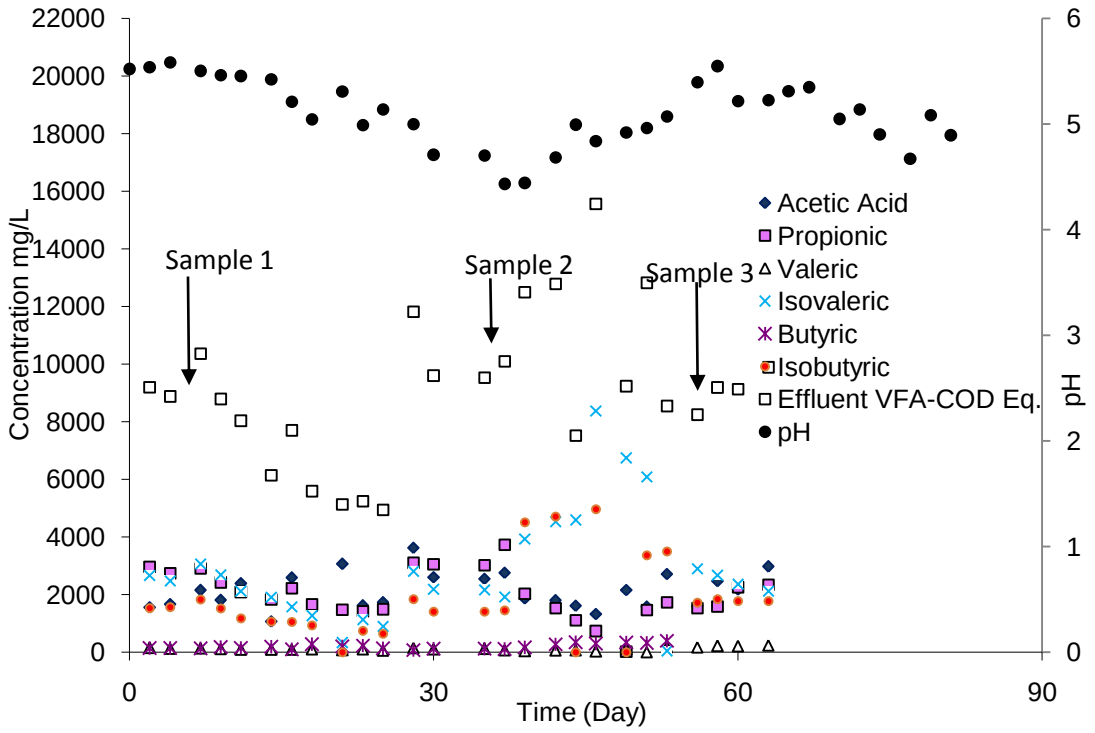


Figure 7.10 : VFA components and pH values in fermenter. Sample numbers show samples that were taken for microbiological analyses.

Although reactors average pH is 5.11, it was observed that there was a significant methane production in fermenter, which may a proof of methanization over hydrogen [82].

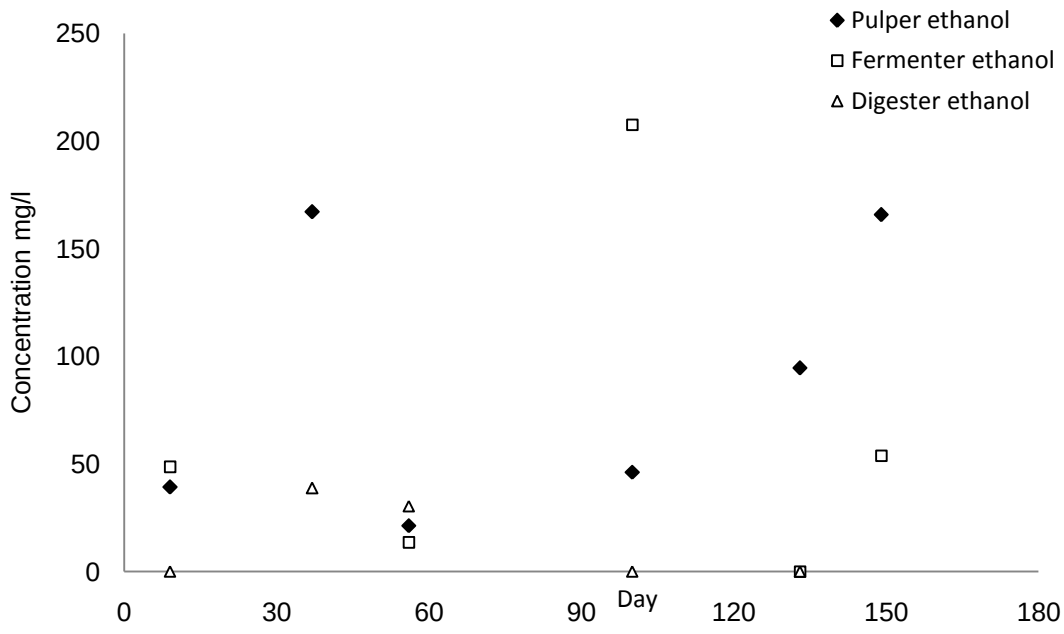


Figure 7.11: Ethanol values in pulper, fermenter and digester.

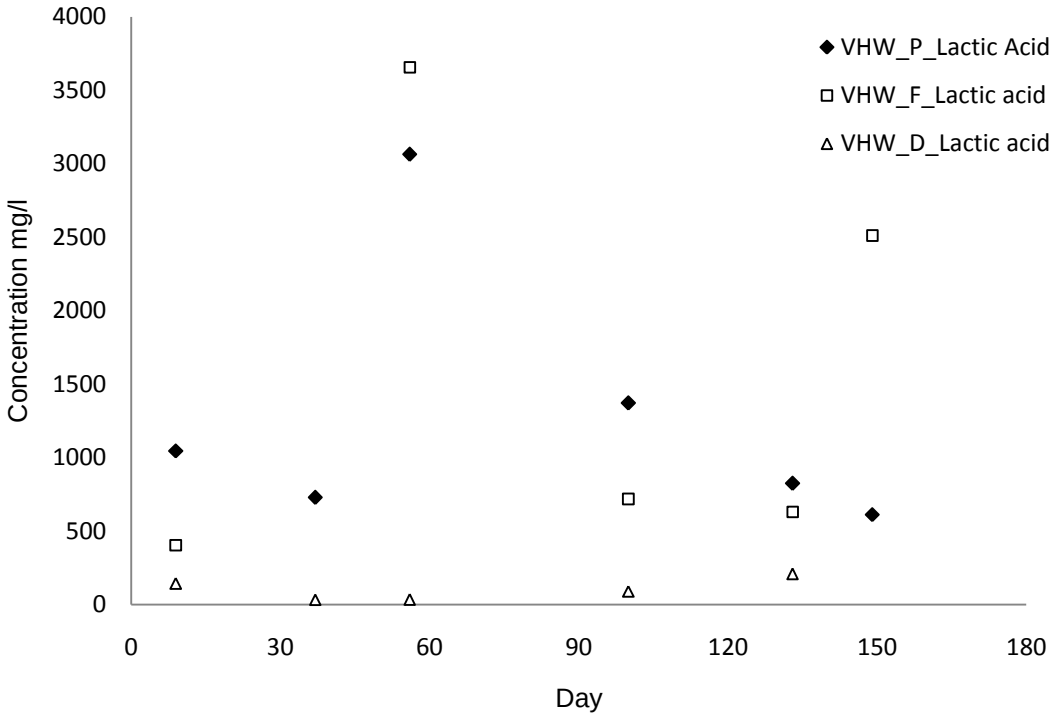


Figure 7.12 : Lactic acid values in pulper, fermenter and digester.

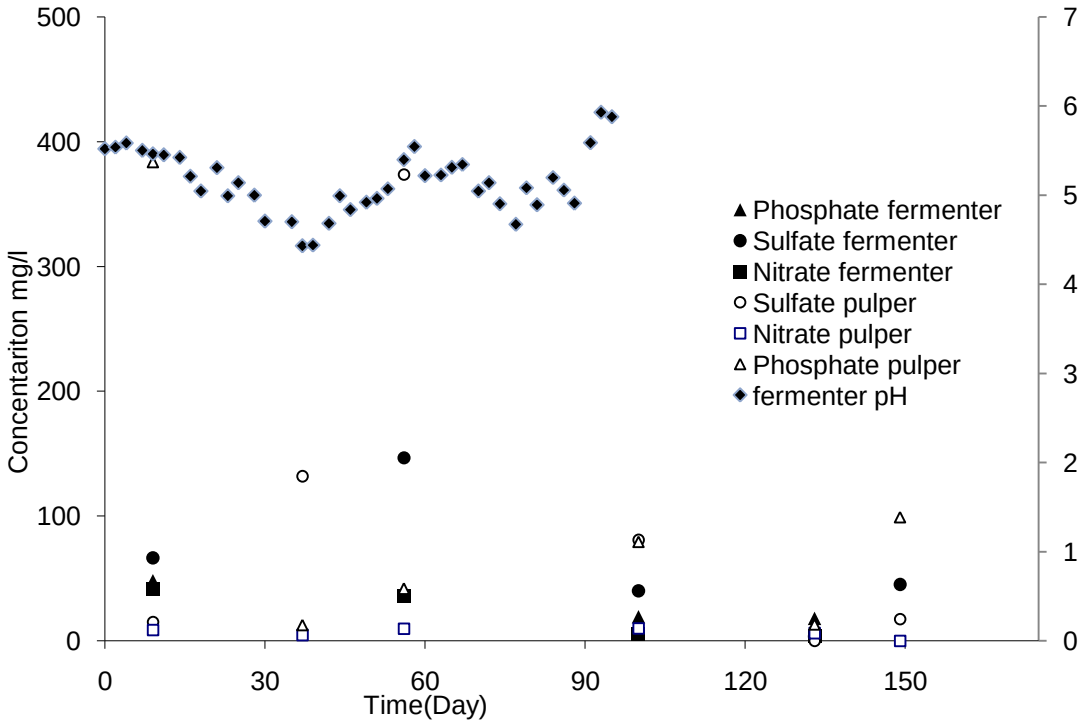


Figure 7.13 : Phosphate, Sulfate, Nitrate and pH values in pulper and fermenter.

7.3.3 Digester

Evaluating periods with soluble COD and total COD parameters, digester influent soluble COD and total COD values were 26560±8000 and 10500±2300 mg/l respectively (Figure 7.13, 7.14). sCOD/tot COD ratio in influent flow of digester with all periods is 0.40. In 133 days operating days, digester's total COD and soluble COD removal performance were 41% and 94% respectively. All system performance for total COD, soluble COD and TVS removal were 43%, 93% and 36% respectively.

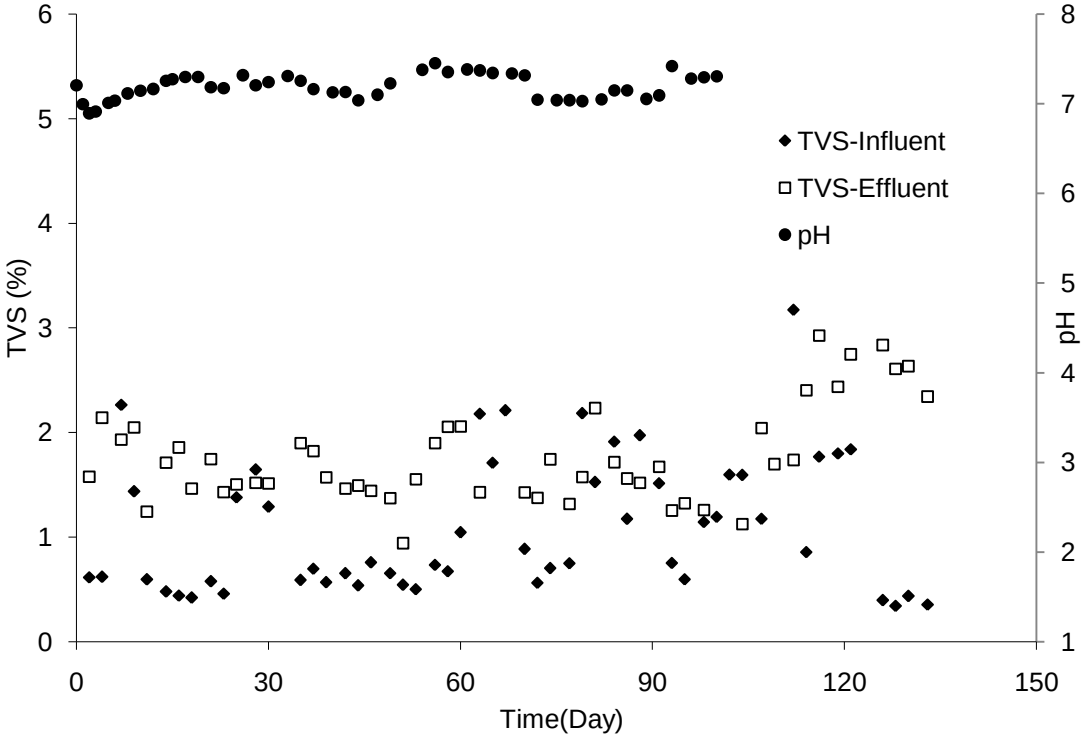


Figure 7.14 : Influent and effluent TVS and pH values in digester.

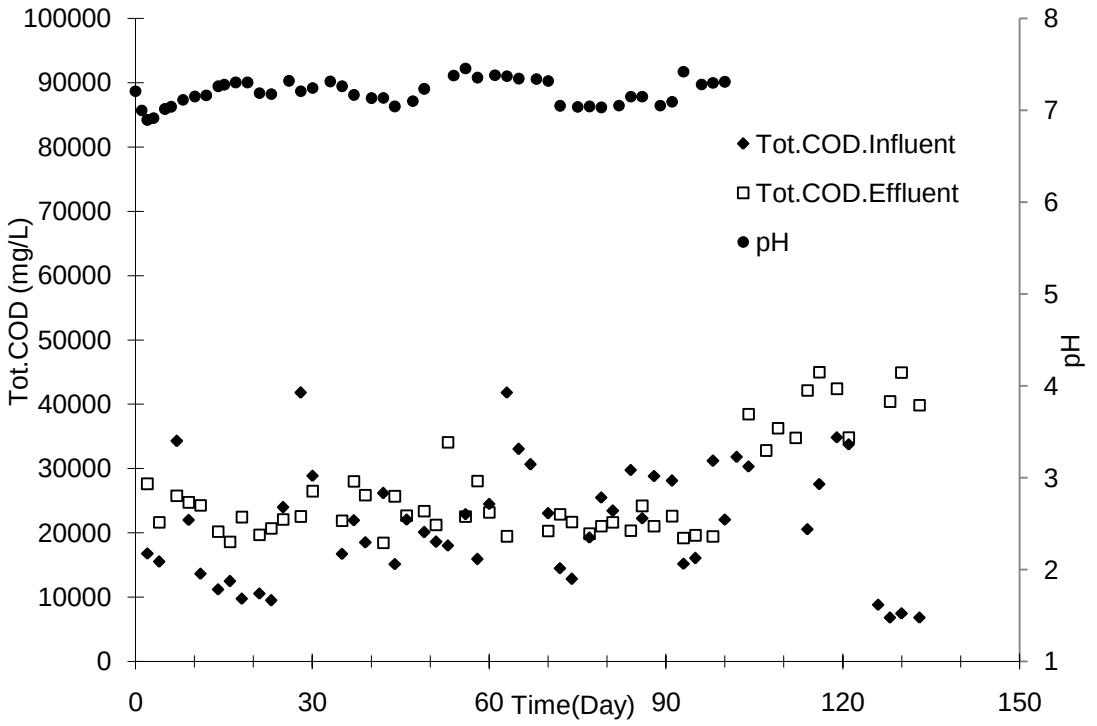


Figure 7.15 : Influent and effluent Tot.COD and pH values in digester.

VFA and COD values show that digester performance was approximately 91%, 95%. At the end of the 120 days operation time, mixing problems caused sampling errors.

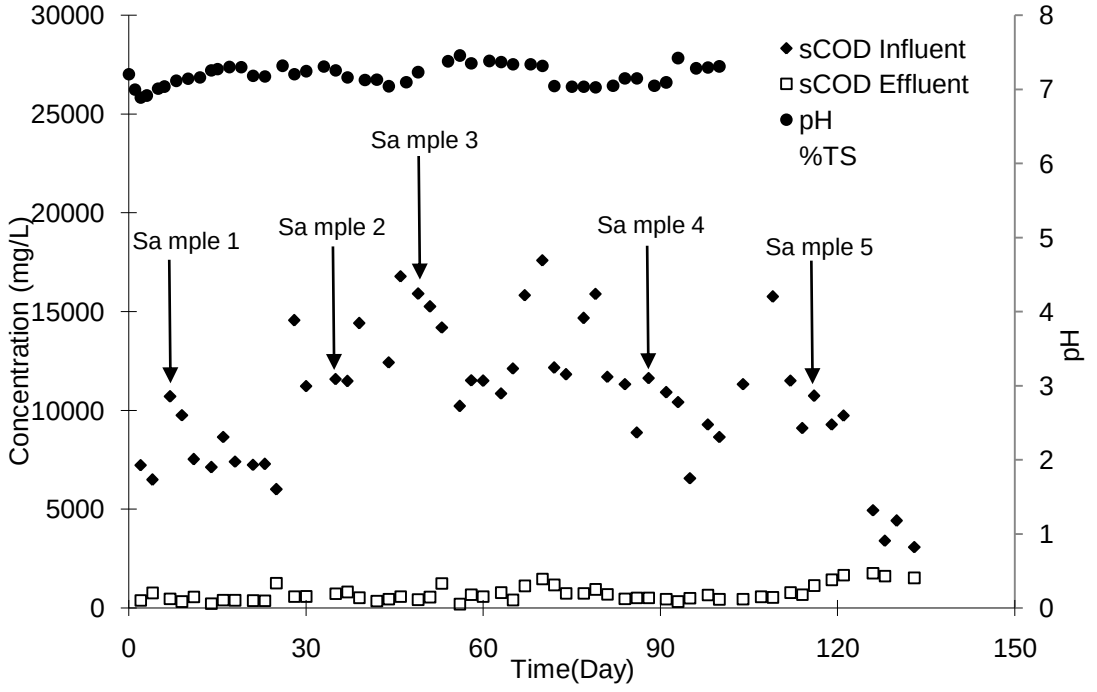


Figure 7.16 : Influent and effluent soluble COD and pH values in digester. Sample numbers show samples that were taken for microbiological analyses

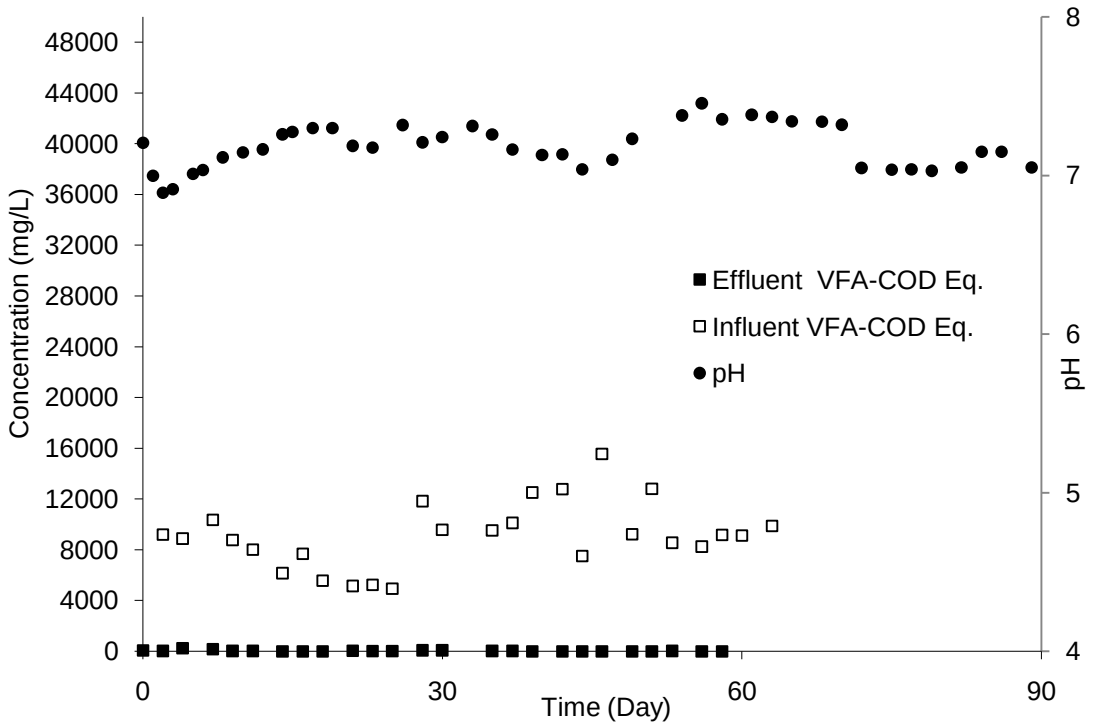


Figure 7.17 : Influent and effluent total VFA and pH values in digester.

8. POPULATION DYNAMICS OF VEGETABLE HALL WASTES IN TWO–STAGE DIGESTER

By conducting a molecular culture-independent methodology, the polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE); and cloning-sequencing was applied in order to characterize the reactors' sludge fed with vegetable hall wastes. The operational conditions of the reactors were described as in Chapter 4. The PCR amplified V6-V8 and V2- V3 regions of 16S rRNA for bacteria and archaea, respectively were analyzed by DGGE to visualize the prominent bacteria in the reactor sludge. DGGE analysis was also used to observe the population dynamics together with diversity along the operational period of the reactors. Moreover, 16S rRNA clone libraries were assessed to gain more insight to the identification of the microorganisms.

Archaeal PCR amplifications were repeated two times for understand the reason of low-light samples, which may result of PCR biases. In 37^{th}, day of pulper sample no products were observed when checked for on agarose gels, indicating that no archaeal population found in this sample. Thus, in 133^{th} day of fermenter sludge sample has low archaeal population.

8.1 DGGE analysis of 16S rRNA gene fragments.

8.1.1 PCR amplification

Extracted DNA's were amplified with Bac968F-GC [75]. and Bac1401R [75]. primers for bacterial population, Arc109 (t) F [76]. and Arc515-GC [75]. primers for archaeal population to generate 433bp and 406bp DNA fragments respectively.

8.1.2 DGGE Result

The DGGE profiles derived from DNA amplicons of the reactor's sludge were given in Figure 8.1 and Figure 8.2. Population shifts of bacterial communities were assessed by comparing the DGGE banding patterns of 15 samples taken during the

operation of all reactors (1-150 days). (Figure 8.1-8.2). In this figures, numbers and Tuzla indicated the representative sampling days, and the marker for the DGGE analysis. DGGE profiles of the bacterial PCR amplification products obtained from the pulper, fermenter and digester reactors' sludge at different time intervals were shown in Figure 4.3. The prominent bands in the DGGE pattern should give the dominant members of the microbial community. So, the dominant isolations, such as F11, R21, N30, N3, M1, M14 and O2 bands in fermenter, P11, P12 and R21 bands in digester profiles were represented by the dark bands in the corresponding DGGE patterns. However, other important players could have been underestimated for complex environments. Such a statement could be derived from the PCR biases and and might cause the weakening or absence of the bands in the patterns as stated on previous study. These differences between the DGGE profiles and the 16S clone library could be due to the bias of the PCR amplification.Another important observation was the detection of multiple bands corresponding to one species. Marked bands were identified in table 8.1 for bacteria and table 8.2.for archaea. Biodiversity analysis, theorically can be said that each band represents a unique species, with band density according to species amount in a sample. But, the limitiations in the DNA extraction, PCR ampification and chance of chimeric product formation from mixed populations,create a failure on this assumption [79]. Thus,commigration of different sequences to same band, could give band two identifications.

Bacterial population shifts seen in the fermenter DGGE profile were mostly linked to changes in the substrate composition. Existence of bacterial variety between fermenter and digester is that the pH, feed composition and hydraulic retention time difference of the reactors. In first 5 profile of Figure 8.1 showed bacterial diversity of raw waste.

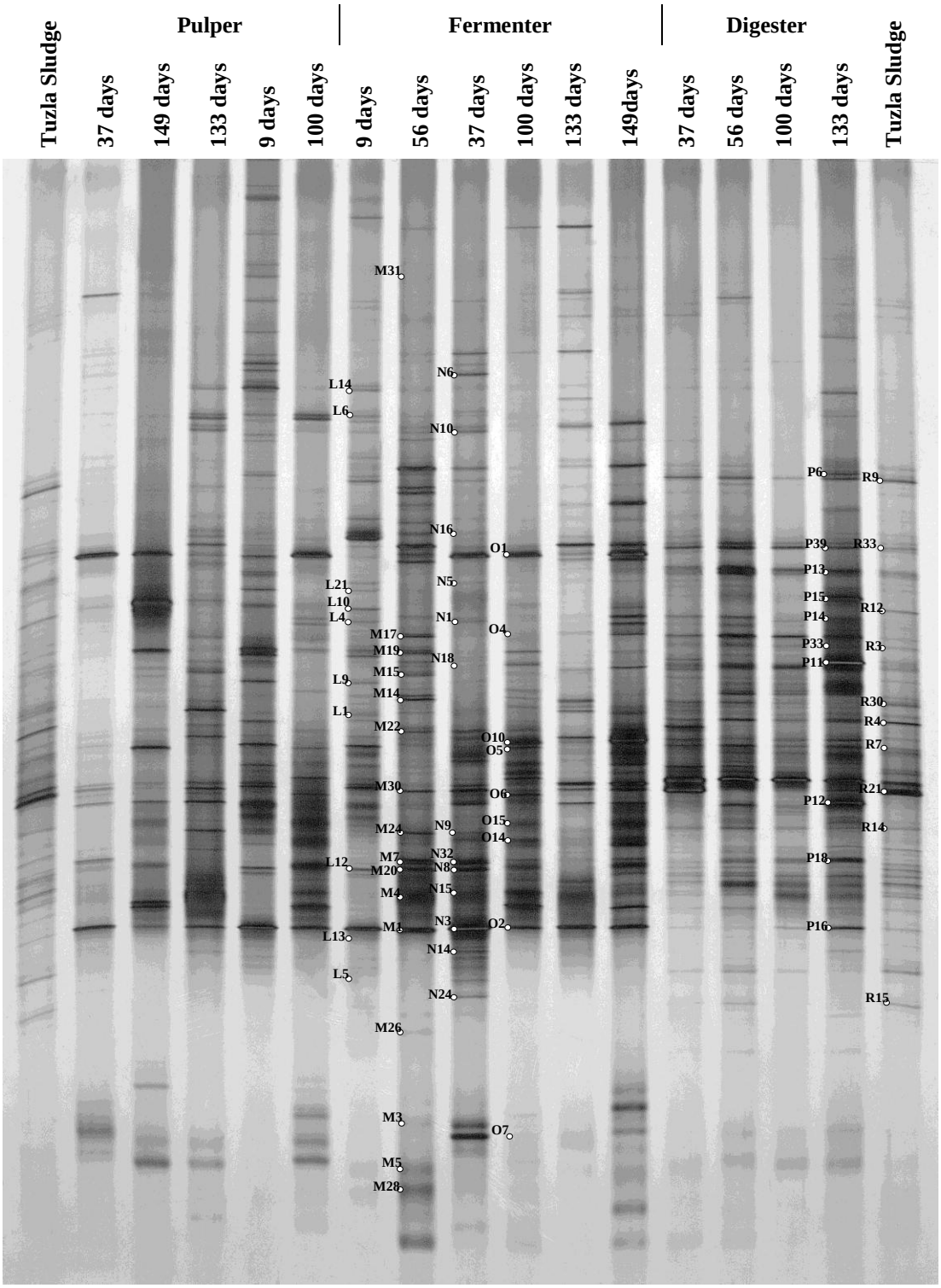


Figure 8.1 : DGGE analysis of the bacterial community during vegetable hall waste fermentation and digestion. 16S rRNA gene amplified with primers 968F-GC and 1401R from DNA samples extracted at different times, as indicated. The formamide-urea denaturing gradient ranged from 35% to 60%. Marked patterns were determined by cloning technique and successfully identified by sequencing. In Table 8.1 sequences were reported as band numbers and sequentially numbered from top to bottom**.**

Table 8.1 : :Sequence similarities to closest relatives and phylogenetic affiliations of DNA, matched with bacterial DGGE bands of VHW wastes

Band no.[a]	Identified Species	Number of clones	% of Total	Similarity(%)[b]	(NCBI accession no.)	Order	Family
L1	*P. freudenreichii subsp. Shermanii*	12	12,5	94%	Y10819	Propionibacteriaceae	Actinobacteria
L4	*Uncultured bacterium clone SJTU_F_15_80*	9	9,4	100%	EF399436a	Bacteroidaceae	Bacteroidetes
L5	*Uncultured bacterium clone SJTU_F_12_63*	2	2,1	89%	EF399177	Prevotellaceae	Bacteroidetes
L6	*Uncultured bacterium mle1-2*	1	1,0	94%	AF280841	Porphyromonadaceae	Bacteroidetes
L9	*Prevotellaceae bacterium WR041*	2	2,1	99%	AB298732	Prevotellaceae	Bacteroidetes
L10	*Uncultured Bacteroidetes clone ATB-LH-6096*	2	2,1	99%	FJ535139	Porphyromonadaceae	Bacteroidetes
L12	*Lactococcus sp. YM05001*	2	2,1	91%	EU689103	Streptococcaceae	Firmicutes
L13	*Uncultured bacterium clone RK*	14	14,6	97%	FJ645714	Synergistaceae	Synergistetes
L14	*clone HAW-R60-B-1249d-G*	2	2,1	97%	FN436190	Bacteroidaceae	Bacteroidales
L21	*Uncultured bacterium clone CE2_c02_1*	2	2,1	89%	EU773759	Carnobacteriaceae	Bacteroidetes
M1	*Uncultured Actinomycetaceae clone A06-09A*	4	4,2	88%	FJ542871	Propionibacteriaceae	Actinobacteria
M3	*Uncultured bacterium clone control_14days-H6*	2	2,1	89%	EF406522	Porphyromonadaceae	Bacteroidetes
M4	*Uncultured bacterium clone SJTU_G_02_82*	8	8,3	99%	EF405367	Veillonellaceae	Firmicutes
M5	*Uncultured bacterium clone 5*	1	1,0	90%	DQ011252	Ruminococcaceae	Firmicutes
M7	*Uncultured rumen bacterium clone L406RC7-D12*	2	2,1	90%	GU304045	Lachnospiraceae	Firmicutes
M14	*CDC Group DF-3 16S LMG 11519*	1	1,0	98%	U41355	Porphyromonadaceae	Bacteroidetes
M15	*Megasphaera paucivorans strain VTT E-032341*	1	1,0	94%	DQ223730	Veillonellaceae	Firmicutes
M17	*Uncultured bacterium clone AS2_aao34d03*	3	3,1	91%	EU772247	Incertae Sedis XIII	Firmicutes
M19	*Uncultured bacterium clone p-2794-24E5*	1	1,0	99%	AF371551	Lachnospiraceae	Firmicutes
M20	*Corynebacterium vitarumen strain NCTC 20294*	1	1,0	99%	X84680	Corynebacteriaceae	Actinobacteria
M22	*Uncultured bacterium clone WSp180*	3	3,1	87%	GQ867397	Ruminococcaceae	Firmicutes
M24	*Corynebacterium vitarumen strain NCTC 20294*	1	1,0	91%	X84680	Corynebacteriaceae	Actinobacteria
M26	*Uncultured bacterium clone H-200*	1	1,0	85%	EU622657	Porphyromonadaceae	Bacteroidetes
M28	*Acidaminococcus fermentans DSM 20731*	1	1,0	91%	CP001859	Veillonellaceae	Firmicutes
M30	*Uncultured bacterium clone SJTU_F_08_47*	1	1,0	95%	EF398877	Ruminococcaceae	Firmicutes
M31	*Uncultured bacterium clone SJTU_D_03_28*	1	1,0	95%	EF400909	Lachnospiraceae	Firmicutes
N1	*Ruminococcus flavefaciens strain FD-1*	1	1,0	93%	AM920691	Ruminococcaceae	Firmicutes
N3	*Uncultured bacterium clone RL117_aae93a02*	1	1,0	89%	EU778066	Prevotellaceae	Bacteroidetes
N5	*Uncultured bacterium clone A4-70*	1	1,0	98%	GQ897970	Clostridiaceae	Firmicutes
N6	*Uncultured Actinomycetaceae clone A06-09A*	5	5,2	92%	FJ542871	Propionibacteriaceae	Actinobacteria
N8	*Uncultured Ruminococcus sp. clone A03-03A*	3	3,1	99%	FJ542832	Ruminococcaceae	Firmicutes
N9	*Corynebacterium vitarumen strain NCTC 20294*	9	9,4	99%	X84680	Corynebacteriaceae	Actinobacteria
N10	*Uncultured bacterium clone SJTU_G_02_82*	2	2,1	99%	EF405367	Veillonellaceae	Firmicutes
N14	*Corynebacterium vitarumen strain NCTC 20294*	4	4,2	95%	X84680	Corynebacteriaceae	Actinobacteria
N15	*Bacterium enrichment culture clone DPF06*	1	1,0	89%	GQ377126	Ruminococcaceae	Firmicutes
N16	*Lactobacillus sp. JCM 20147*	1	1,0	91%	AB507169	Lactobacillaceae	Firmicutes
N18	*Lactobacillus parabuchneri strain: YIT 0272*	1	1,0	99%	AB429372	Lactobacillaceae	Firmicutes
N24	*Uncultured bacterium clone 5*	1	1,0	90%	DQ011252	Ruminococcaceae	Firmicutes
N32	*CDC Group DF-3 16S LMG 11519*	1	1,0	98%	U41355	Porphyromonadaceae	Actinobacteria

[a] Bands are numbered according to Figure 8.1

[b] Identity represents the % identity shared with sequences in the Genbank database

Table 8.1 : (Continue)

Band no.a	Identified Species	Number of clones	% of Total	Similarity(%)b	(NCBI accession no.)	Order	Family
O1	*Lactobacillus delbrueckii strain KLDS 1.9201*	2	2,1	99%	EU676001	Lactobacillaceae	Firmicutes
O2	*Lactobacillus parabuchneri strain: YIT 0272*	11	11,5	98%	AB429372	Lactobacillaceae	Firmicutes
O5	*Uncultured bacterium clone SJTU_G_02_82*	1	1,0	99%	EF405367	Veillonellaceae	Firmicutes
O6	*Lactobacillus delbrueckistrain KLDS 1.9201*	1	1,0	99%	EU676001	Lactobacillaceae	Firmicutes
O7	*Uncultured rumen bacterium clone: T20H60F21*	4	4,2	93%	AB269959	Lachnospiraceae	Firmicutes
O10	*Bacterium LB01*	2	2,1	99%	EF053126	Veillonellaceae	Firmicutes
O14	*Uncultured bacterium gene clone:A58*	1	1,0	89%	AB494782	Lachnospiraceae	Firmicutes
O15	*Uncultured Clostridia bacterium clone S44*	2	2,1	95%	EU887963	Ruminococcaceae	Firmicutes
P6	*Uncultured bacterium clone BUD12*	2	2,1	92%	DQ447172	Thermotogaceae	Firmicutes
P11	*Unidentified eubacterium clone vadinHA60*	2	2,1	90%	U81720	Desulfobacteraceae	Proteobacteria
P12	*Uncultured bacterium clone PS7*	1	1,0	99%	DQ984664	Desulfobacteraceae	Proteobacteria
P13	*Uncultured bacterium clone Er-LAYS-2*	7	7,3	97%	GU180160	Syntrophaceae	Proteobacteria
P14	*Uncultured bacterium clone ST7*	2	2,1	90%	DQ347893	Intrasporangiaceae	Actinobacteria
P15	*Uncultured bacterium clone C55_D6_H_B_F08*	1	1,0	99%	EF559014	Peptococcaceae	Firmicutes
P16	*Uncultured bacterium clone Eb48*	1	1,0	85%	EF063623	Micrococcineae	Actinobacteria
P18	*Uncultured bacterium clone S3-17*	3	3,1	90%	GQ898497	Actinobacteria	Firmicutes
P33	*Uncultured Unclassified clone QEDV2BD01*	1	1,0	93%	CU919999	Veillonellaceae	Firmicutes
P39	*Uncultured bacterium clone BUD12*	2	2,1	89%	DQ447172	Thermotogaceae	Firmicutes
R3	*Uncultured bacterium clone BUD12*	1	1,0	90%	DQ447172	Thermotogaceae	Firmicutes
R4	*Soehngenia saccharolytica strain DSM 12858*	4	4,2	99%	GQ461828	Incertae Sedis XI	Firmicutes
R7	*Soehngenia saccharolytica strain DSM 12858*	2	2,1	93%	GQ461828	Incertae Sedis XI	Firmicutes
R9	*Uncultured bacterium clone RK*	3	3,1	99%	FJ645714	Synergistaceae	Synergistetes
R12	*Uncultured bacterium clone Er-LAYS-2*	2	2,1	92%	GU180160	Syntrophaceae	Proteobacteria
R14	*Uncultured compost bacterium clone 1B10*	6	6,3	99%	DQ346489	Porphyromonadaceae	Bacteroidetes
R15	*Uncultured bacterium clone BS3*	1	1,0	97%	AF087055	Incertae Sedis XIII	Firmicutes
R21	*Uncultured bacterium clone:Hados.Sed.Eubac.6*	2	2,1	85%	AB355075	Incertae Sedis XI	Firmicutes
R30	*Uncultured bacterium clone SJTU_C_06_21*	1	1,0	92%	EF404201.	Incertae Sedis XIII	Firmicutes
R33	*Uncultured Clostridium sp. clone 16IIISN*	1	1,0	90%	EU887804	Lachnospiraceae	Firmicutes

[a] Bands are numbered according to Figure 8.1
[b] Identity represents the % identity shared with sequences in the Genbank database

All bacterial samples were amplified in same set and same master mix in PCR. Thus, band density of pulper samples were high that could be indicate high bacterial activation inside raw waste. Moreover, this amount of micro flora could significantly affect the microbial diversity in fermenter. Digester diversity increased with every month with no band and diversity loss in sludge. Dendrograms in Figure 8.8 and 8.9 showed the similarity of bacterial and archaeal ecology of each sample. Amplification of the V2-V3 region of the archaeal 16S rRNA genes, followed by DGGE analysis of the DNA isolated from each of the samples, were shown in Figure 8.2. Population amount that can be estimated comparing the density of the bands and archaeal diversity were also remarkable in pulper samples. 2 archaeal sequences were identified as a bacterial sequence in homology searches in pulper sample like fermenter samples in previous study. Table 8.2 showed identifications of marked bands for archaeal isolations. Digester archaeal diversity was increased with each month without band losses.

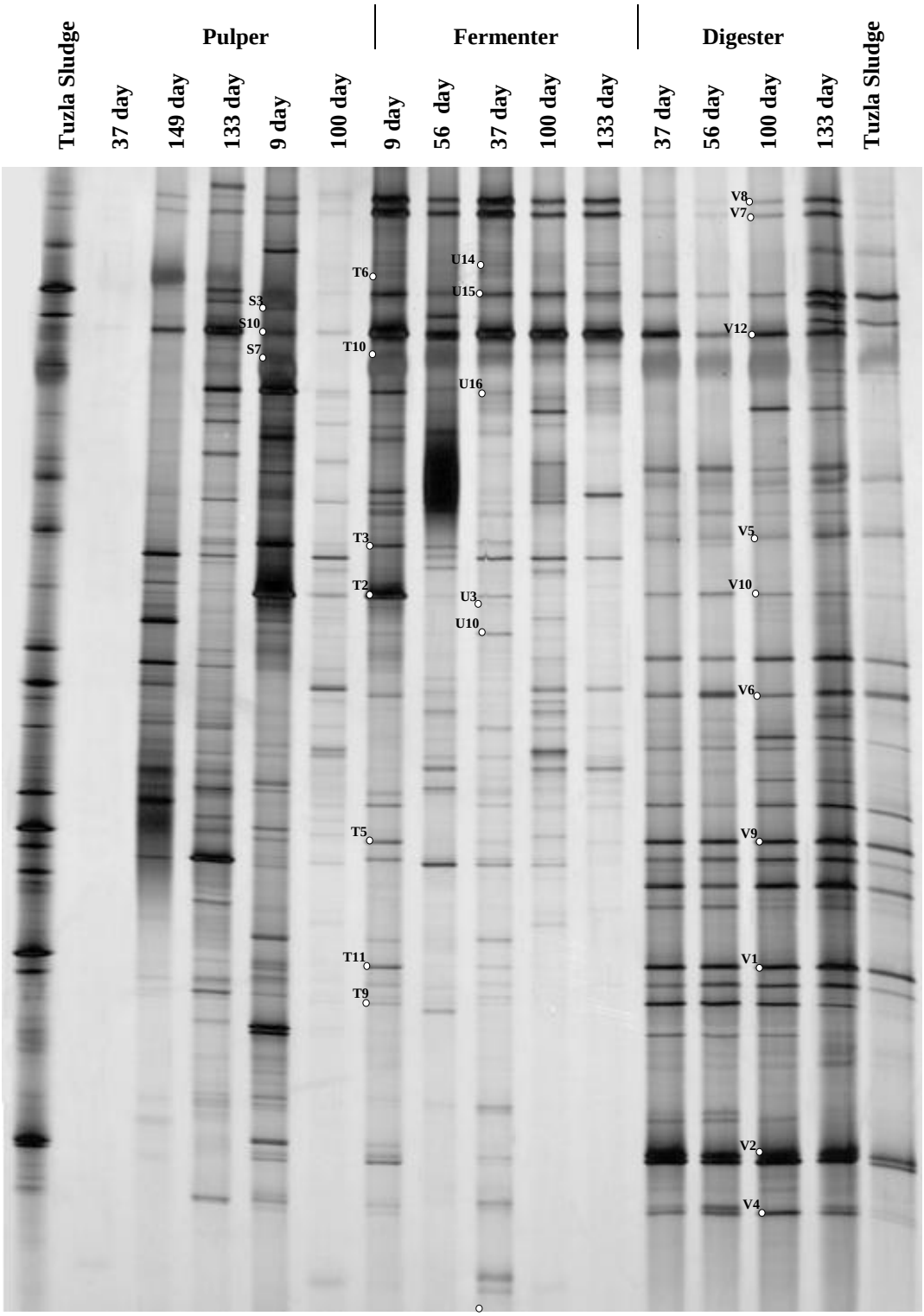


Figure 8.2 : DGGE analysis of the archaeal community during vegetable hall waste fermentation and digestion. 16S rRNA gene amplified with primers 109(t)F and 515R-GC from DNA samples extracted at different times, as indicated. The formamide-urea denaturing gradient ranged from 30% to 55%. Marked patterns were determined by cloning technique and successfully identified by sequencing (Table 7.2) were reported as band numbers and sequentially numbered from top to bottom

Table 8.2 : :Sequence similarities to closest relatives and phylogenetic affiliations of DNA, matched with archaeal DGGE bands of VHW wastes

Band no.[a]	*Identified Species*	Number of clones	% of Total	Similarity(%)[b]	(NCBI accession no.)	Order	Family
S3	*Uncultured Methanobacteriales archaeon clone A82*	22	22,9	98%	DQ464414	Methanobacteriaceae	Methanobacteria
S7	*Uncultured Methanobacteriales archaeon clone A82*	32	33,3	96%	DQ464414	Methanobacteriaceae	Methanobacteria
S10	*Uncultured archaeon clone HNA-02*	1	1,0	99%	AB509221	Methanosarcinaceae	Methanomicrobia
T2	*Uncultured Methanobacteriales archaeon clone A82*	2	2,1	99%	DQ464414	Methanobacteriaceae	Methanobacteria
T3	*Uncultured Methanobacteriales archaeon clone A82*	28	29,2	98%	DQ464414	Methanobacteriaceae	Methanobacteria
T5	*Uncultured archaeon gene clone:YC-E2*	2	2,1	90%	AB288256	Methanosarcinaceae	Methanomicrobia
T6	*Uncultured Methanobacteriaceae archaeon clone KR-L01-B01*	1	1,0	93%	FJ579568	Methanobacteriaceae	Methanobacteria
T9	*Uncultured archaeon ACE2_A*	1	1,0	97%	AF142977	Methanosarcinaceae	Methanomicrobia
T10	*Uncultured archaeon clone ATB-KS-0162*	1	1,0	97%	EF686890	Methanospirillum	Methanomicrobia
T11	*Uncultured Methanosaeta sp. clone KA1*	1	1,0	99%	DQ085318	Methanosaetaceae	Methanomicrobia
U15	*Uncultured Methanobacteriaceae archaeon clone KR-L01-B01*	14	14,6	95%	FJ579568	Methanobacteriaceae	Methanobacteria
U3	*Methanobrevibacter sp. ATM*	9	9,4	99%	AF242652	Methanobacteriaceae	Methanobacteria
U10	*Methanobrevibacter acididurans strain ATM*	1	1,0	99%	NR_028779	Methanobacteriaceae	Methanobacteria
U14	*Methanobacteriaceae archaeon clone KR-L01-B01*	1	1,0	96%	FJ579568	Methanosphaera	Methanobacteria
U16	*Uncultured archaeon gene clone:K08_2_5*	1	1,0	99%	AB541578	Methanocorpusculum	Methanomicrobia
V1	*Uncultured archaeon clone QEED1BH111*	17	17,7	98%	EF512457	Methanosaetaceae	Methanomicrobia
V2	*Uncultured Methanosaeta sp. clone KA1*	42	43,8	99%	DQ085318	Methanosaetaceae	Methanomicrobia
V4	*Uncultured archaeon ZAR109*	7	7,3	99%	FJ982799	Methanocaldococcaceae	Methanococci
V5	*Uncultured archaeon clone A_LC_1*	1	1,0	99%	GQ453608	Methanomicrobiaceae	Methanomicrobia
V6	*Uncultured methanogenic archaeon clone peat2_A6*	1	1,0	98%	FJ586827	Methanosarcinaceae	Methanomicrobia
V7	*Uncultured methanogenic archaeon clone decelerated_CH4_C7*	1	1,0	88%	DQ447172	Methanocellaceae	Methanomicrobia
V8	*Uncultured archaeon clone KAB187-14*	1	1,0	89%	AB541623	Methanomicrobiaceae	Methanomicrobia
V9	*Uncultured rumen archaeon clone T2PL03_D12*	15	15,6	96%	AB366595	Methanobacteriaceae	Methanobacteria
V10	*Uncultured Methanosarcinales archaeon clone QEEF1DA071*	1	1,0	94%	FJ586827	Methanomicrobiales_incertae_sedis	Methanomicrobia
V12	*Uncultured archaeon clone K08_21_4*	2	2,1	89%	CU916832	Methanosaetaceae	Methanomicrobia

[a] Bands are numbered according to Figure 8.1

[b] Identity represents the % identity shared with sequences in the Genbank database

8.1.2.1 Fermenter Analysis

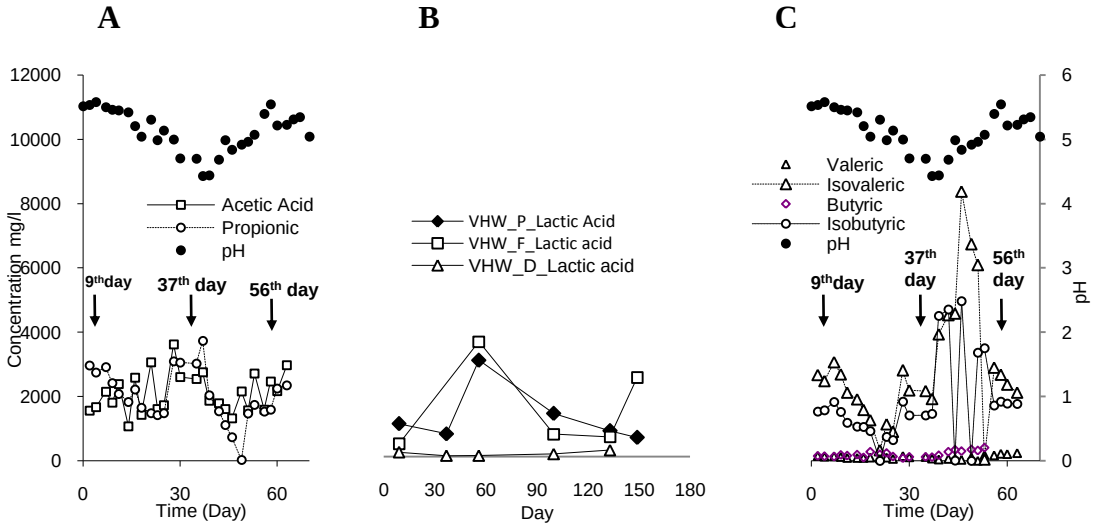


Figure 8.3 : VFA composition of fermenter feeding with vegetable hall wastes. (A:acetic, propionic acid, B:lactic acid, C:valeric, butric, isovaleric, isobutric acid)

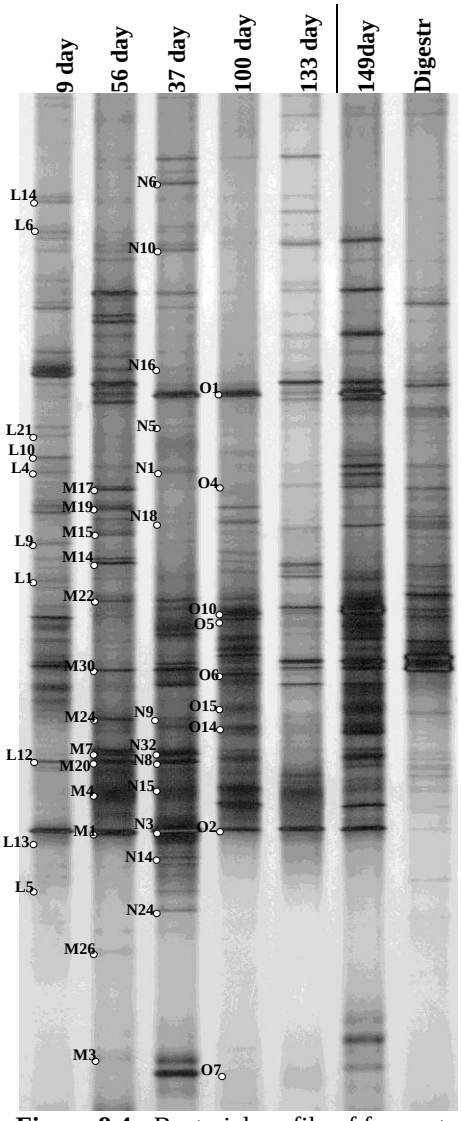


Figure 8.4 : Bacterial profile of fermenter

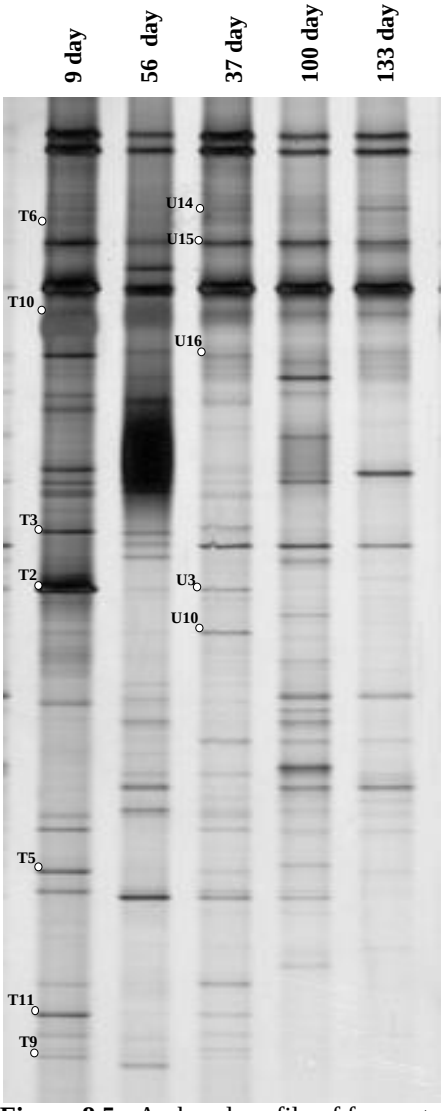


Figure 8.5 : Archaeal profile of fermenter

As shown on Figure 7.2, organic loading rates of the fermenter were not changable. Tempreture and pH values and mixing conditions were stable. Due to substrate composition and biodiversity, fermenter's microflora was changed significantly. Some species preserve their existince though changable composition such as M1, M30, O1, O2. Solubilization rates were stated in Figure 7.6 amd 7.8. Vegetabe hall wastes were made up of mostly cellulosic compods and organic components that need to be hydrolyzed.. The nature of the substrate (particulate organic materials of VHW like cellulose, hemicellulose, pectin, and lignin) contains active sites for extracellular enzymes and permits liquefaction. Thus, hydrolytic organisms belonged *Bacteriodales* were became dominant species (%36) inside fermenter sludge. O1, O10, N3 dominant bands were identified as *Lactobacillaceae, Veillonellaceae, Prevotellaceae* respectively. In early stages of the fermentation (1 month) process, *Synergistaceae* order was dominant inside reactor. This was the reason of equal values of VFA's inside the reactor with production and consumption.In 40th day of operation, although system was operated with pH adjustment, the pH of the fermenter was dropped to 4,5 from 6.This made a significant diversity change in microbial populations. Acetate and propionic acid concentrations rapidly dropped, but lactic acid and isobutric acid concentrations were increased inside reactor. In 37th day sample *Actinomycetales* phlum was dominant with %49 cover and *Clostridiales* was second dominant with *%19.* After pH dropped, at 56th day of operation, diversity changed to *Clostridiales* dominance with %54 cover, and *Actinomycetales* percentage dropped to %9. Fermenter archaeal diversity had a decreasing trend after 133 day opeartion. Dissapering of the bands were stable and no bands occured with significant density. The archaeal diversity showed fermenter was operated with intended conditions. Moreover, *Methanobacteriaceae* with %57 percentage was dominant order inside the fermenter at day 37. It was found that raw vegetable hall waste contains archaeal communities such as *Methanobacteriaceae* with %98 percentage.and *Methanosarcinaceae* with %2 percentage. Existence of archaeal diversity could be linked to micro-anoxic zones inside raw waste.

8.1.2.2 Digester Analysis

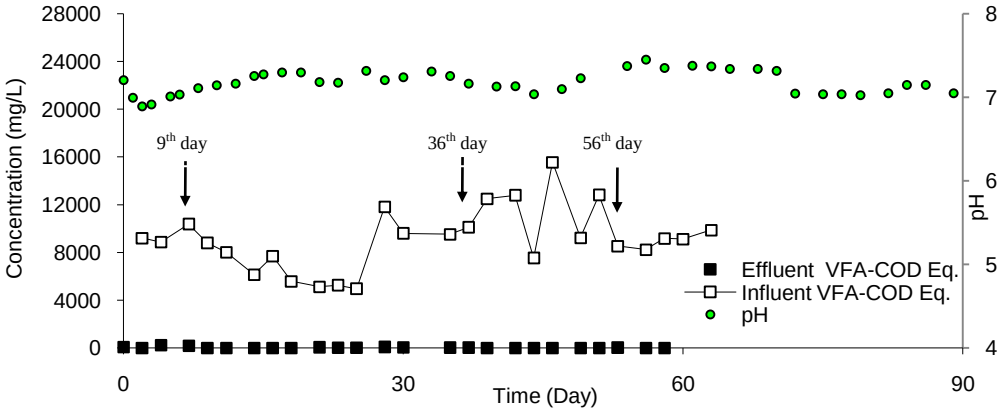


Figure 8.6 : Influent and effluent total VFA and pH values in digester.

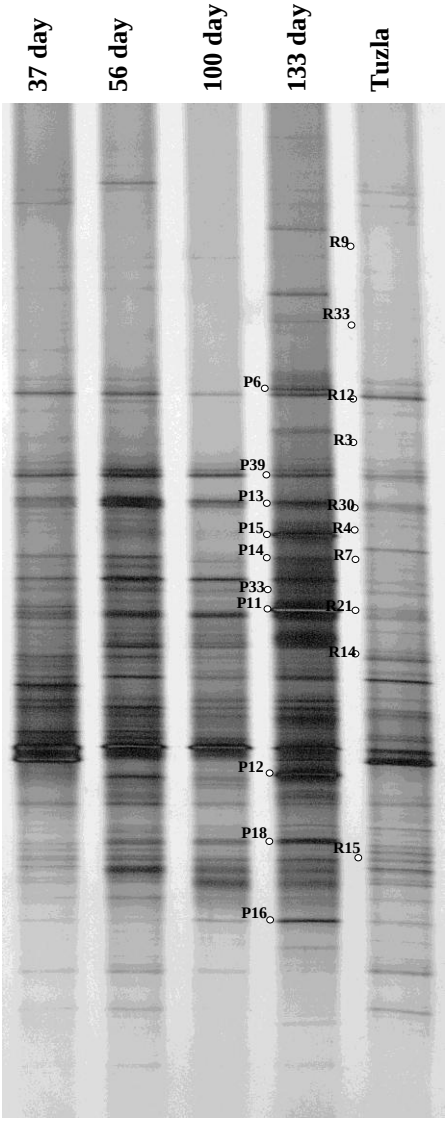


Figure 8.7 : DGGE profile archaeal population in digester

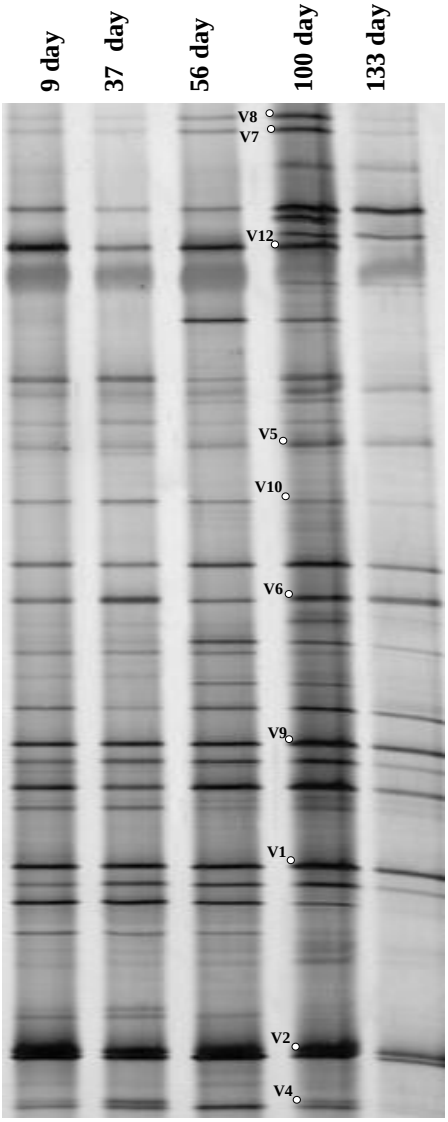


Figure 8.8 : DGGE profile archaeal population in digester

Long HRT values inside the reactor made the digester to worked with high performance.As stated before, all VFA's and soluble COD's were degraded into last step of methanization process. Ecology of the digester was very similar to Tuzla. Dominant bands such as P11 and P12 were belonged to *Desulfobacteraceae* order, R12 was belonged to *Syntrophaceae*. DNA based microbioogical studies give information about existing and dissappering species inside samples. Activation of the species was not able to determine.

8.1.2.3 Dendrogram of Bacterial Profiles

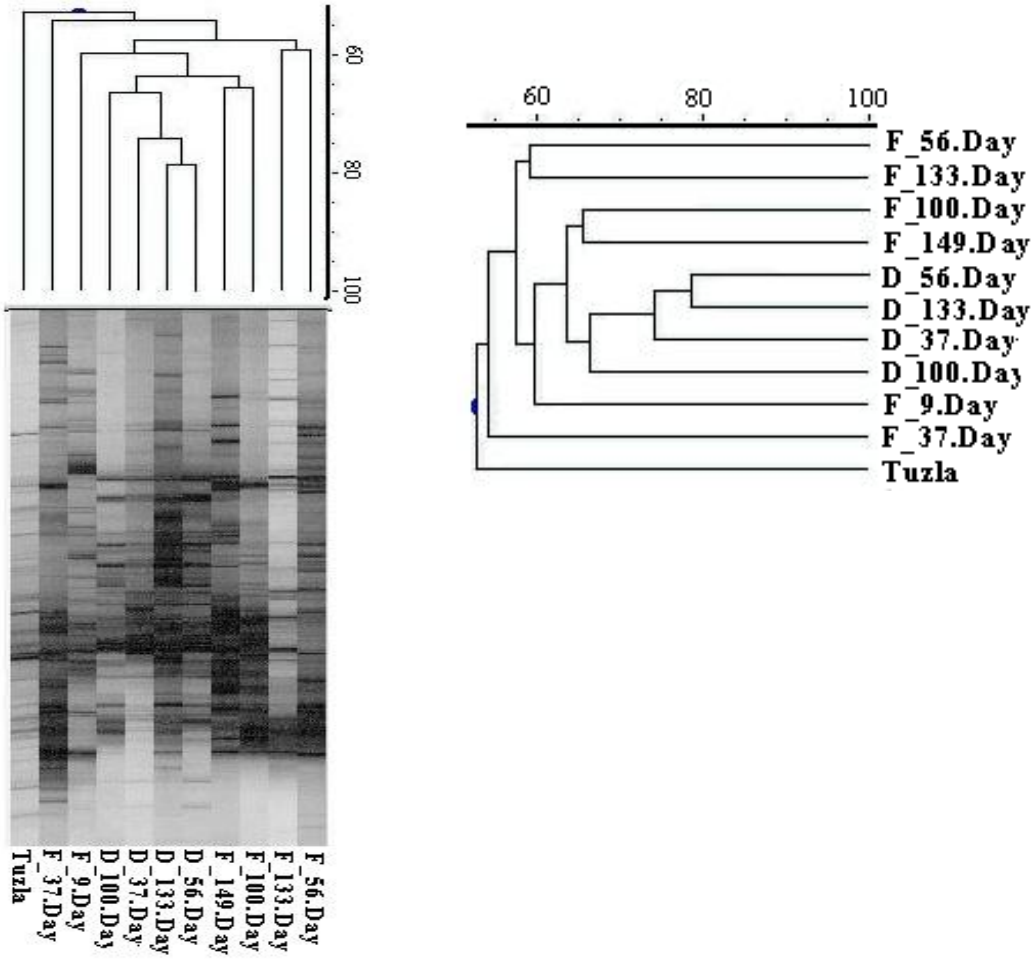


Figure 8.9: Illustrations of the cluster analysis of the bacterial PCR-DGGE profiles of vegetable hall waste fermentation and digestion. Dendrograms were based on the Dice coefficient of similarity (weighted) and obtained with the UPGMA clustering algorithm. Samples are indicated by reactor name and operation day. (D_37.day means; 37th day in digester)

Cluster analysis of the PCR-DGGE bacterial profiles of fermentation and digester was given in Figure 8.9. Cluster analyzes showed that 9th day and 37th day of fermenter sludge's microflora were still not acclimated and similar to inoculum (Tuzla) diversity more than fermenter acclimated sludge. *Synergistaceae* like order

that could found in mostly digester sludge took 2 months to extinct from fermenter sludge. After pH drop in 35th day of fermenter, microflora took a new shape. 100th and 149th day of fermenter were %65 similar to digester ecology. The first month of the operation of fermenter was %55-60 similar to digester microflora. Digester diversity was very stable but, %55 similar to inoculums diversity. It was not a longstanding acclimation to have the 133th day microflora in digester.

8.1.2.4 Dendrogram of Archaeal Profiles

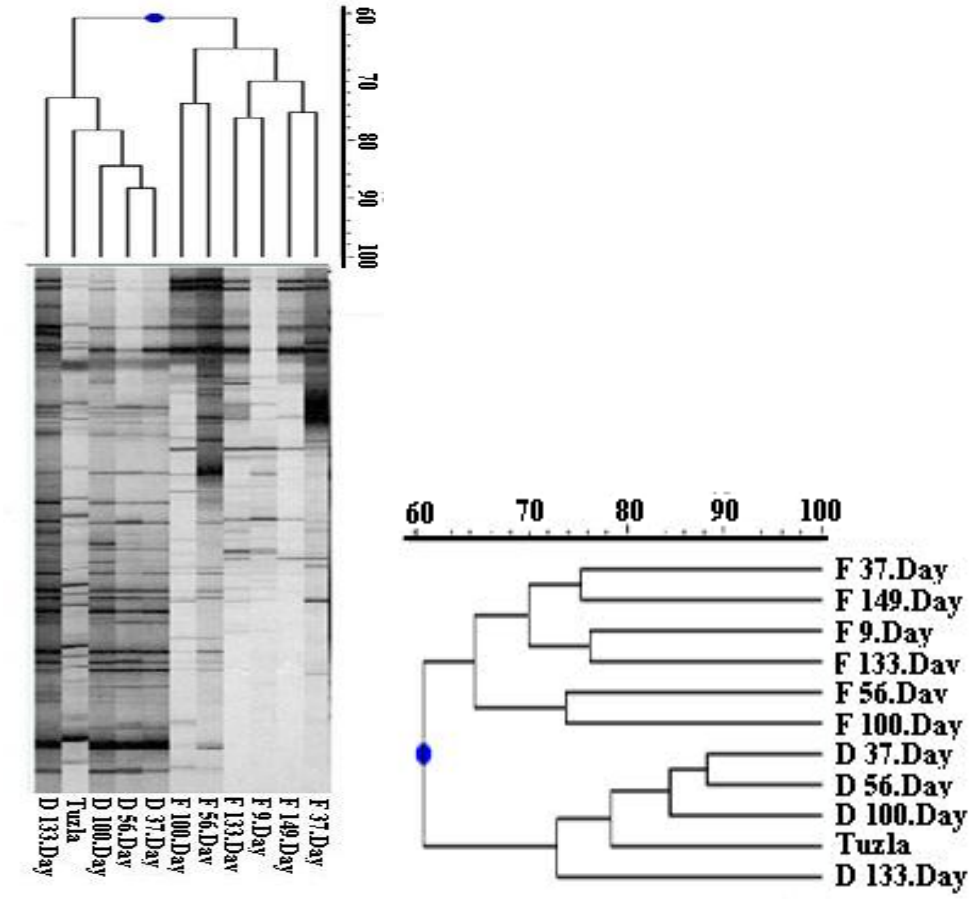


Figure 8.10 : Illustrations of the cluster analysis of the archaeal PCR-DGGE profiles of vegetable hall waste fermentation and digestion. Dendrograms were based on the Dice coefficient of similarity (weighted) and obtained with the UPGMA clustering algorithm. Samples are indicated by reactor name and operation day. (D 37.day means; 37th day in digester)

Fermenter and digester archaeal profiles were significantly different from each other (%60 similarity) since the first month of the operation. Although, it could be said that fermenter archaeal diversity had a decreasing trend, dendogram showed that 9th and 133th day of the fermenter had % 76 similarities. 37th and 149th day of fermenter had %73 similarity. Digester archeal diversity had a stable trend with high similarity. Tuzla inoculum diversity was not changed and continues with %77 simlarity in digester. Although fermenter ecology was changeable, digester was strongly stable.

8.1.2.5 Canonocial Correspondence Analysis(CCA) of Bacterial Profiles

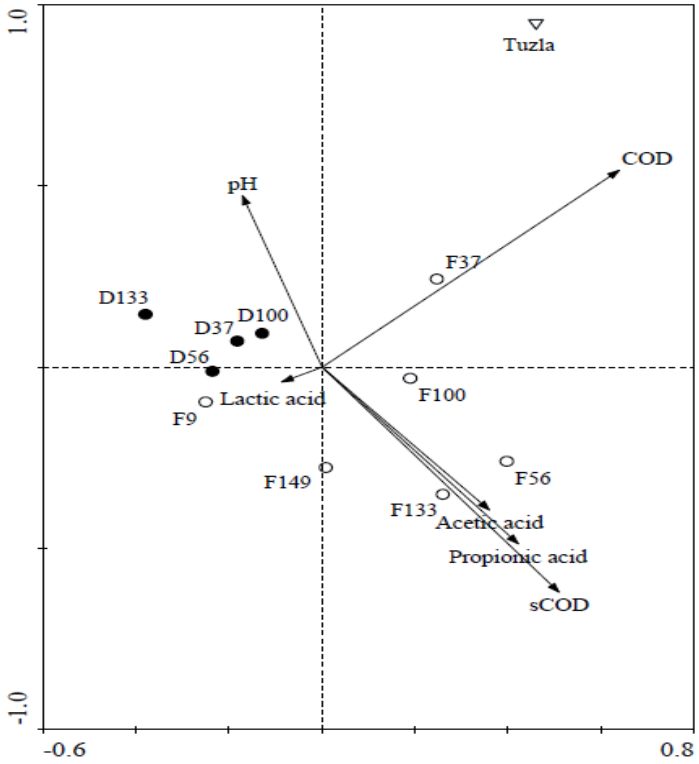


Figure 8.11: CCA diagrams for ordination of environmental variables such as volatile fatty acids (acetic acid, propionic acid, lactic acid), COD, sCOD, pH and of the 11 fermenter and digester samples from bacterial DGGE fingerprints. D37 indicates digester sample on day 37, F9 indicates fermenter sample on day 9.

CCA of DGGE fingerprints of fermenter and digester bacterial communities showed that acetic acid, propionic acid, lactic acids and sCOD concentrations were strongly and positively correlated with changes in the structure of anaerobic bacterial communities especially F56, F100, F133 and F149. The pH of the fermenter reactor has lower and negatively correlated with community structure. Lactic acid concentrations showed a small correlation with bacterial communities. Lactobacillus species ratio that observed from cloning results also showed the same results. Fermenter ecology was rapidly changed after 30 days operation from Tuzla inoculum. After 60 days operation bacterial community structure in fermenter showed a stable property that all samples accumulated in a small area with positive gradient.

In first 37 operation days digester ecology was differentiate form inoculums sludge. After that all samples were accumulated in a area. These results showed that bacterial diversity of digester was not changed much when analyzed with the environmental variables. The pH of the reactor was positively correlated with digester community structures.

8.1.2.6 Canonocial Correspondence Analysis(CCA) of Archaeal Profiles

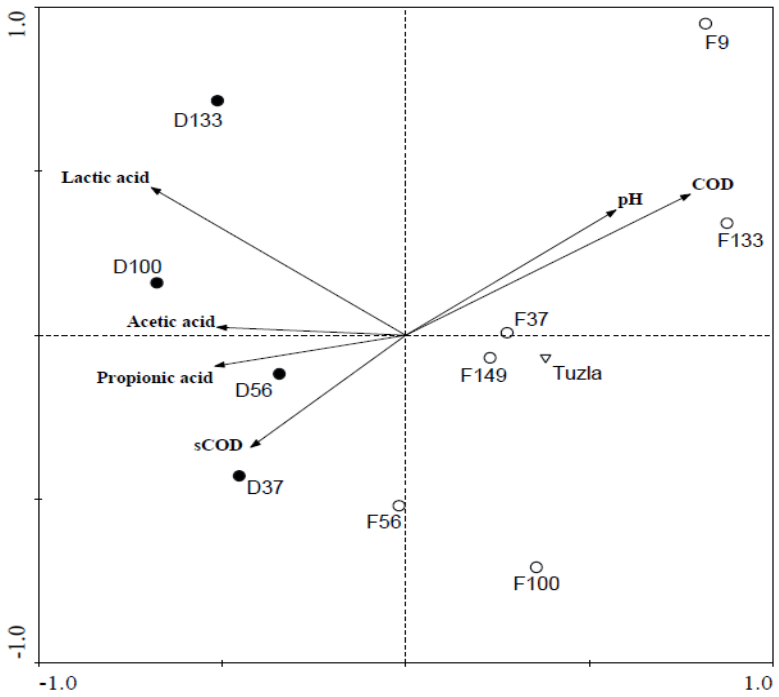


Figure 8.12: CCA diagrams for ordination of environmental variables such as volatile fatty acids (acetic acid, propionic acid, lactic acid), COD, sCOD, pH and of the 11 fermenter and digester samples from archaeal DGGE fingerprints. D37 indicates digester sample on day 37, F56 indicates fermenter sample on day 56.

CCA of DGGE fingerprints of fermenter and digester archaeal communities showed that lactic acid, pH, COD values were highly correlated acetic acid, propionic acid, and soluble COD values were lower correlation with fermenter and digester samples. Fermenter ecology was very differing from each other. It was interesting that inoculums (Tuzla) ecology was rapidly changed after 9 days operation, but after 30 day operation it had the close diversity with inocolum archaeal community. After that, fermenter archaeal community was not stable, all samples were in positive gradient, but community structures were very differentiated and separated. Fermenter archaeal samples were highly and positively correlated with pH and COD especially F37, F133, F149 and F9 samples. F56 ecology was more close the digester (D37) ecology.

Digester archael ecology was changed with the same ratio and clearly different from fermenter's ecology. Community structures were highly correlated with pH, COD, sCOD, lactic acid values, and lower correlation with acetic acid and propionic acid concentrations. All samples were clearly different from inoculums diversity.

8.2 The clone library of the fermenter and digester sludge:

There are potential experimental errors starting from extraction of the nucleic acids through the sequencing of the clones. To prevent those potential errors, many precautions were taken in the process of experimental works. For the clone libraries, PCR is the crucial step due to primer selectivity in the amplification and have the variable rRNA gene copy numbers.

Vegetable hall waste study's biomass samples collected at the days of 9, 37, 56 and 149 from fermenter, 94 and 117 from digester for bacterial analysis, were subjected to cloning and sequencing analysis. Thus, for archaeal analysis, biomass samples collected at the days of 9 for pulper, 9, 37 for fermenter and 100 for digester, were subjected to cloning and sequencing analysis. In this respect, 96 bacterial and clones were randomly picked for each sample and these clones were screened by RFLP. 21 out of 1056 clones were found as a shorter fragment compared to the 16S rRNA length and were eliminated for further analysis.

For bacterial cloning, 120 transformants from fermenter, 84 transformants from digester were selected for approximately 1500 bp sequencing using RFLP screening. For archaeal cloning 15 transformants from pulper, 33 transformants from fermenter, 15 transformants from digester were selected for approximately 500 bp sequencing using RFLP screening Based on these partial 16S rRNA sequences with DGGE technique, 264 clones were compared with each other, and a total of 95 isolate were attained for bacteria, 51 isolates were attained for archaeal. Representative clones for each isolate was sequenced completely. The total distributions for the fermenter and digester reactors were 96 and 51 respectively. Six of these sequences were detected as chimera and were taken aside for further analysis. .96 16S rRNA archaeal gene clones selected from each reactor were subjected to screening and subsequenty to cloning and sequencing. It was found that all clones were closely related to the lineages of *Euryarchaeota*; including *Methanobacteria* and *Methanomicrobia* phylum and *Methanococci* in minor amounts. Table 8.1-8.2 lists the percentage of similarities between transformants sequences of all three reactors and BLAST database after analysis using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi). A homology search showed that the main groups could be classified into 3 major phylotypes in fermenter that belong to *Firmucutes*, *Bacteroidetes* and *Actinobacteria* phylums. In addition, some minor clones were in *Synergistetes amd protobacteria*

phylums (Tables 6.1-6.2). Thus, with the results of homology search, digester ecology could be consisted of 3 main groups such as *Firmicutes*, *Proteobacteria* and *Actinobacteria*. In addition, some minor clones were in *Synergistetes* phylum (Tables 8.1-8.2).

8.2.1 The clone library of the fermenter sludge

Bacterial Diversity

9th day

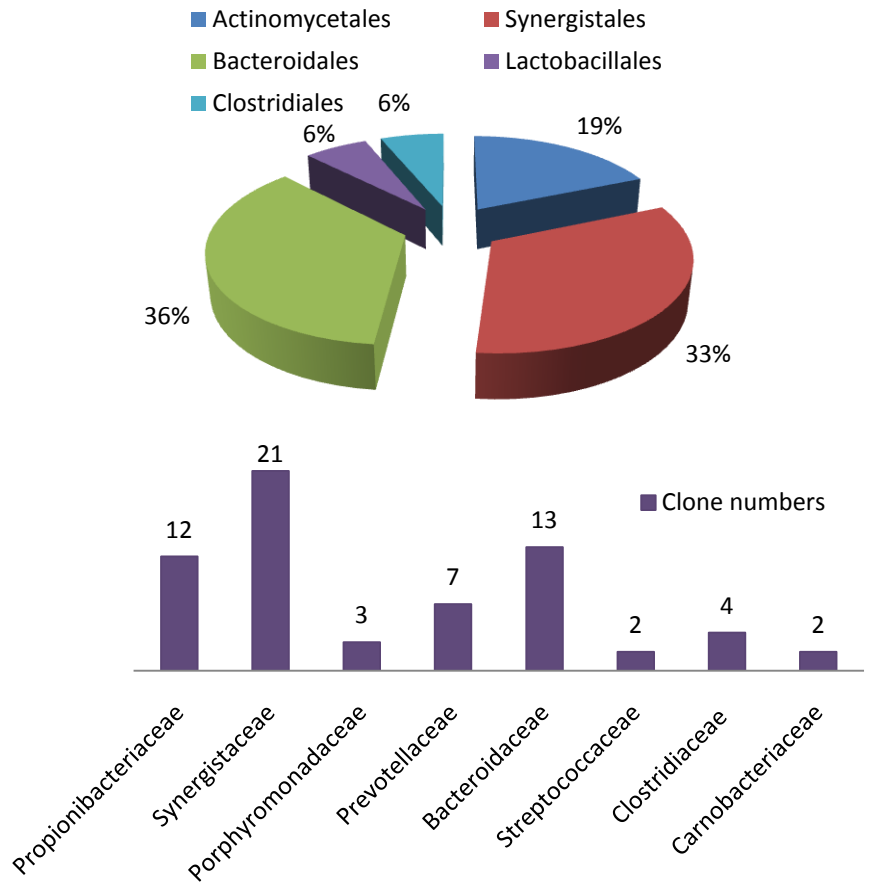


Figure 8.13: Phylogenetic distribution of bacterial 16S rRNA clones derived from 68th day of the fermenter reactor

Phylogenetic distribution of 16S rRNA clones derived from the 9th day of the fermenter reactor was given in Figure 8.11. Phylogenetic analysis of the 9th day clones revealed a community clearly dominated by diverse populations of *Bacteroidales* (36%) and *Synergistales* (33%) of all clones; Table 6.1; but *Actinomycetales* (19%), *Lactobacillales* (6%), and Clostridiales (6%) were detected as well (Figure 8.11). The most abundant isolate 97% identical to *Uncultured bacterium clone RK*. It was belonged to *Synergistaceae* order and isolated from anaerobic sludge granules.The second most abundant isolate 99% identical to

Propionibacterium freudenreichii. The other abundant clone was the *Uncultured bacterium clone SJTU_F_15_80* with %100 identity and was closely was in *Bacteroidaceae* order.

37th day

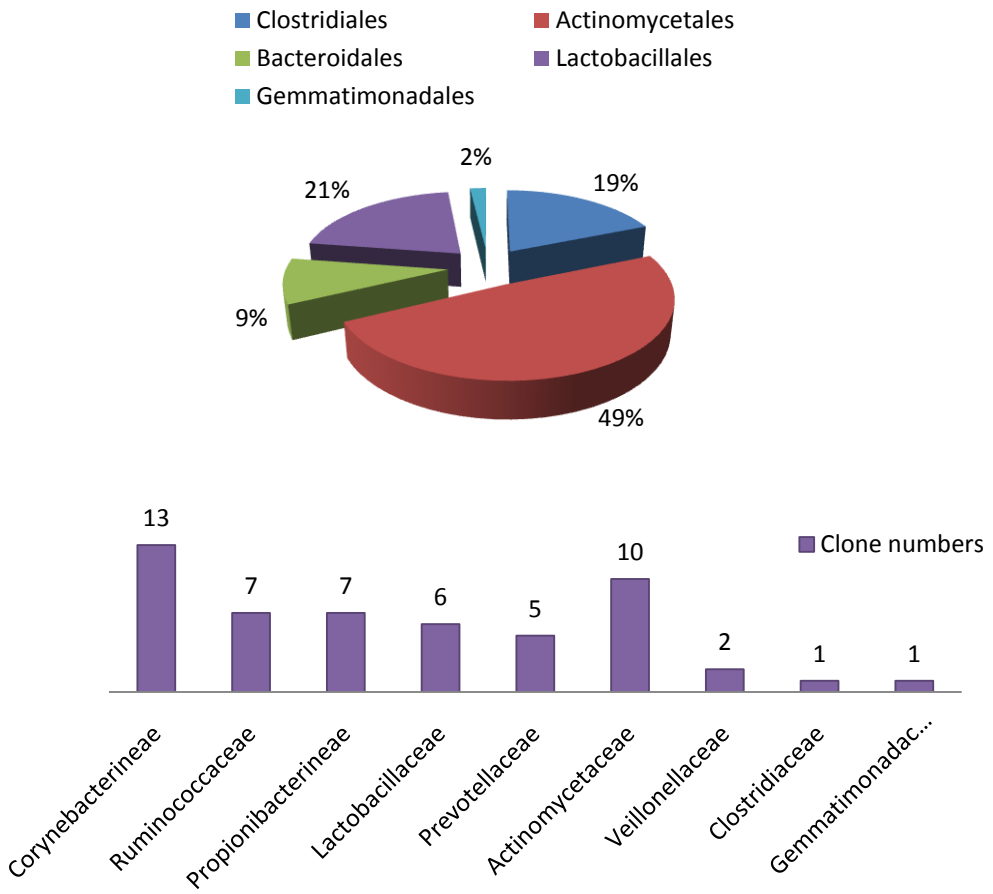


Figure 8.14: Phylogenetic distribution of bacterial 16S rRNA clones derived from 37th day of the fermenter reactor

Cloning of the 37th day of fermenter sludge clearly showed that *Actinomycetales* was the main dominant phylum in this clone library. The clones belonging to this phylum mainly *Corynebacterium vitarumen,* phylogenetically were scattered over several genus. This species DNA exhibits 51% homology with the DNA of *Corynebacterium diphtheriae* and it shares with certain other *corynebacteria* the capacity to hydrolyze sucrose and trehalose, as well as glucose, galactose, fructose, maltose, and mannose [196]. The second most abundant isolate 99% identical to *Lactobacillus parabuchneri strain: YIT 0272,* they were known as lactic acid producers.Beside this, clones belonging to *Bacteroidales, Clostridiales, Gemmatimonadales* detected a minor amount which accounts as %9, %2, %19 of the total library (Figure 8.12) respectively.

56th day

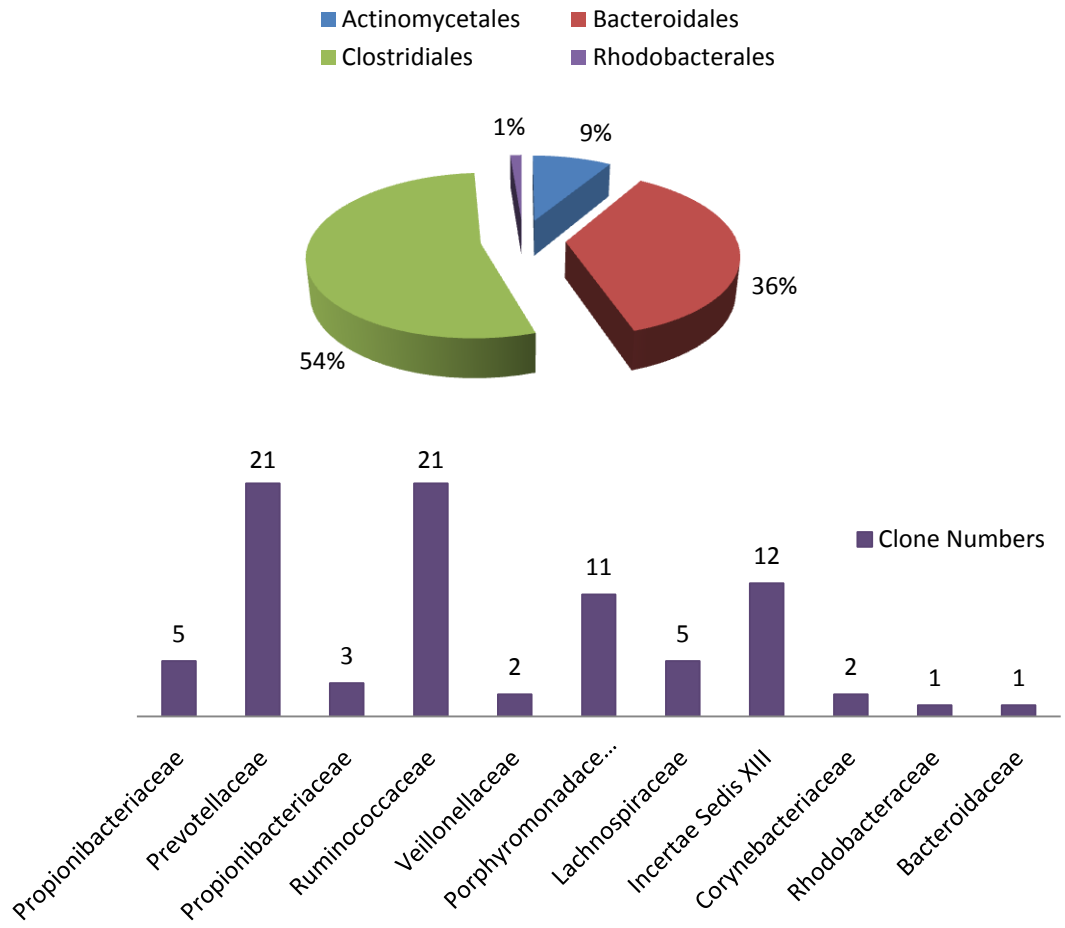


Figure 8.15: Phylogenetic distribution of bacterial 16S rRNA clones derived from 56th day of the fermenter reactor

Phylogenetic distribution of 16S rRNA clones derived from the 56th day of the fermenter reactor was given in Figure 8.13. Phylogenetic analysis of the 56th day clones revealed a community clearly dominated by diverse populations of *Clostridiales* (54% of all clones; Table 8.13);but *Bacteroidales* (35%), *was* detected as well. The most abundant isolate 99% identical to *Uncultured bacterium clone SJTU_G_02_82*, from *Veillonellaceae* order. The second most abundant isolate 99% identical to *Uncultured bacterium clone:1406* from *Prevotellaceae* order.

149th day

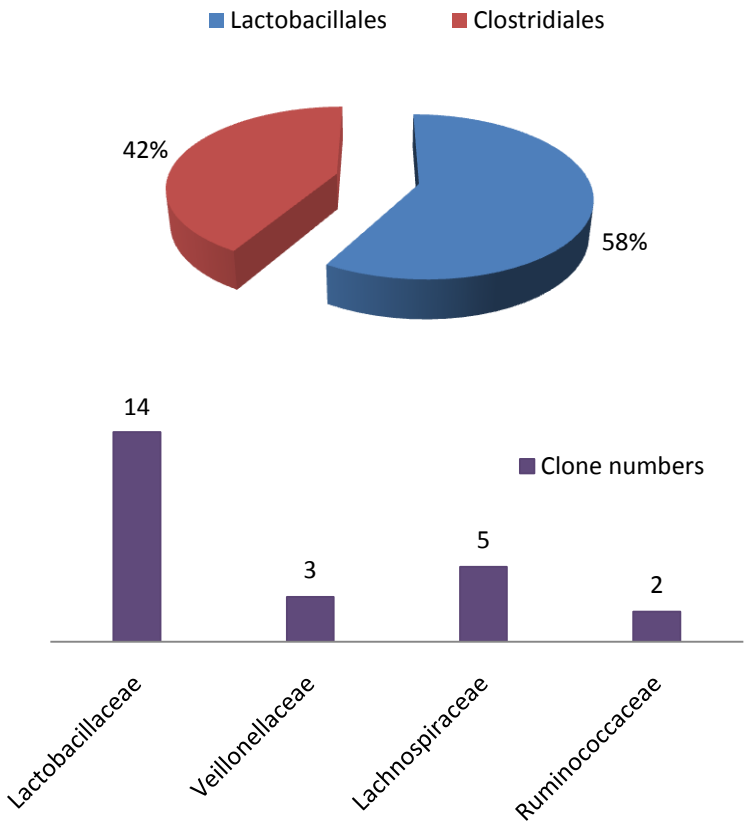


Figure 8.16: Phylogenetic distribution of bacterial 16S rRNA clones derived from 149th day of the fermenter reactor

Phylogenetic distribution of 16S rRNA clones derived from the 149th day of the fermenter reactor was given in Figure 8.14. Phylogenetic analysis of the 149th day clones revealed a community clearly dominated by diverse populations of *Lactobacillaceae* (58% of all clones; Table 6.13);but *Clostridiales* (42%), was detected as well (Figure 6.13).The most abundant isolate 98% identical to *Lactobacillus parabuchneri strain: YIT 0272, a* lactate-producing bacterium. The second most abundant isolate 93% identical to *Uncultured rumen bacterium clone: T20H60F21* from *Lachnospiraceae* order.

Archeal Diversity

9th Day

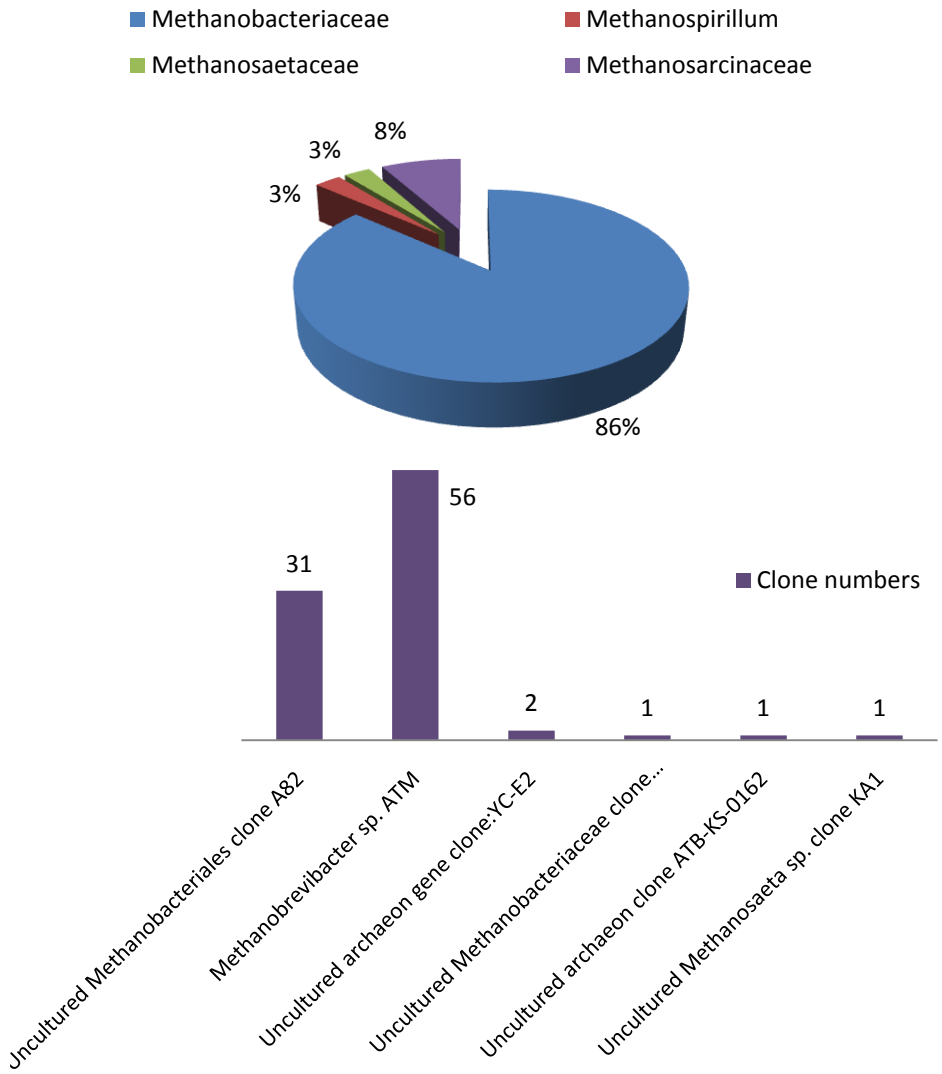


Figure 8.17: Phylogenetic distribution of archaeal 16S rRNA clones derived from 9th day of the fermenter reactor

The archaeal composition of the 37th day of digester sludge was given in Table 8.2. Archaeal methanogenic community showed high diversity in first month of the reactor. Archaeal diversity was not stable and had a decreasing trend in fermenter .The major member of the Archaea group detected in the digester sludge was *Methanobacteriaceae* (86%). and mainly *Methanobrevibacter sp. ATM*. The second dominant archaeal species in the 9th day of fermenter sludge were *Methanosarcinaceae* (8%).The other members of the archaeal community belongs to *Methanosaetaceae* and *Methanospirillum* with an abundance of 3% and %3 respectively.

37th Day

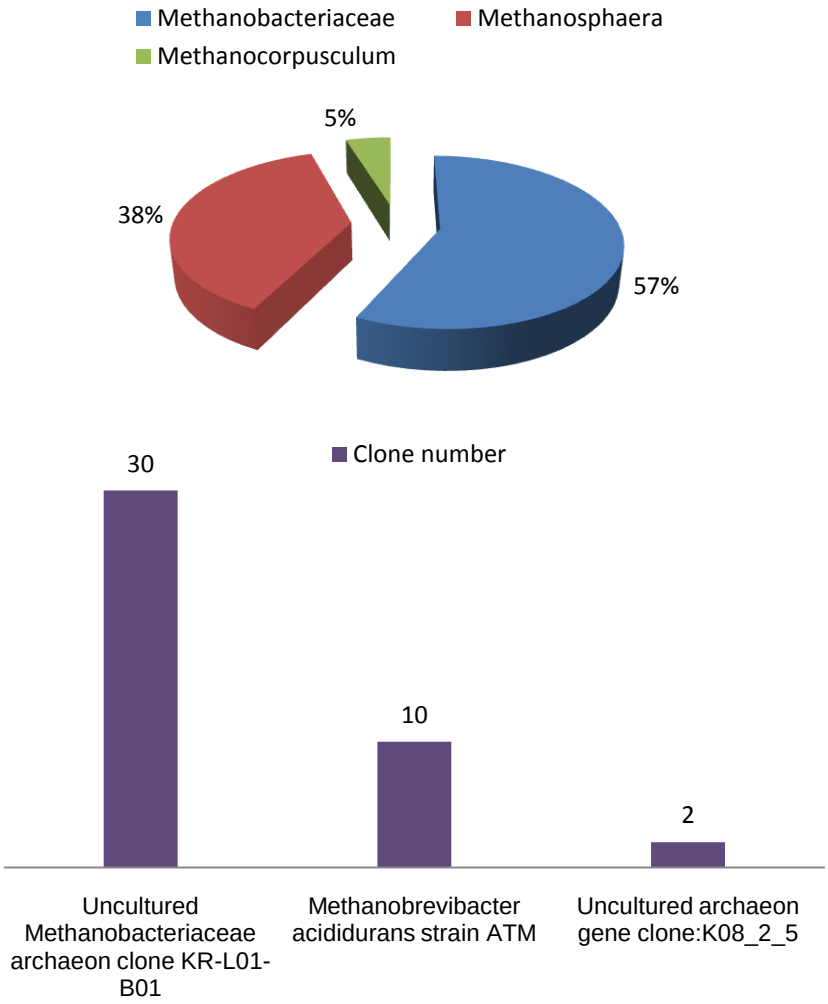


Figure 8.18: Phylogenetic distribution of archaeal 16S rRNA clones derived from 37th day of the fermenter reactor

The archaeal composition of the 37th day of digester sludge was given in Table 8.2..The major member of the Archaea group detected in the digester sludge was *Methanobacteriaceae* (57%). and mainly *Uncultured Methanobacteriaceae archaeon clone KR-L01-B01*. The second dominant archaeal species in the 37th day of fermenter sludge were *Methanosphaera* (8%).The other members of the archaeal community belongs to *Methanocorpusculum* with an abundance of 5%.

8.2.2 The clone library of the digester sludge

Bacterial Diversity

9th Day

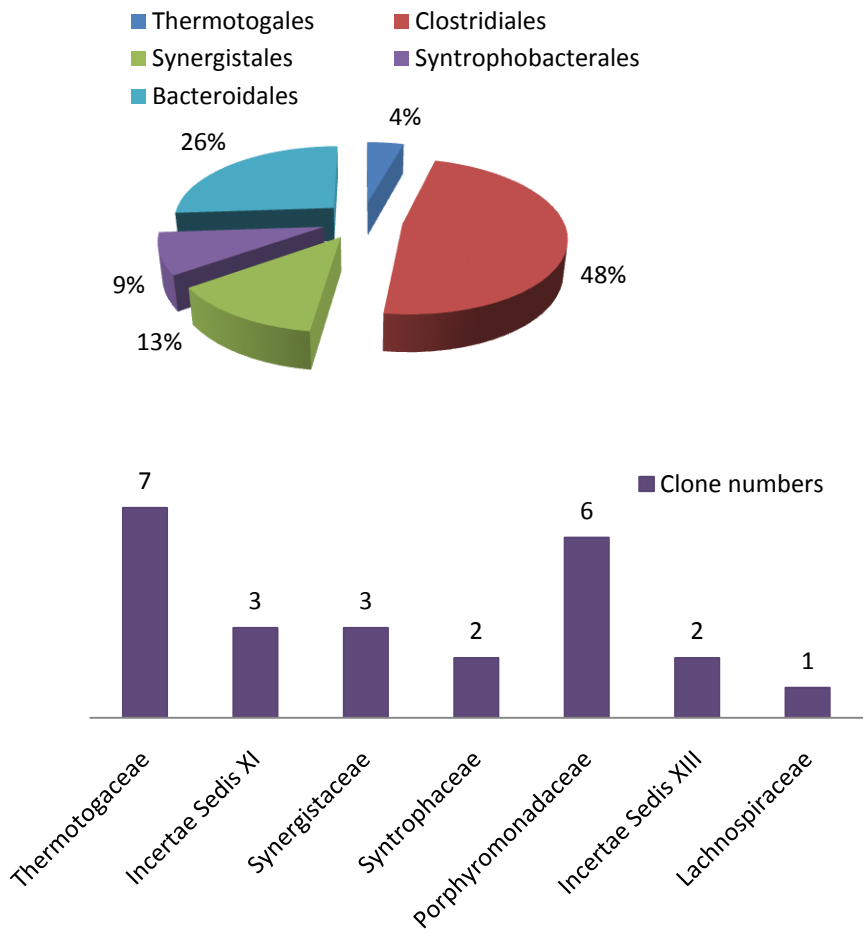


Figure 8.19: Phylogenetic distribution of bacterial 16S rRNA clones derived from 9th day of the fermenter reactor

Phylogenetic distribution of 16S rRNA clones derived from the 9th day of the digester reactor was given in Figure 8.16. Phylogenetic analysis of the 9th day clones revealed a community clearly dominated by diverse populations of *Clostridiales* (48%) and *Bacteroidales* (26%) of all clones; Table 6.1; but *Synergistales* (13%), *Syntrophobacterales* (9%), and Thermotogales (4%) were detected as well (Figure 8.16). The most abundant isolate 99% identical to *Soehngenia saccharolytica strain DSM 12858.* It was belonged to *Incertae Sedis XI* order and isolated from anaerobic sludge granules. The second most abundant isolate 99% identical to *Uncultured compost bacterium clone 1B10* and was in *Porphyromonadaceae* order. The other

abundant clone was the *Uncultured bacterium clone RK* with 99% identity and was in *Synergistaceae* order.

133th day

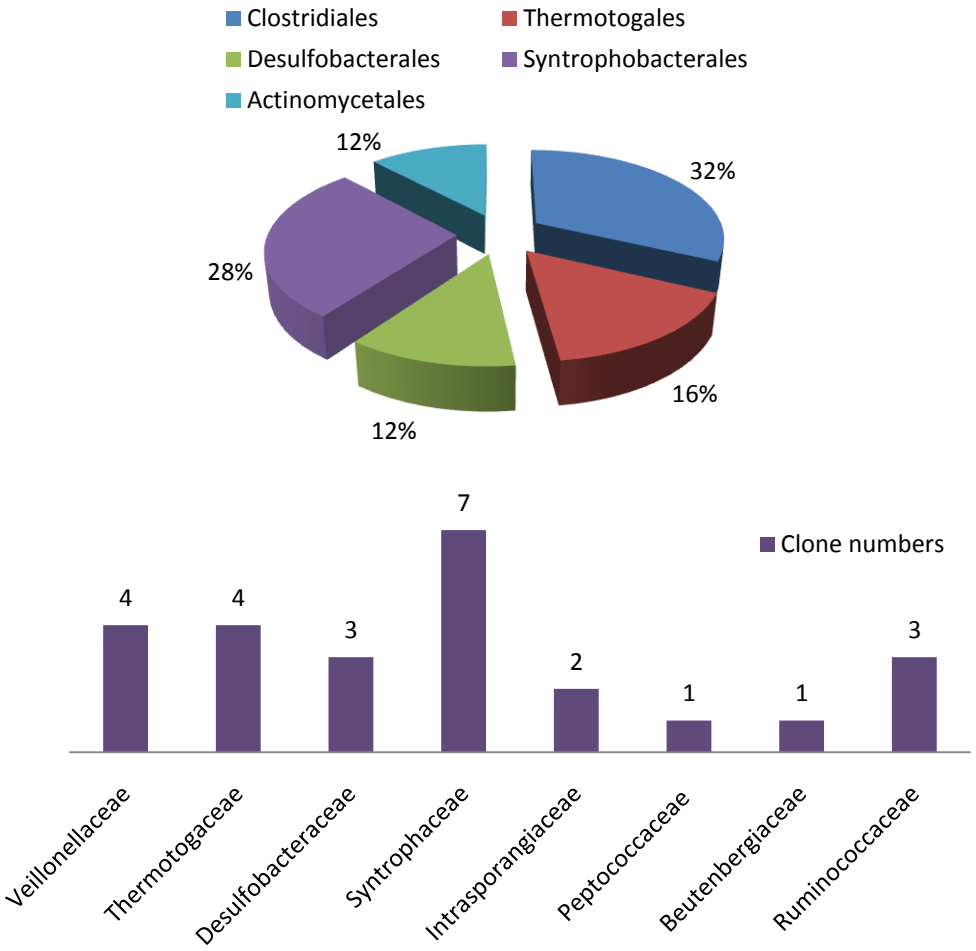


Figure 8.20: Phylogenetic distribution of bacterial 16S rRNA clones derived from 133th day of the digester reactor

Cloning of the 133th day of digester sludge clearly showed that *Syntrophobacterales and Clostridiales phylum* were the main dominant phylum in this clone librarywith %32 and %28 respectively. The clones belonging to this phylum mainly *Syntrophaceae,* phylogenetically were scattered over several genus. The second dominant bacterial species in the 133th day of digester sludge were *Thermotogales* (16%).The other members of the bacterial community belongs to *Desulfobacterales* and *Actinomycetales*with an abundance of %12 and %12 respectively.

Archaeal Diversity
100th day

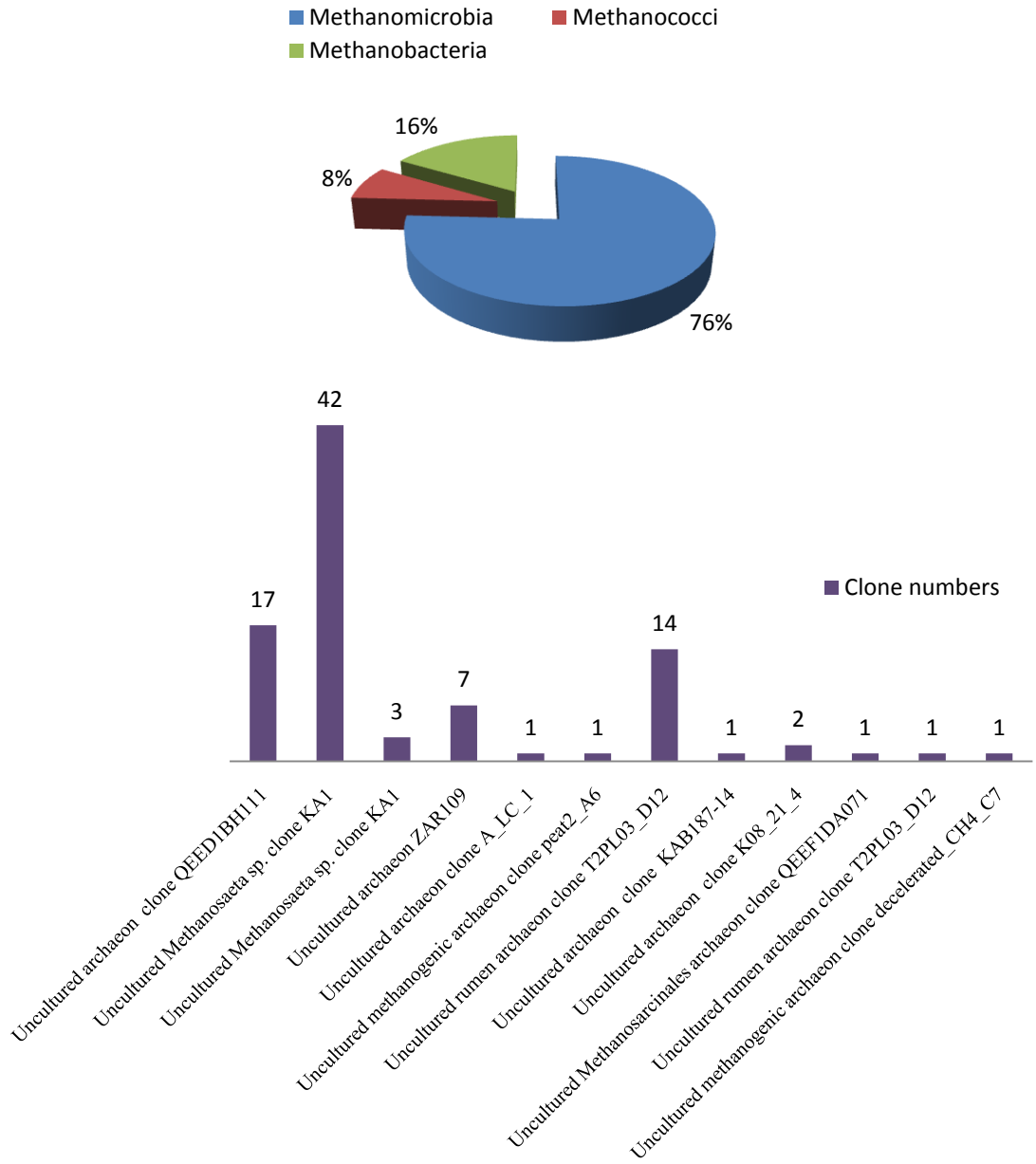


Figure 8.21: Phylogenetic distribution of archaeal 16S rRNA clones derived from 100th day of the digester reactor

The archaeal composition of the 100th day of digester sludge was given in Table 8.2..The major member of the Archaea group detected in the digester sludge were *Methanomicrobia* (76%). and mainly *Uncultured Methanosaeta sp. clone KA1*. The second dominant archaeal species in the 100th day of digester sludge were *Methanobacteria* (8%).The other members of the archaeal community belongs to *Methanococci*with an abundance of 8%.

8.2.3 Phylogenetic Tree of bacterial clones

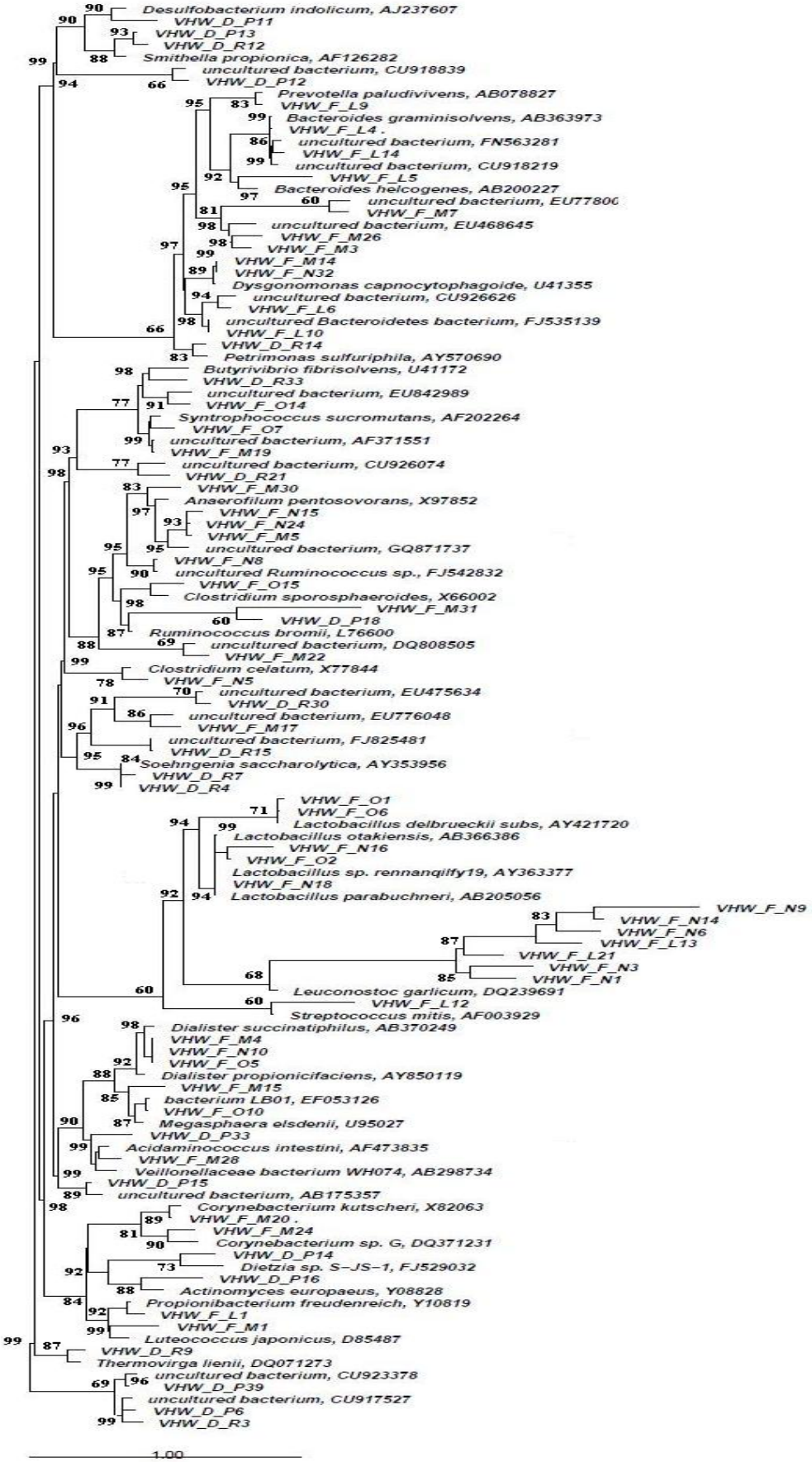


Figure 8.22 : Phylogenetic relationships inferred from the alignment of partial archaeal 16S rRNA gene sequences of 47 isolated from fermenter and 20 from digester. GenBank accession numbers of reference sequences are reported. The DHW stands for dinner hall wastes, F and D represents fermenter and digester reactors, letters with numbers represents band numbers in Figure 8.1. 16S rRNA gene sequences belong to each isolation were aligned using CLUSTALX (editor 4.1) in ARB. The tree was constructed using the neighbor-joining method. The bar indicates 100% sequence divergence. Bootstrap values (expressed as percentages of 1,000 replications) are reported at each node.

8.2.4 Phylogenetic Tree of archaeal clones

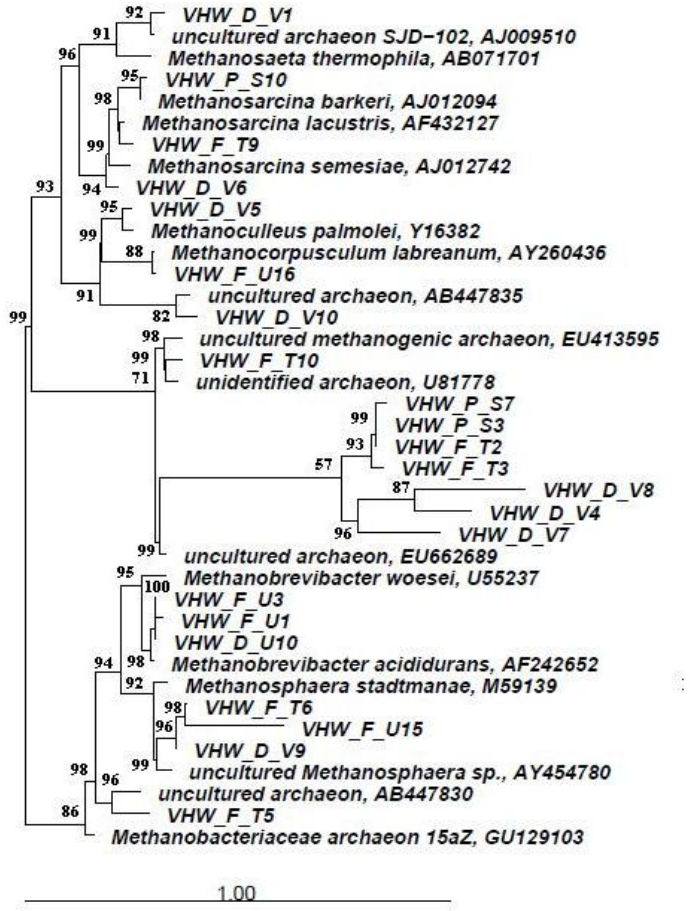


Figure 8.23: Phylogenetic relationships inferred from the alignment of partial archaeal 16S rRNA gene sequences of 15 isolated from fermenter and 10 from digester. GenBank accession numbers of reference sequences are reported. The DHW stands for dinner hall wastes, F and D represents fermenter and digester reactors, letters with numbers represents band numbers in Figure 8.1. 16S rRNA gene sequences belong to each isolation were aligned using CLUSTALX (editor 4.1) in ARB. The tree was constructed using the neighbor-joining method. The bar indicates 100% sequence divergence. Bootstrap values (expressed as percentages of 1,000 replications) are reported at each node.

9. COMPARISION OF DIGESTER POPULATIONS WITH DIFFERENT SUBSTRATES (DINNER HALL AND VEGETABLE HALL WASTES)

9.1 DGGE Results

The DGGE profiles derived from DNA amplicons of the digester's sludge were given in Figure 9.1 and Figure 9.2. Population shifts of bacterial communities were assessed by comparing the DGGE banding patterns of 12 samples taken during the operation of digesters (1-133 days). (Figure 9.1-9.2). In this Figures, numbers and Tuzla indicated the representative sampling days, and the marker for the DGGE analysis.DGGE profiles of the bacterial and archeal PCR amplification products obtained from the digesters sludge at different time intervals that were shown in Table 4.2 and 4.3. The prominent bands in the DGGE pattern should give the dominant members of the microbial community. So, the dominant isolations, such as F31, F20, F3 bands for dinner hall waste digester sludge, R21, R9, P33, P14 bands for vegetable hall digester profiles were represented by the dark bands in the corresponding DGGE patterns.

Digester of two different studies were fed with fermenters outflows at a rate of 200L each day. Substrate composition that feed digesters are different as shown on table 5.5, 5.8, 5.9, 7.8 and 7.10. Due to this data's and clone libraries, it was clear that pH and composition of inflow determined the digester's microflora. Dinnerhall waste consist significant amount of lipids and proteins. Proteins were degraded more slowly than carbohydrates [101]. Thus, high carbonhydrate concentration in reactors, supress the synthesis of exopeptidases, which was enables the protein hydrolysis in reactor [180].

These results showed, a very poor protein degradation inside fermenter and took all protein load into digester of dinner hall wastes. Moreover, research of dinnerhall wastes digester was done with pH adjustment in digester, but in vegetable hall wastes pH adjustment was performed for fermenter. These difference could made significant microflora difference that also observed in clone libraries and DGGE results. According to Figure 9.1, dinner hall waste digester had a similar ecology to Tuzla inoculum sample more than vegetable hall waste digester except samples that inhibition occured.

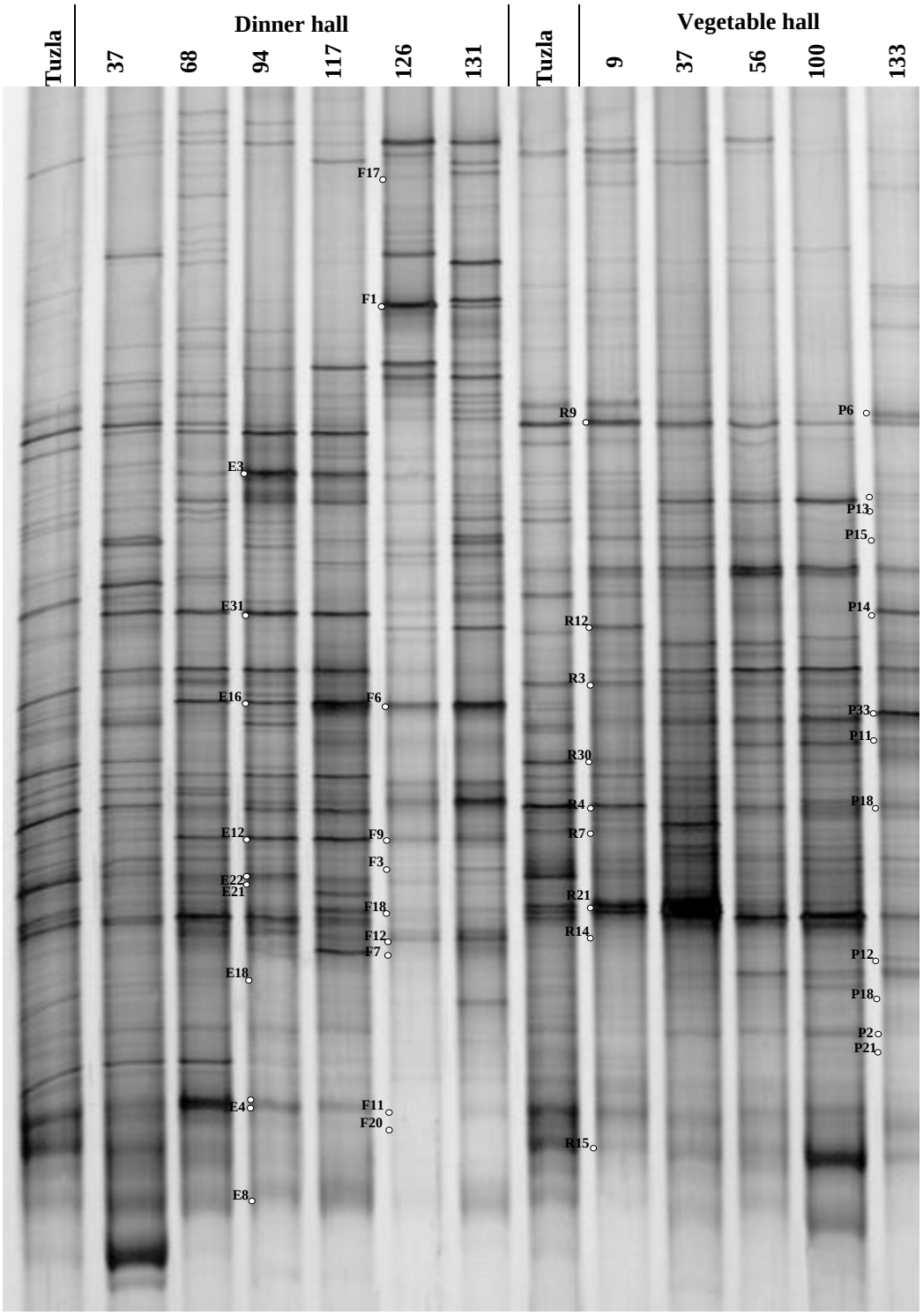


Figure 9.1 : DGGE profiles of the bacterial 16S rRNA of the digester sludge's.

It was observed that vegetabe hall wastes had a more stable microflora and dominance of R21 band had a 85% similarity to *Incertae Sedis XI* continued its dominance in each sample. Loading rates are different for two substrates. A increasing trend 3% to 12 %TS was observed in dinner hall wastes , 1% to 4% in vegetable hall wastes.

9.1.1 Dendrogram of Bacterial Profiles

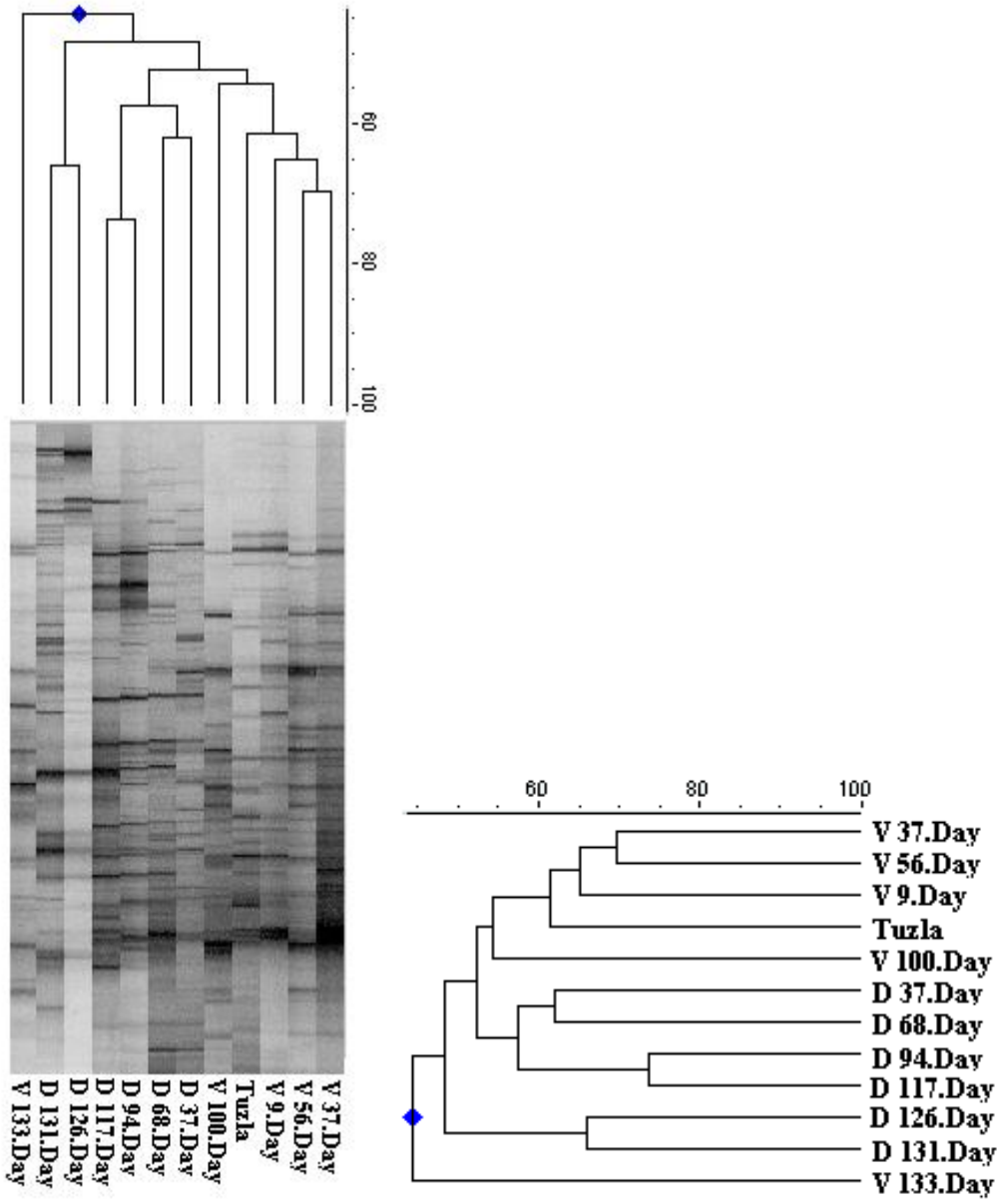


Figure 9.2: Illustrations of the cluster analysis of the bacterial PCR-DGGE profiles of dinner hall waste and vegetable hall waste digesters. Dendrograms were based on the Dice coefficient of similarity (weighted) and obtained with the UPGMA clustering algorithm. Samples are indicated by reactor name and operation day. (V_37.day means 37th day in digester that feed with vegetable hall wastes)

Cluster analyses showed that digester diversity were clearly different from each other with %43 similarities. 133 day of VHW digester could be said as acclimated sludge and had more similarity to dinner hall waste acclimated sludge. VHW sludge was more close to inoculums diversity. DHW digester micro flora after inhibition had %48 similarity to other samples.

9.1.2 Principal Component analysis (PCA) of Bacterial Profiles

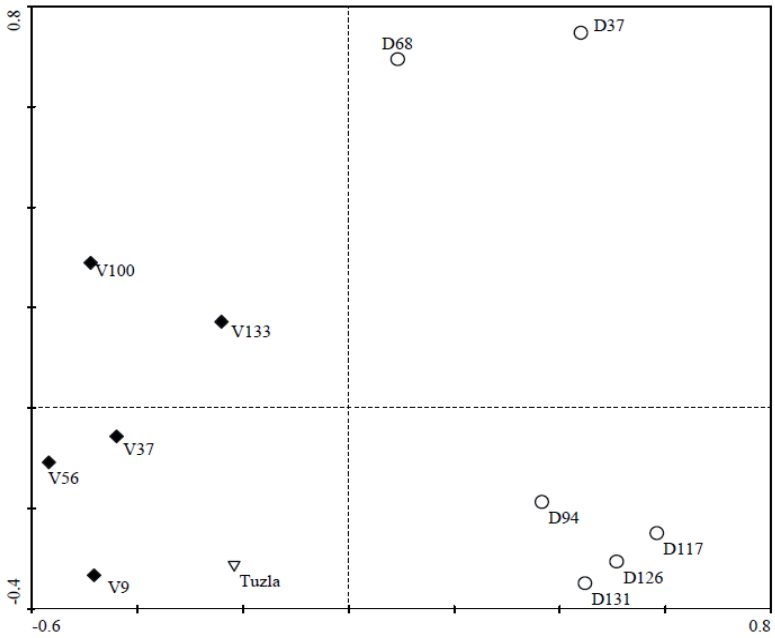


Figure 9.3: Principal-component analysis (PCA) scatter plot of bacterial denaturing gradient gel electrophoresis profiles (Fig. 9.1). The numbers of days of operation are also indicated; for example, D94 indicates digester sample of dinner hall wastes on day 94, V9 indicates digester sample of vegetable hall wastes on day 9.

Operational changes in the bacterial communities were monitored using principal-component analysis (Fig. 9.3). Based on visual inspection of the raw data, there was a clear difference in the DGGE profiles between ecology of dinner hall waste and vegetable hall waste digester that depended on the time and type of substrate. PCA1 revealed two different groups; the first group consisted of bacterial communites in dinner hall waste digester, and the second groups consisted of bacterial communities in vegetable hall waste digester, respectively.

Both digesters (dinner hall and vegetable hall) exhibited a noticeable and regular separation from each other in the first principal component (PC1). Furthermore, for each sample the PC2 value was increasingly far from the Tuzla sample as operation time increased for vegetable hall wastes. Acclimation period of dinnner hall wastes

was clearly seen in figure 9.3 as D37 and D68, after that there was a rapid change in the bacterial composition, but stayed stable afterwards, also in D117, D126, D131 in spite of the inhibition. Vegetable hall waste digester showed a increasing diversity view in figure 9.3. Like archaeal communities, it have closer diversity to Tuzla sludge.

This could explain the effect of %TS loading rate and substrate, to the microbial communities. Low TS loadings and celulosic feeding had lower effect on inoculum sludge microorganism structure. Archaeal populations in both digesters were similar to inoculums diversity except dominance. DHW digester had more archaeal diversity than VHW sludge. V2 band in VHW sludge was 99% to *Methanosaetaceae* order and dominant in all VHW samples although; it was slowly vanished from DHW sludge. J3 band %97 close to *Methanobacteriaceae* was dominant in both VHW and DHW sludge *with J8 which was %95 was close to Methanosaetaceae* order.

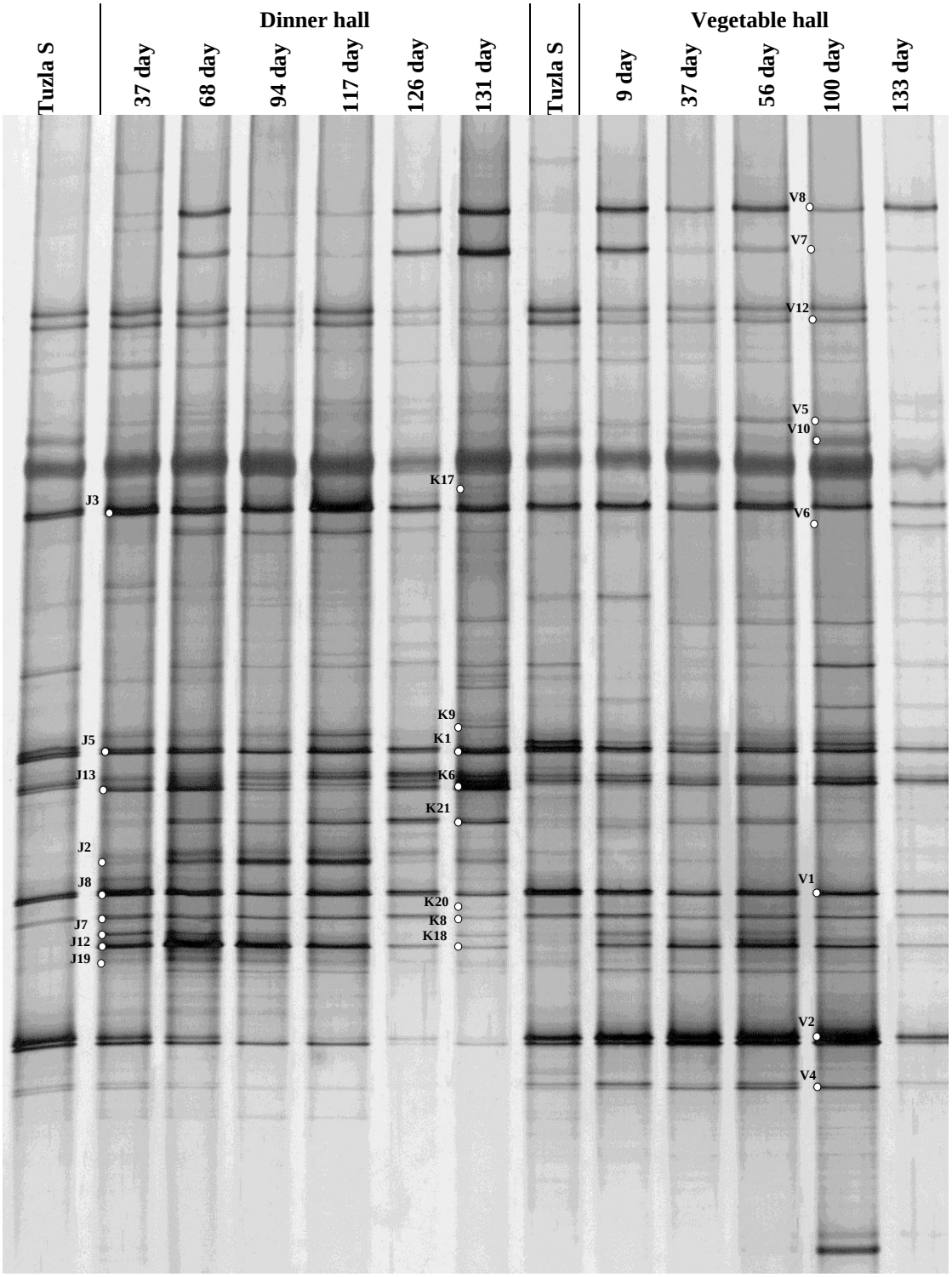


Figure 9.4 : DGGE profiles of the archaeal 16S rRNA of the digester sludges. Marked patterns with numbers indicate bands recovered and sequenced by cloning technique in Table 6.2 and 8.2.

9.1.3 Dendrogram of Archaeal Profiles

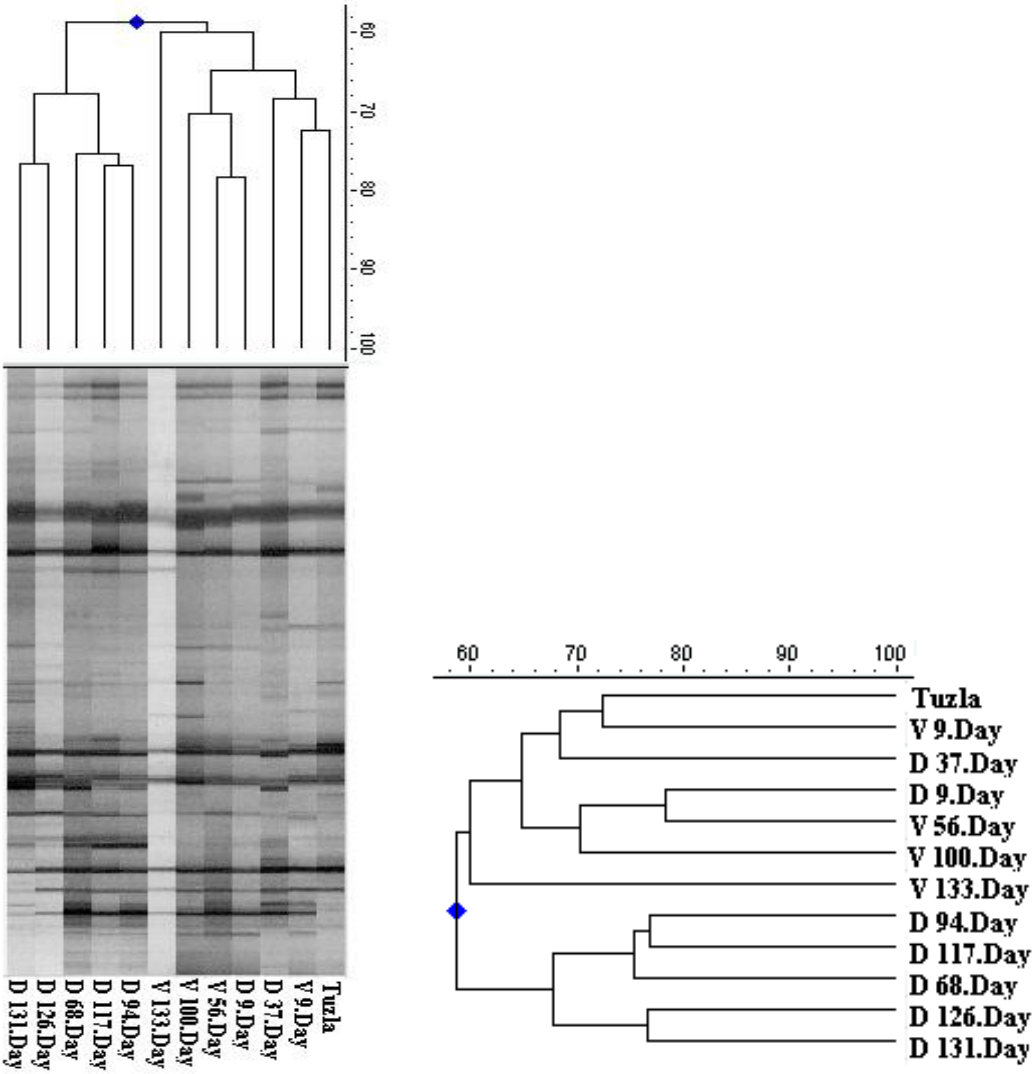


Figure 9.5: Illustrations of the cluster analysis of the archaeal PCR-DGGE profiles of dinner hall waste and vegetable hall waste digesters. Dendrograms were based on the Dice coefficient of similarity (weighted) and obtained with the UPGMA clustering algorithm. Samples are indicated by reactor name and operation day. (D_37.day means 37th day in digester that feed with dinner hall wastes)

Archaeal communitie99s were similar in both digesters. D9, V9 were showed a close similarity (68%and 72%) to inoculums. 68th day of VHW digester showed 60% similarities to inoculums and other samples. DHW samples showed closer diversity (70%- 75%) with each other than DHW.

9.1.4 Principal Component analysis (PCA) of Archaeal Profiles

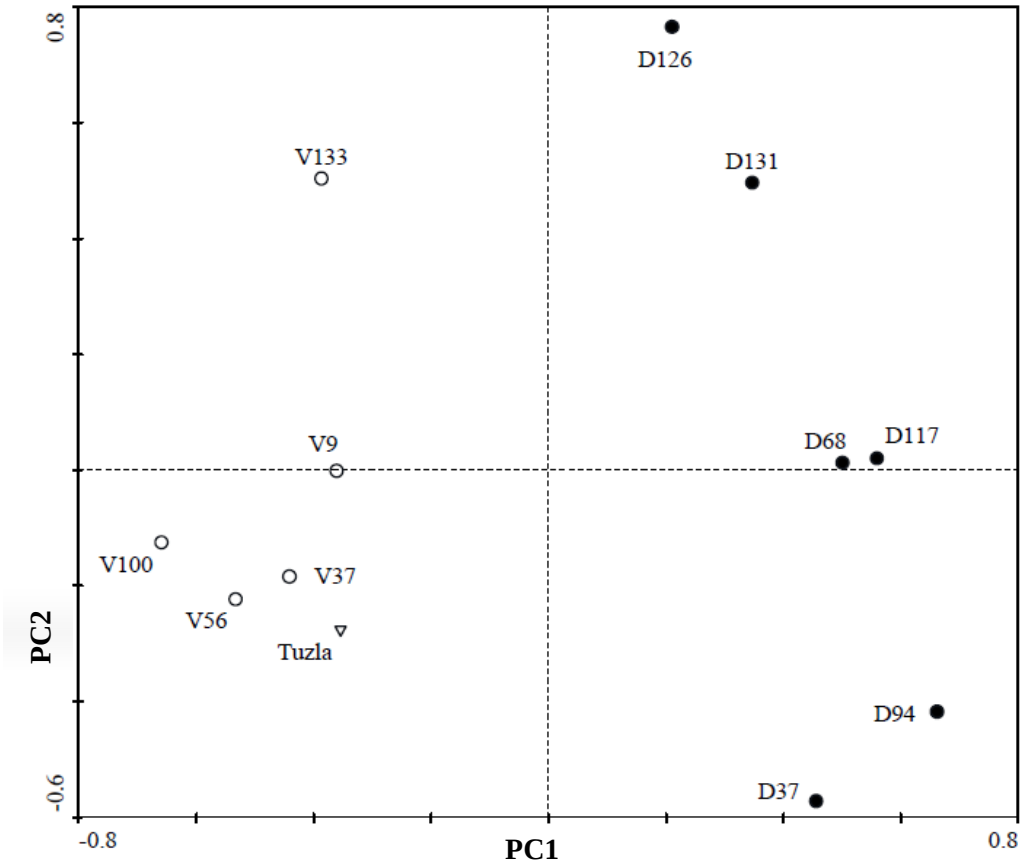


Figure 9.6: Principal component analysis (PCA) scatter plot of archaeal denaturing gradient gel electrophoresis profiles of digesters that fed with two different substrates (Fig. 9.3). The numbers of days of treatment are also indicated; for example, D94 indicates digester sample of dinner hall wastes on day 94, V9 indicates digester sample of vegetable hall wastes on day 9.

Operational changes in the archaeal community were monitored using principal-component analysis (Fig. 9.3). Based on visual inspection of the raw data, there was a clear difference in the DGGE profiles between ecology of dinner hall waste and vegetable hall waste digester that depended on the time and type of substrate. PCA revealed two different groups; the first group consisted of archaeal communites in dinner hall waste digester, and the second groups consisted archaeal communities in vegetable hall waste digester, respectively. Both digesters (dinner hall and vegetable hall) exhibited a noticeable and regular separation from each other in the first principal component (PC1). Furthermore, for each sample the PC1 value was increasingly far from the Tuzla sample as operation time increased for dinner hall wastes. The highest second principal component (PC2) for the both digesters corresponded to positive values after 120 day operation, while the first acclimation

period values were negative (Figure 9.3). This could explain archaeal communities differences due to loading rates and pH change. Coincidentally, the greatest change in PC2 occurred for dinner hall wastes after inhibition period. Furthermore, DGGE bands displayed different dynamics for the two digesters. Vegetable hall waste digester ecology was more close to Tuzla sludge composition and significantly differ from dinner hall waste digester structure according to PC1 result. In vegetable hall digester ecology diversity was decreased in first 2 months than showed a strictly increasing value from -0.2 to +0.5.

10. CONCLUSION AND RECOMMENDATIONS

This research studied the archaeal and bacterial community composition in the two stage digester treating vegetable hall and dinner hall wastes. Organic acids are important constituents of two-stage anaerobic digestion because they directly influence the pH during fermentation. Thus, acidic pH and the mesophilic temperature promote growth of microorganisms, which are supposed to metabolize rapidly reducing sugars. Therefore, differences in temperature, HRT, and substrate type caused the changes in the structure of the microbial community in the acidogenic fermenter [180]. Dinner hall and vegetable hall wastes fermenter sludge micro flora were significantly different due to pH adjustment and substrate composition. Fermentation process of dinner hall wastes implies changes in composition and activity of the microbial community concomitant with high carbohydrate amounts, increasing organic loading rates and low pH values of substrate. Three phases can be linked in this process: Early phase: equalization of lactic acid production by *Veillonellaceae* order that converted them into acetate and butyrate. Middle phase: Increased loading rates, made *Lactobacillaceae* order as a dominated bacterium with 74% dominance and 7000 mg/l lactic acid values. Late phase: *Lactobacillaceae* order found as the dominating bacterial order with 99% in fermenter microflora. Lactic acid concentrations inside the reactor was 10.000 mg/lt at 9% TS loading rate. Vegetable hall wastes fermenter was operated with pH adjusment and more stable loadings. Diversity was different from DHW fermenter, but similar in itself. Performance of digester for both reactors are high with a significant VFA consumption. A rapid pH decrease in the DHW digester was caused a significant change in digester microflora. This change was not reversal as shown in DGGE profiles. *Methanosaetaceae* order was dominant in DHW digester and *Methanomicrobiaceae* order in VHW digester for archaeal community. Archaeal populations came from inoculum were eliminated by pH adjustment as observed in both fermenters. Digester results showed that proper acidification needed for an effective methanization microflora in digester. Thus, adaquate mixing needed for homogeneous substrate dispersion inside reactors which was became important with

high loading rates. The results showed that the two-stage anaerobic reactor system is effective and efficient for conversion of biodegradable organic wastes such as; dinner hall and vegetable hall wastes to biogas.

In conclusion, this study predicts a plausible and unprecedented research for biomethanization of solid wastes with two-stage system. Although the research was not extended to more than 6 months, it could provide a basis for future development of strategies aimed at maximizing the production of biogas and conversion performance.

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