ANALYSIS OF MICROBIAL COMMUNITIES ASSOCIATED WITH ANAEROBIC SOLVENT DEGRADATION IN SEQUENCING BATCH REACTORS BY TRADITIONAL AND MOLECULAR TOOLS

by

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Submitted to the Institute of Environmental Sciences in partial fulfillment of the requirements for the degree of

Doctor

of

Philosophy

in

Environmental Technology

Boğaziçi University

2008

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DATE OF APPROVAL 27.11.2008

To the memory of my parents, Nesibe-Muammer Ayman

ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my thesis supervisor Prof. Dr. Bahar K. İnce for her guidance, friendly approach and encouragement throughout this study. I will always be indebted to her for having me in her research group and giving me the chance to have a life-time experience of academic environment along with friends and colleagues. She has always supported me both in academic and personal terms. She has been more than just a supervisor for me and I must say that I feel lucky to be her student and to know her.

I would also like to thank Prof. Dr. Orhan İnce, who has opened the doors of working with such a nice group in Istabul Technical University, for taking over the part of the second supervisor, for sharing his experiences and expanding my vision. I appreciate his many useful comments on this work, but even more so, I appreciate his advice, comments, and willingness to discuss any questions or ideas that I have had.

I would also like to thank my jury members for spending their valuable time to evaluate this thesis.

Many thanks go in particular to Gökhan Türker and Erkin Gözdereliler for their support during the work and helping me run the experiments.

I would also like to thank to all members of the Microbial Ecology Group (MEG), Mustafa Kolukirik, Ozge Eyice, Zeynep Cetecioglu, Sukriye Celikkol as well as the people of academic and technical stuff of the Molecular Biology and Genetics Research Center (MOBGAM) of Istanbul Technical University.

This study was supported by TUBITAK Project No:106Y241, “Determination of Interaction between Anaerobic Treatment of Organic Solvent Containing Industrial Wastewater with Its System Dynamics Using Molecular Tools”. The financial support partly provided by the Boğaziçi University Research Fund to the projects 06Y102D, 06HY105 and 08M108 is greatly acknowledged. I also kindly acknowledge Boğazici

University (BU) administrative personnel especially working in Research Fund Projects dealing with funds, administration and bureaucratic matters. I also acknowledge BU technical personnel who helped me fixing troubles in bioreactor set-up.

Scholarship given by TUBITAK, NATO-A2 for a research project at Nijmegen University, Microbiology Department, Netherlands is also gratefully acknowledged.

I express my heartiest thanks to my dear friend Suna Erses for her endless support and friendship. I would like to thank Gamze Gökmen Sözak and Işıl Gültekin for their friendship and support. I owe very special thanks to Gülhan Özkösemen for her help during my laboratory studies.

The most special thanks go to my dear husband Erdinç Öz and my sisters Aygün and Ayla Ayman for their endless love, support, patience and understanding. Without their encouragement and understanding it would have been impossible for me to finish this work. Words fail me to express my appreciation to my husband whose dedication, love, incrediable patience and persistent confidence in me, has taken the load off my shoulder. He kept me away from family responsibilities and encouraged me to concentrate on my study and helped me whenever I need. Without him, the work would have been much more difficult.

Finally, I would like to thank everybody who was important to the realization of the thesis, as well as expressing my apology that I could not mention personally one by one.

ABSTRACT

In recent years, discharges of the organic solvents have been subjected to stringent environmental regulations because of their undesirable effect on living organisms in aquatic environments and human health. Organic solvents such as methanol, isopropanol and toluene are found in wastewaters of several industries such as refineries, paint and pharmaceutical manufacturers. Treatment of solvent-containing wastewaters using anaerobic reactors has gained much attention in recent years as a cost-effective option compared to other treatment methods. However, there are still some concerns in the application of anaerobic treatment processes for this kind of wastewaters due to possible detrimental effect of the compounds present in the waste streams on both activity and microbial community playing crucial role in anaerobic digestion. Defining the effects of solvents on both microbial community structure and activity changes in anaerobic reactors may lead to improvements in the understanding of interactions in the bioreactors, thereby obtaining better reactor performance.

This study presents results from microbiological investigations of several lab-scale anaerobic wastewater treatments systems treating selected organic solvents using molecular techniques. Firstly, the importance of initial seed sludge quality in terms of activity and microbial composition has been investigated. Within the scope of the thesis, effects of methanol, propanol and toluene on microbial diversity and activity have been examined for acclimated and unacclimated sludge using particularly FISH, DGGE techniques and SMA tests, respectively. In addition, effect of solvents on activity and microbiology sludges taken from single-phase and two-phase anaerobic reactors was also evaluated to find out the role of phase differentiation to solvent response.

Within the scope of the dissertation, it was found that initial seed sludge characteristics in terms of activity and microbial community is of great importance in the performance of anaerobic reactors. Besides abundance of major methanogenic Achaeal groups in anaerobic sludges, a diverged bacterial groups (major or minor) having different metabolic capability may also play an important role in reactor performance. When the results of effects of selected solvents on methanogenic activity test and FISH studies for

the sludges taken from single-phase and methane reactor were compared, it was found that acetoclastic and methanogenic activity did not show a significant change for the sludges taken from different phases whereas FISH results of methanogens did change. Vitality of the methanogens under IC_{50} concentrations of methanol and isopropanol was higher in the sludge taken from methane reactor compared to single phase reactor. On the other hand, methanogens showed a better response to toluene in the sludge taken from single phase reactor. The results are attributed to different microbiological characteristics of the sludge taken from different phases. In the studies with anaerobic sequencing batch reactor (ASBR) fed with solvents, microbial diversity changed significantly throughout the course of the operation where different wastewater compositions including organic solvents were fed to the anaerobic reactors. In ASBRs fed with solvent-containing synthetic wastewater, percentage of active population in the reactor decreased over the period of operation of the reactor. At the beginning, the decrease in the active population did not affect the reactor performance. However, reactor performance failed as a result of the decrease of the active population to a critical level. *Methanomicrobiales* which are hydrogenotrophic methanogens was the most negatively affected group by the all selected solvents and completely lost their activity in the ASBR fed with the solvents. The most pronounced effect on active total methanogenic community has been caused by isoproponal, toluene and methanol, respectively.

ÖZET

Son yıllarda, organik solventlerin alıcı ortama deşarjları, insan hayatı ve doğal yaşam üzerine olumsuz etkileri nedeniyle çevre yönetmeliklerince sınırlandırılmıştır. Metanol, toluen ve propanol gibi organik bazlı solventler ilaç, petrol, boya vb. endüstrilerin atıksularında önemli miktarlarda bulunmaktadır. Diğer arıtım metodları ile karşılaştırıldığında, organik solvent içeren atıksuların anaerobik şartlarda arıtılması çevresel ve ekonomik açıdan bir alternatif seçenek olarak önem kazanmıştır. Bununla birlikte, solventlerin anaerobik arıtımda rol oynayan mikrobiyal topluluğun yapısına ve aktivitesi üzerine muhtemel olumsuz etkileri nedeniyle, anaerobik arıtma metodlarının solvent içeren atıksulara uygulanması konusunda hala bazı önemli noktaların aydınlatılmasına ihtiyaç vardır. Anaerobik reaktörlerin daha verimli işletebilmesi için, solventlerin anaerobik arıtımda rol oynayan mikrobiyal topluluğun yapısına ve aktivitesine etki eden faktörlerin anlaşılması önem taşımaktadır. Solventler gibi spesifik bazı kirleticilerin, anaerobik reaktörlerde mikrobiyal topluluğun yapısına ve aktivitesine yaptığı etkileri tanımlayarak, biyoreaktörlerde hakim proseslerin anlaşılması ve böylece bu sistemlerden daha iyi performans elde edilmesi sağlanabilir.

Bu tez kapsamında, solvent arıtan laboratuvar ölçekli anaerobik reaktörlerde, moleküler teknikler kullanılarak reaktörlerdeki mikrobiyal popülasyonun dinamikleri incelenmektedir. Öncelikle, aktivite ve mikrobiyal kompozisyon açısından aşı çamurunun kalitesinin anaerobik reaktörlerin performansındaki etkisi incelenmiştir. Ayrıca bu tez kapsamında, metanol, toluen ve isopropanol gibi solvent içeren atıksuların anaerobik arıtımda, reaktörlerin mikrobiyal populasyon dinamikleri FISH ve DGGE gibi moleküler yöntemler yardımıyla incelenmiştir. Bu çalışmalara ek olarak, seçilmiş solventlerin tek fazlı ve iki fazlı anaerobik reaktörlerden alınan çamurun aktivitesine ve mikrobiyolojisine etkisi incelenerek faz ayrımının rolü incelenmiştir.

Bu çalışmada, anaerobik aşı çamurunun metanojenik aktivitesi yanında bakteriyel çeşitlilik açısından çamurun mikrobiyal kalitesinin anaerobik reaktörlerin performansında büyük önemi olduğu gösterilmiştir. Aşı çamurunun, metanojenik arkeyal topluluk yanında, çeşitli metabolik fonksiyonlara sahip majör veya minor bakteriyal gruplar içermesinin

reaktör performansına olumlu etki yaptığı bulunmuştur. Tek fazlı ve metan reaktöründen alınan çamurlarda solventlerin metanojenik aktivite ve mikrobial kompozisyona yaptığı etkiler karşılaştırıldığında, aktivite test sonuçlarının farklılık göstermemesine karşılık, kantitatif metanojenlerin oranı değişiklik göstermiştir. Metan reaktöründen alınan çamurda, metanol ve isopropanolün metanojenlerin rölatif miktarına ve aktivitesine etkisi tek fazlı reaktörle karşılaştırıldığında daha az olarak tespit edilmiştir. Bununla birlikte metanojenler tek fazlı reaktörden alınan çamurda toluene daha iyi tepki göstermiştir. Bu sonuçlar, tek fazlı ve iki fazlı reaktörün metan fazından alınan çamurun mikrobiyolojik karakteristiğindeki farklılıklardan kaynaklanmış olabileceği düşünülmektedir. Seçilmiş solventlerle beslenen ardışık kesikli anaerobik reaktörlerde (AKAR), mikrobiyal çeşitlilik reaktörlere beslenen değişik atıksu kompozisyonlarına bağlı olarak önemli bir değişiklik göstermiştir. Bu reaktörlerin işletilmesi sırasında, solventlerin etkisine bağlı olarak aktif populasyonun oranı önemli miktarda azalmıştır. Başlangıçta, aktif popülasyondaki bu düşüş reaktör perfomansına yansımamakla birlikte, düşüşün devam etmesi reaktörlerin verimini önemli derecede etkilenmiştir. Hidrojenotrofik *Methanomicrobiales,* solventlerden en fazla etkilenen grup olarak tespit edilmiştir. Aktif metanojenik populasyona en önemli etkiyi sırasıyla isoproponal, toluene ve metanol neden olmustur

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Symbol	Explanation	Units
ASBR	Anaerobic Sequencing Batch Reactor	
COD	Chemical Oxygen Demand	mg/L
DGGE	Denaturing Gradient Gel Electrophoresis	
EGSB	Expanded Granular Sludge Bed	
EDTA	Ethylenediamine Tetraacetic Acid	
EtBr	Ethidium Bromide	
FISH	Fluorescent in situ Hybridization	
HRT	Hydraulic Retention Time	
OTU	Operational Taxonomic Unit	
PBS	Phosphate Buffered Saline	
PCR	Polymerase Chain Reaction	
PFA	Paraformaldehyde	
rDNA	Ribosomal DNA	
rRNA	Ribosomal RNA	
SDS	Sodiumdodecylsulfate	
SMA	Specific Methanogenic Activity	$mLCH_4/gVSS/d$
SRB	Sulphate Reducing Bacteria	
SRT	Sludge Retention Time	
SS	Suspended Solids	mg/L
SSU	Small Subunit	
TAE	Tris-Acetic Acid-EDTA	
TS	Total Solid	mg/L
TVS	Total Volatile Solid	mg/L
VFA	Volatile Fatty Acids	mg/L
VSS	Volatile Suspended Solids	mg/L

1. INTRODUCTION

Anaerobic wastewater treatment is now one of the key technologies in environmental biotechnology as a cost-effective alternative compared to other treatment methods and has gained popularity for a wide range industrial effluents such as pharmaceutical, refineries, etc. containing organic solvents (Enright et al.,2005; 2007a; 2007b). Organic solvents such as methanol, toluene and isopropanol are extensively used to dissolve the compounds required for certain processes in the industries. In recent years, discharges of the compounds have been subjected to stringent environmental regulations because of their undesirable effect on living organisms in aquatic environments. Increased application of anaerobic digestion to a broader range of wastewaters including organic solvents would provide significant environmental and economic benefits for the industries (Ince et al., 2002; Oz et al., 2003; 2004). However, there are still some concerns in the application of anaerobic treatment processes for solvent containing wastewaters. The processes are mostly limited by the extent of inhibitory effects of different compounds found in waste streams on both activity and microbial community structure playing role in anaerobic wastewater treatment.

Understanding of the microbial ecology of the anaerobic reactors plays an important role in the controlling of start-up and operation of the reactors. The composition of the microbial community in the bioreactors is determined by physical, chemical and biological selective pressures. This selection plays an important role in degradation process and resisting to changes in environmental conditions and disturbances. However, wastewater treatment systems have been generally designed and operated without fully understanding of the microbial community structure and function involved in the degradation process (Ince et al., 1995). The ecology of microbial populations and communities capable of catabolizing specific reactions remains largely unexplored in engineered reactor systems. In order to be able to start-up and operate anaerobic reactors treating different industrial wastewaters properly and effectively, it is becoming much more important to understand the factors affecting both microbial diversity and activity of the biomass in the reactors (Ince et al., 2005). Therefore, evaluation of the microbial community together with other operatioanal parameters is important from a point of view of process engineering. Recent

developments with the integration of microbial ecology and molecular biology are rapidly evolving and provide a new insight into the interrelations between microorganisms and their environment in bioreactors (Roest, 2007; McHugh et al., 2004; Amann et al., 1990; Hugenholtz et al., 1998). More recently, the microbial ecology of anaerobic reactor systems has been investigated in detail using several molecular techniques such as FISH, DGGE etc. (Delbes et al., 2001; Gerardi, 2003; Collins et al., 2003). Despite the gained experience on the matters in recent years, much more study should be carried out to define microbial community interactions inside the reactors treating specific pollutants such as solvent-containing wastewaters inside the bioreactors. In this manner, investigations should also include changes in quantification of different group of microorganisms under specific compounds in anaerobic systems. Only a limited number of studies cover the microbial ecology of anaerobic reactors solvent-containing wastewaters (Enright et al., 2005; 2007a; 2007b).

In the light of the discussion above, anaerobic treatment systems can be more efficiently used for the wastewaters containing the solvents if the microbial community is defined better in terms of activity and vitality in the presence of organic solvents. This study was, therefore, carried out to investigate possible inhibitory effects of individual and mixture of different solvents on mixed culture. Furthermore, it is aimed to investigate the effects of individual and mixture of different organic solvents on methanogenic and non-methanogenic activity. In addition, effect of the organic solvents on the species playing role in anaerobic treatment of the solvents in anaerobic sequencing batch reactors was investigated by using molecular techniques such as Denaturant Gradient Gel Electrophoresis (DGGE) and Fluorescent in situ Hybridization (FISH). The information on structure and functionality of bioreactor microbiota that can be gained from the studies will be pivotal to a better understanding and predictability of anaerobic wastewater treatment processes treating solvent containing wastewaters and reactor performance provide potential identification of general and system-specific indicator populations, allowing improved diagnostics and reactor predictability.

2. THEORETICAL BACKGROUND ON ANAEROBIC PROCESSES

2.1. Fundamentals of Anaerobic Degradation

Anaerobic degradation which is a multistage process in which biodegradable complex organic materials are converted to the end products CH_4, CO_2 and trace amounts of hydrogen in the absence of oxygen involves a consortium of microorganisms. Several models have been proposed to explain the biochemical steps in anaerobic digestion such as nine-stage model (Harper and Pohland, 1986), six-stage model (Lester et al., 1986) and three-stage model (Gerardi, 2003). Biochemical reactions in the nine-stage model of anaerobic digestion are listed below (Harper and Pohland, 1986) and shown diagrammatically in Figure 2.1 and Figure 2.2. As can be seen from the figures, the biochemistry and microbiology of anaerobic digestion is a complex biogenic process involving a number of microbial populations, linked by their individual substrate and product specifities (Hutnan et al., 1999).

1. Hydrolysis of organic polymers to intermediate organic monomers,
2. Fermentation of organic monomers,
3. Oxidation of propionic and butyric acids and alcohols by obligate H_2 producing acetogens,
4. Acetogenic respiration of bicarbonate by homoacetogens,
5. Oxidation of propionic and butyric acids and alcohols by sulphate reducing bacteria (SRB) and nitrate reducing bacteria (NRB),
6. Oxidation of acetic acid by SRB and NRB,
7. Oxidation of hydrogen by SRB and NRB,
8. Acetoclastic methane formation,
9. Methanogenic respiration of bicarbonate.

Anaerobic digestion involves numerous interactions between four major metabolic groups that are generally accepted as present in anaerobic digesters; hydrolytic-fermentative bacteria, proton-reducing acetogenic bacteria, hydrogenotophic methanogens, and acetoclastic methanogens (Zinder et al., 1984). Therefore, the steps of anaerobic

digestion process can be classified into four major phases including hydrolysis, acidogenesis, acetogenesis and methanogenesis.

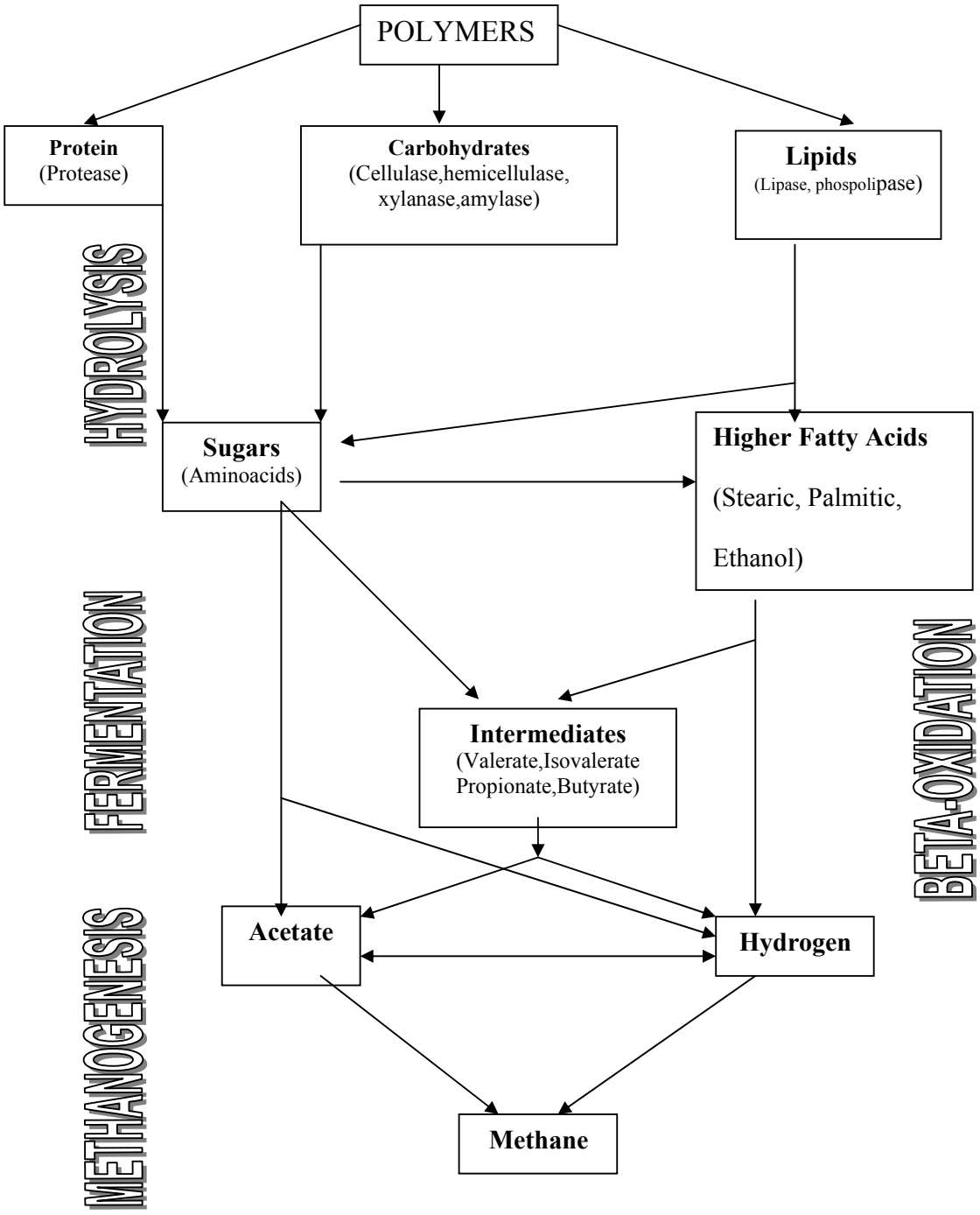


Figure 2.1. Anaerobic breakdown of organic polymers (Stronach et al., 1986)

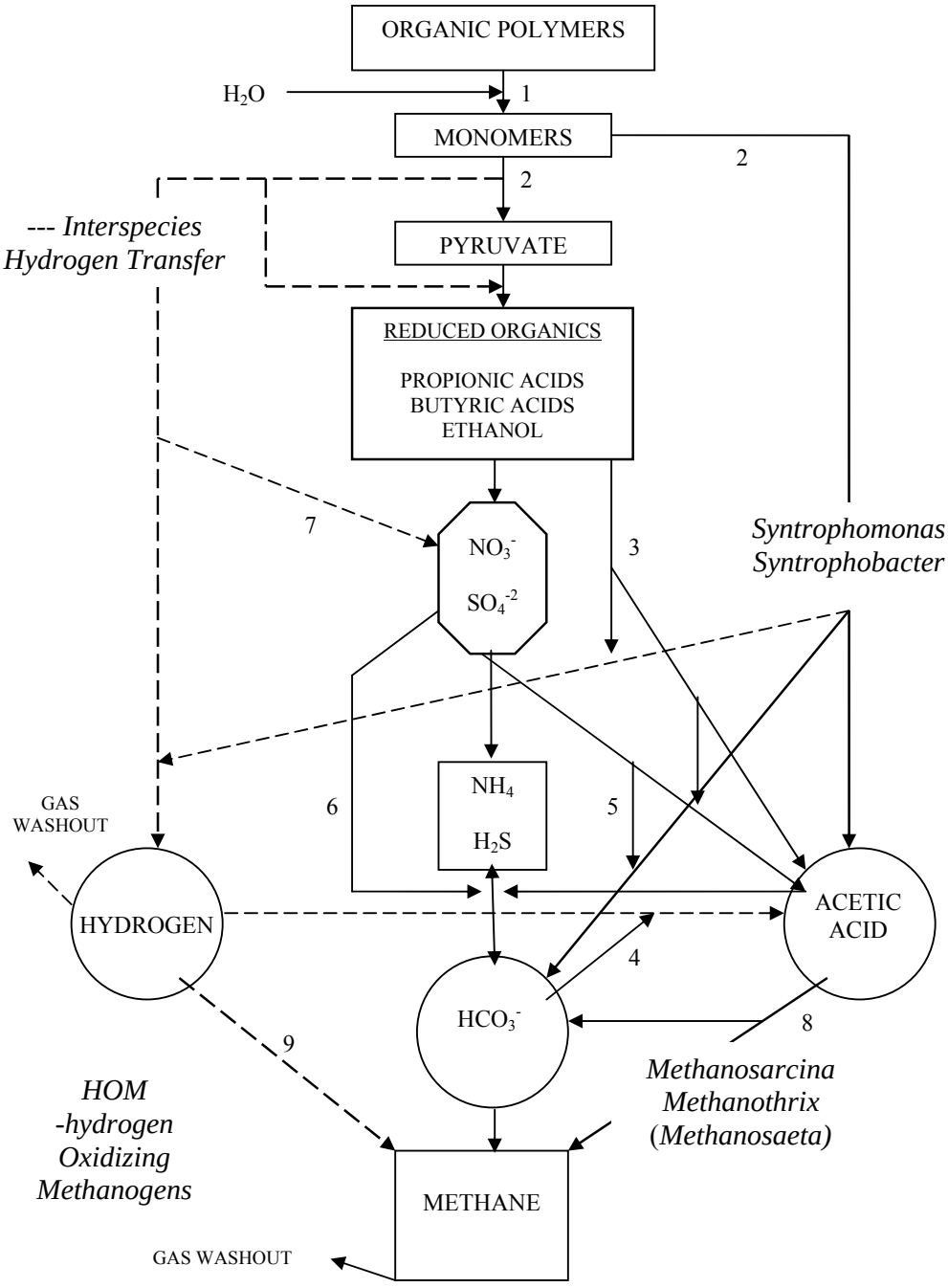


Figure 2.2. Substrate conversion patterns associated with the anaerobic digestion (Harper and Pohland, 1986).

2.1.1. Hydrolysis

Hydrolysis is the first step in anaerobic digestion and includes the enzme-mediated reactions by which complex organic matter such as carbonhydrates, proteins, and lipids are hydrolyzed into simpler and soluble products which can transport across the cell membrane. The reaction rates of the step are influenced by pH, cell residence time and the waste constituents in the reactor. In an anaerobic digestion process where a substantial portion of the waste stream contains complex organic compounds, the hydrolytic bacteria and their enzymes are of paramount importance for the succeeding steps in the anaerobic degradation sequence (Stronach et al., 1986). In this case, hydrolysis can be rate-limiting step in overall conversion of complex substrates (Pavlostathis and Giraldo-Gomez, 1991). The strictly anaerobic genus *Clostridium* includes many species that are highly active in the production of extracellular hydrolytic enzymes and is responsible for degradation of compounds containing cellulose and starch while Bacillus play role in the degradation of proteins and fats (Lema et al., 1991; Noike et al., 1985). The types of hydrolytic microorganisms are reported namely as, the cellulytic (*Clostridium thermocellum*), proteoytic (*Clostridium bifermentas, Peptococcus*), lipolytic (genera of clostridia and micrococci) and aminolytic (*Clostridium butyricum*, *Bacillus subtilis*) bacteria (Hungate, 1982; Payton and Haddock, 1986). The hydrolytic bacteria may also break down the some intermediate products to simple volatile fatty acids, carbon dioxide, hydrogen, ethanol (Eastman and Ferguson, 1981).

The hydrolysis of the compounds is carried out by specific extracellular enzymes such as amylases, proteinases, lipases and nucleases. Lipases convert lipids to long-chain fatty acids. A population density of 10^4 - 10^5 lipolytic bacteria per mL of digester fluid has been reported. Clostridia and the micrococci appear to be responsible for most of the extracellular lipase producers. The long-chain fatty acids produced are further degraded by β-oxidation to produce acetyl-CoA. Proteins are generally hydrolyzed to amino acids by proteases, secreted by *Bacteroides, Butyrivibrio, Clostridium, Fusobacterium, Selenomonas,* and *Streptococcus.* The amino acids produced are then degraded to fatty acids such as acetate, propionate, and butyrate, and to ammonia as found in *Clostridium, Peptococcus, Selenomonas, Campylobacter,* and *Bacteroides.* Polysaccharides such as cellulose, starch, and pectin are hydrolyzed by cellulases, amylases, and pectinases. The

majority of microbial cellulases are composed of three species: (a) endo-(3-l, 4-glucanases; (b) exo-*p*-l, 4-glucanases; (c) cellobiase or *p*-glucosidase. These three enzymes act synergistically on cellulose effectively hydrolyzing its crystal structure, to produce glucose. Microbial hydrolysis of raw starch to glucose requires amylolytic activity, which consist of 5 amylase species: (a) alpha-amylases (b) *p*-amylases (c) amyloglucosidases (d) debranching enzymes (e) maltase. Pectins are degraded by pectinases, including pectinesterases and depolymerases. Xylans are degraded with xylanase and xylosidase to produce xylose. Hexoses and pentoses are generally converted to C_2 and C_3 intermediates and to reduce electron carriers (e.g., NADH) via common pathways.

2.1.2. Acidogenesis

In the acidogenesis phase, the breakdown products of the hydrolysis phase including amino acids, sugars and long chain fatty acids are converted to carbon dioxide, hydrogen, alcohols, ammonia and short-chain fatty acids such as acetic, butyric, propionic, valeric acid. The products of the process vary with the type of bacteria as well as with temperature, pH, and the composition of the influent feed. The process utilizes single amino acids, pairs of amino acids or a single amino acid with a non-nitrogenous compound. The catabolism of these organic compounds is carried out by a large number of both obligatory and facultatively anaerobic microorganisms and single amino acids are converted under anaerobic conditions by *Clostridia, Mycoplasmas* and *Streptococci* while butanol, butyric acid, acetone and iso-propanol are generally produced by the bacteria of the genera *Clostridium* and *Butyribacterium*, for example *Clostridium butyricum* produces butyrate, *Clostridium acetobutylicum* mainly acetone and butanol and *Clostridium butylicum* produces butanol in addition to hydrogen, carbondioxide and iso-proponol. The genera *Clostridium* and *Butyribacterium* are involved in the production of butyrate, buthanol, butyric acid, acetone and iso-propanol.

2.1.3. Acetogenesis

The short chain fatty acids other than butyric, propionic acid and other higher fatty acids are converted to acetic acid, carbondioxide and hydrogen by the process by obligate hydrogen producing acetogenic bacteria (OHPA). This process is called as β-oxidation in which a molecule is removed from fatty acids having more than two carbons at each reaction step until all fatty acids are converted to acetate molecules. The activity of the organisms playing role in the step is of paramount importance, since methanogens can not utilize the fatty acids other than acetic acid. Since conversion of butyric and propionic acid need energy input and only proceed at very low hydrogen partial pressure, the step is slow and energetically unfavaroble and can be rate limiting step of the soluble part of anaerobic digestion. Bacteria producing acetic acid are *Methanobacterium bryantii, Desulfovibrio Syntrophobacter wolinii* (responsible for acetic acid production from propionic acid) (Malina et al., 1992; Stronach et al., 1986), *Syntrophomonas wolfei* (responsible for acetic acid production from butyric, caproic and valeric acids) (Malina et al., 1992; Gujer et al., 1983), *Syntrophus buswellii*.

2.1.4. Methanogenesis

In the final stage of anaerobic digestion, the end products of the previous step are converted into methane and carbon dioxide by methanogens via two conversion mechanisms including decarboxylation of acetic acid and reduction of carbon dioxide in the absence of other electron acceptors such as oxygen, nitrate, and sulfate except bicarbonate and protons as terminal electron acceptors (Garcia et al., 2000; De Bok et al., 2004; Stams et al., 2006). Methanogenesis is considered the rate-limiting step in the whole anaerobic digestion process due to the slow growth rate of the methanogens (Malina et al., 1992). Therefore, activity of the methanogens is important for maintaining efficient anaerobic digestion and avoiding the accumulation of H_2 and short chain fatty acids. Phylogenetically, methanogens belongs to Archaea domain, a group of microbes that are distinguished from true bacteria by a number of characteristiristics, including the possesion of membrane lipids composed of isoprenoids ether-linked to glycerol or other carbonhydrates, a lack of peptidoglycan containig muramic acid, a distinctive ribosamal

RNA sequences (Woese, 1987). This group also includes some extreme halophiles and some extremely thermophilic, sulfur-dependent microbes (Woese, 1987) and phylogenetically distinct from eukaryotes and true bacteria. Methanogens which are represented in several orders (Figure 2.3) and families are strictly anaerobic organisms and gain their energy by producing CH_4 and CO_2 from simple substrates such as H_2, CO, formate, and a few alcohols. Alternatively, methanogens produce CH_4 by the reduction of the methyl groups in acetate, methanol, trimethylamine, and dimethylsulfide, part of which are oxidized to CO_2 to generate the electrons necessary for reduction of the methyl group to CH_4. Some methanogens are able to use H_2 as second substrate to reduce the methyl, for example in methanol. The substrates including CO_2-type, methyl substrates and acetate converted to methane by various methanogenic *Archaea* are listed in Table 2.1. All reactions are thermodynamically exergonic at standard conditions allowing occur in nature, if substrate concentrations are sufficiently high.

In the first class of substrate, CO_2-type substrates including CO_2, formate and carbon monoxide are reduced to methane. Although the reduction of CO_2 to CH_4, shown in Eq. 2.1, is generally H_2 dependent, other substrates in this class can supply the electrons for CO_2 reduction. Hydrogenotrophic methanogens which produce methane from carbondioxide–type substrates are found among several methanogenic taxa including members of the order *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales* and *Methanosarcinaceae*. Most hydrogenotrophic methanogens that can reduce CO_2 to methane use H_2 as the primary electron donor. Many hydrogenotrophic methanogens are also able to use formate and secondary alcohols, such as 2-propanol, 2-butanol, and cyclopentanol as electron donors. A small number of methanogens can also use ethanol.

$$CO_2+4H_2\rightarrow CH_4+2H_2O \qquad \Delta G°=-131kJ/mol \qquad (2.1)$$

The second class of methanogenic substrates are methyl group substances which are converted to methane via two conversion mechanisms. First mechanism is the formation of methane by reducing methyl group substances using an external electron donor such as H_2. In the conversion equations methanol (CH_3OH) is used as a model methyl substrate, as given in Eq. 2.2. Alternatively, the methyl group substances can be oxidized to CO_2 in

order to generate the electrons needed to reduce other molecules of CH_3OH to CH_4 in the absence of H_2, shown in Eq. 2.3. Methanogens that are able to use methylated compounds, or methylotrophicmethanogens, are limited to the order *Methanosarcinales*, except for *Methanosphaera* species, which belong to the order *Methanobacteriales*.

$$CH_3OH+H_2 \rightarrow CH_4+H_2O \qquad \Delta G°=-113kJ/mol \qquad (2.2)$$

$$4CH_3OH \rightarrow 3CH_4+CO_2+2H_2O \qquad \Delta G°=-319kJ/mol \qquad (2.3)$$

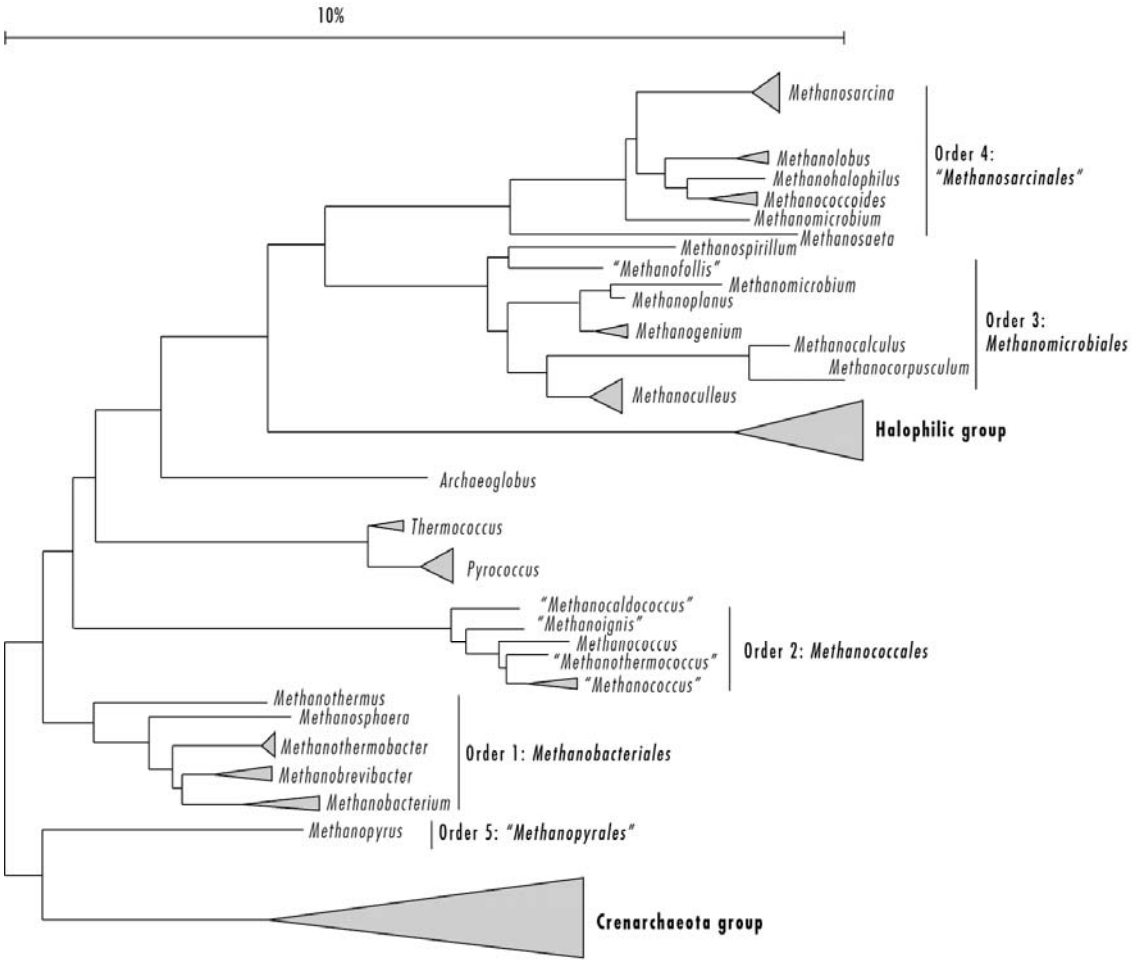


Figure 2.3. Phylogeny of methanogens, domain Archaea. (Non-methanogens are indicated by their group names, large triangles) (Garcia et al., 2000).

Table 2.1. Substrates converted to methane by various methanogenic *Archaea* (Madigan et al., 2002)

Substrates and Reactions	**Organisms**
I. CO_2-type substrates (Carbon dioxide with electrons derived from H_2, certain alcohols, or pyruvate; Formate, Carbon monoxide)	
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$ $4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$ CO_2+4isopropanol$\rightarrow CH_4$ + 4acetone + $2H_2O$ $4\ CO + 2H_2O \rightarrow CH_4 + 3\ CO_2$	Most methanogens Many hydrogenotrophic methanogens Some hydrogenotrophic methanogens *Methanothermobacter* and *Methanosarcina*
II. Methylated C1 compounds (Methanol, Methylamine, Dimethylamine, Trimethylamine, Methylmercaptan, Dimethylsulfide)	
$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$ $CH_3OH + H_2 \rightarrow CH_4 + H_2O$ $2\ (CH_3)_2\text{-}S + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ H_2S$ $4\ CH_3\text{-}NH_2 + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_3$ $2(CH_3)_2\text{-}NH + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_3$ $4\ (CH_3)_3\text{-}N + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_3$ $4CH_3NH_3Cl + 2H_2O \rightarrow 3CH_4 + CO_2 + 4\ NH_4Cl$	*Methanosarcina* and other methylotrophic methanogens *Methanomicrococcus blatticola* and *Methanosphaera* Some methylotrophic methanogens Some methylotrophic methanogens Some methylotrophic methanogens Some methylotrophic methanogens Some methylotrophic methanogens
III. Acetate	
$CH_3COOH \rightarrow CH_4 + CO_2$	*Methanosarcina* and *Methanosaeta*

The third type of substrate that is used by methanogens is acetate. Acetate is a major product of the fermentation and accounts for two-thirds of the methane production in anaerobic bioreactors. Acetate is catabolized by cleavage, with the carboxyl group being oxidized to CO_2 and the methyl group being reduced to CH_4. The CH_4-producing reaction is generated by the activity of methyl-CoM reductase, which converts methyl-CoM (methyl-coenzymeM) and HS-HTP (N-7-mercaptoheptanoyl-O-phospho-L-threonine) to CH_4 and a heterodisulfide consisting of HS-HTP and CoM-SH. This reaction is universal to all methanogens, independent of primary substrate. The subsequent reduction of the heterodisulfide to CoM-SH and HS-HTP is coupled to the generation of a proton motive force. This reaction is the most important one for energy conservation and is universal for all methanogens. In the first step, acetate has to be converted to acetylcoenzyme A (acetyl-CoA), which requires the expenditure of energy. Formation of acetyl-CoA occurs by two different reactions (Ferry, 1992). In *Methanosarcina* spp., acetate is first phosphorylated with ATP by an acetate kinase producing acetyl-P and ADP. Subsequently, the acetyl-P is converted by a phosphotransacetylase with CoA-SH to acetyl-CoA and phosphate. In summary, conversion of acetate to acetyl-CoA requires one energy-rich phosphate bond of ATP in *Methanosarcina* spp. In *Methanosaeta* spp., on the other hand, acetate is activated using an acetyl-CoA synthetase, which converts acetate, CoA-SH, and ATP to acetyl-CoA, AMP, and pyrophosphate. In summary, this reaction requires two energy-rich phosphate bonds of ATP. This means that *Methanosaeta spp.* use more energy for acetate activation than *Methanosarcina spp*. Acetate is catabolized by the members of only two genera of methanogens, that is *Methanosarcina* and *Methanosaeta*, which belong to the families of *Methanosarcinaceae* and *Methanosaetaceae*, respectively. *Methanosarcina* has a low affinity for acetate and dominate when acetate concentrations are high. On the other hand, *Methanosaeta* has a high affinity for acetate and dominate when acetate concentrations are as low. *Methanosaeta*, which uses only acetate as substrate, is a superior acetate utilizer in that it can use acetate at concentrations as low as 5–20 μM, while *Methanosarcina* requires a minimum concentration of about 1 mM. Members of the family *Methanosarcinaceae*, including *Methanosarcina* spp. are also able to utilize H_2/CO_2, methanol, metyl amines and pyruvate besides acetate as energy substrate for CH_4 production. The difference between the organisms in terms of acetate affinity depends probably on the differences in the first step of acetate metabolism. *Methanosarcina* uses the low-affinity acetate kinase (AK)-phosphotransacetylase (PTA) system to activate acetate to acetyl-CoA. On the other hand,

Methanosaeta uses the high-affinity adenosine monophosphate (AMP)–forming acetyl-CoA synthetase.

Table 2.2. Characteristics of methanogenic Archaea (Madigan et al., 2002).

Genus	Morphology	Substrate for methanogenesis
Methanobacteriales		
Methanobacterium	Long rods	H_2+CO_2, formate
Methanobrevibacter	Short rods	H_2+CO_2, formate
Methanosphaera	Cocci	Methanol+H_2
Methanothermus	Rods	H_2+CO_2, can also reduce S^0; hyperthermophile
Methanococcales		
Methanococcus	Irregular cocci	H_2+CO_2, pyruvate+CO_2, formate
Methanomicrobiales		
Methanomicrobium	Short rods	H_2+CO_2, formate
Methanogenium	Irregular cocci	H_2+CO_2, formate
Methanospirillum	Spirilla	H_2+CO_2, formate
Methanoplanus	Plate-shaped cells	H_2+CO_2, formate
Methanocorpusculum	Irregular cocci	H_2+CO_2, formate, alcohols
Methanoculleus		H_2+CO_2, alcohols, formate
Methanosarcinales		
Methanosarcina	Large irregular cocci in packets	H_2+CO_2, methanol, methylamines, acetate
Methanolobus	Irregular cocci in aggregates	Methanol, methylamines
Methanohalobium	Irregular cocci	Methanol, methylamines;halophilic
Methanococcoides	Irregular cocci	Methanol, methylamines
Methanohalophilus	Irregular cocci	Methanol, methylamines, methyl sulfides; halophile
Methanothrix	Long rods to filaments	Acetate
Methanopyrales		
Methanopyrus	Rods in chains	CO_2, hyperthermophile, growth at 110 °C

2.2. Environmental Factors Affecting Anaerobic Treatment Processes

It is generally assumed that the rate limiting step of the anaerobic treatment process is methanogenesis. By removing the metabolic products of syntrophic acetogens, methanogens play a regulative role in maintaining the overall efficiency of the process. However, low growth rate of methanogenic *Archaea* can make the anaerobic system sensitive to environmental changes (Xing et al., 1997) and disturbances in populations from one trophic level may affect the entire community (Raskin et al., 1996). The most important factors which may affect the performance of anaerobic systems are listed below.

2.2.1.Temperature

Temperature is a significant parameter affecting microbial systems in several ways including ionization equilibrium, solubility of substrates, substrate removal rate and other constants such as specific growth rate, decay biomass yield, and half-saturation constant. Anaerobic treatment has been reported under a wide range of temperatures including psycrophilic, mesophilic and thermophilic. Anaerobic digestion is carried out in the mesophilic range at temperatures from 25°C to up to 40°C with the optimum at approximately 35°C. Psycrophilic bacteria function between 0-20°C. Thermophilic digestion operates at temperature ranges of 50-65°C. At temperatures between 40^{o}C and 50^{o}C, methane-forming organisms are inhibited. Digester performance decreases significantly somewhere near 42^{o}C, as this represents the transition from mesophilic and thermophilic range (Gerardi, 2003). Because of their slower growth as compared with acidogenic bacteria, methanogenic bacteria are very sensitive to small changes in temperature, which leads to a decrease of the maximum specific growth rate while the half-saturation constant increases. Temperature fluctuations become more important in high loading rates. Thus, a mesophilic digester must be designed to operate at temperatures between 30°C and 35°C for their optimal functioning.

2.2.2.pH

Most methanogenic Archaea function in a pH range between 6.7 and 7.4 (Table 2.3), but optimally at pH between 7.0-7.2. It is reported that pH below 6.0 are inhibitory to methanogenic *Archaea* whereas acid forming bacteria can live at this pH and continue to produce volatile fatty acids despite low pH, therefore aggravating the environmental conditions further (Pohland, 1987; Malina et al., 1992). Under normal conditions, this pH reduction is buffered by the bicarbonate that is produced by methanogens. However, under adverse environmental conditions, instability is observed due to the destruction of the bicarbonate buffer system under the excess production and accumulation of organic fatty acids eventually stopping the production of methane. An increase in volatile acid levels thus serves as an early indicator of system upset. Monitoring the ratio of total volatile acids (as acetic acid) to total alkalinity (as calcium carbonate) has been suggested to ensure that it remains below 0.1.

Table 2.3. Optimum pH for some methanogenic *Archaea* (Gerardi, 2003)

Genus	Optimum pH Range
Methanothermus	6.5
Methanogenium	7.0
Methanolacinia	6.6-7.2
Methanomicrobium	6.1-6.9
Methanospirillium	7.0-7.5
Methanococcoides	7.0-7.5
Methanohalobium	6.5-7.5
Methanolobus	6.5-6.8
Methanothrix	7.1-7.2

2.2.3. Nutrients

Methanogens need trace amounts of elements called as micronutrients besides nitrogen and phosphorus for their fundamental requirements of bacterial metabolism

(Speece et al., 1983). The most significant micronutrients considered as necessary for various conditions of active methanogenesis are iron, nickel, magnesium, calcium, sodium, barium, tungstate, molybdate, selenium and cobalt (Henze et al., 1983). Some of the elements such as selenium, tungsten and nickel are important in the enzyme systems of acetogenic and methanogenic bacteria (Stronach et al., 1986).

2.2.4. Mixing

Mixing ensures the absolute contact between the reactor contents and the biomass. It is also minimizes the inhibitory effects of local build-up of VFAs and other digestion products. Another advantage of mixing is that it avoids settling which could lead to reduction of substrate and microorganism contact. Mixing could be energy consuming process but it is applied most of treatment systems.

2.2.5. Retention Time

The hydraulic retention time (HRT), which depends on wastewater characteristics and environmental conditions, must be long enough to allow sustaining anaerobic bacteria in digesters.

2.2.6.Toxicants

A wide range of toxicants is responsible for the occasional failure of anaerobic digesters. Inhibition of methanogenesis is generally indicated by reduced methane production and increased concentration of volatile acids. Methanogenesis is generally the most sensitive step to inhibitory or toxic material although all groups involved in process can be affected. Bacteria are affected by increasing undesirable environmental conditions. However; methanogens can be acclimatized to these compounds (Speece and Parkin, 1983). Increasing the VFA concentrations and carbon dioxide concentrations cause decreasing the pH, gas production and methane content.

2.2.6.1. Sulfide Inhibition. Introduction of waste streams and/or biological production in the anaerobic digestion may cause the sulfides via reduction of sulfates or other sulphure-contaioning inorganic compounds. Anderson et al., (1986) found that sulphate in the influent of an anaerobic digester could inhibit methanogenesis due to both the competition for acetate and hydrogen by SRBs and the production of sulfide from sulphate reduction by SRBs. While soluble sulfide concentrations between 50 and 100 mg/L can be tolerated in anaerobic treatment with slightly or no acclimation, higher than 200 mg/L soluble sulfides does not show a significant inhibitory effect after some acclimation. Stronach et al., (1986) stated that sulphate concentrations in excess of 200 mg/L had a direct toxic effect on anaerobic systems.

2.2.6.2. Ammonia-Nitrogen Inhibition. Although ammonia is an important buffer in an anaerobic treatment high concentrations of ammonia may cause failure in the system. Ammonia can be present in the form of ammonium ion (NH_4^+) or dissolved ammonium gas (NH_3). Although these forms are in equilibrium with each others at constant pH, at high pH levels the equilibrium shifts the ammonia gas. Ammonia nitrogen concentrations up to 1000 mg/L have no adverse effect on methanogens, whereas in the rage of 1500 and 3000 mg/L may have inhibitory effect on methanogens at higher pH values.

2.2.6.3. Volatile Fatty Acids (VFA) Inhibition. Anaerobic reactor effluents contains, low concentrations of higher fatty acids however it contains higher concentrations of mainly acetic acid, propionic and butyric acids. Studies show that two important fermentation types occur complementary to each other. These two types of fermentations are butyric and propionic acid. During butyric acid fermentation butyrate, acetate, hydrogen and CO_2 are produced, while propionic acid type fermentation produces propionate, acetate and some valerate, with no significant gas production (Dinopolou et al., 1988). The most common inhibition that inhibits the system in the anaerobic reactor is the accumulation of VFA produced by acidogenic bacterial culture. Inhibition is identified by its high accumulation of VFA is the system which is an indicator of failure of methanogenic population. This failure might be caused by negative impact of bad environment conditions namely shock loading, nutrient depletion or infiltration of inhibitory substances. High concentrations of

VFA (i.e.; butyric and propionic acid) in a system is making toxic impact on the microorganisms in the reactor. It is reported that (Ionnati and Fisher, 1983) inhibition of microbial growth was observed at 35 mg/L acetic acid and excess of 3000 gm/l propionic acid concentrations. The same researchers indicated that butyrate has a toxic effect at 1000 mg/L concentrations minimum. The inhibition of VFA at acidic medium can be attributed to the existence of unionized VFA in significant quantities in the system. When the pH value drops, the equilibrium go to the left causing the increasing of unionized VFAs.

2.2.6.4. Heavy Metal Inhibition. Heavy metal may cause toxic effect on anaerobic processes which are influenced by the oxidation–reduction potential, pH and ionic strength and the resultant speciation of the metals or metal complexes. Although heavy metal ions inhibit metabolisms of the organisms and kill them by inactivating their certain enzymes, existence of the heavy metals in trace amounts is essential for the bacterial activity.

2.2.6.5. Organic Chemicals. Many organic chemicals that are a source of food for anaerobic microorganisms at low concentrations can show inhibitory effects at higher concentrations. Organic chemicals which are poorly soluble in water or adsorbed to the surfaces of sludge solids may accumulate to high levels in anaerobic digesters. The parameters that affect the toxicity of organic compounds include toxicant concentration, biomass concentration, toxicant exposure time, cell age, feeding pattern, acclimation, and temperature (Yang and Speece, 1986). The inhibition concentration ranges vary widely for specific toxicants. Blum and Speece (1991) conducted a comparative analysis of the toxicity of a large number of organic compounds to unacclimated mixed cultures. Since the cultures were not acclimated, meaning they are not given time to adapt to inhibition, the compounds probably were not degraded following addition. Table 2.4 summarizes concentrations of organic compounds that reduce gas production by 50% (IC_{50}) with nonacclimated acetate-utilizing methanogens.

Table 2.4. Concentrations of organic compounds that reduce gas production by 50% with nonacclimated acetate-utilizing methanogens (Blum and Speece,1991)

Toxicant	mg/L	Toxicant	mg/L	Toxicant	mg/L	Toxicant	mg/L
Hydrocarbons		1-Pentanol	4700	**Halogenated Alkanes**		Trichloroethane	13
Alkanes		1-Hexanol	1500	Chloromethane	50	Tetrachloroethane	22
Cyclohexane	150	1-Octanol	370	Methylene Chloride	7	1,3-Dichloropropene	0.6
Octane	2	1-Decanol	41	Chloroform	1	5-Chloro-1-pentyne	44
Decane	0.35	1-Dodecanol	22	Carbon tetrachloride	6	**Halogenated Aromatics**	
Undecane	0.61	**Ketones**		1-1-Dichloroethane	6	Chlorobenzene	270
Dodecane	0.23	Acetone	50000	1,2-Dichloroethane	25	1,2-Dichlorobenzene	150
Pentadecane	0.09	2-Butanone	28000	1,1,1-Trichloroethane	0.5	1,3-Dichlorobenzene	260
Heptadecane	0.03	2-Hexanone	6100	1,1,2-Trichloroethane	1	1,4-Dichlorobenzene	86
Nonadecane	0.01	**Miscellaneous**		1,1,1,2-Tetrachloroethane	2	1,2,3-Trichlorobenzene	24
Aromatics		Cateschol	1400	1,1,2,2-Tetrachloroethane	4	1,2,3,4-Tetrachlorobenzene	20
Benzene	1200	Resorcinol	1600	Pentachloroethane	11	2-Chlorotoluene	53
Toluene	580	Hydroquinone	2800	Hexachloroethane	22	2-Chloro-p-xylene	89
Xylene	250	2-Aminophenol	6	1-Chloropropane	60	2-Chlorophenol	160
Ethylbenzene	160	Isopropylether	4200	2-Chloropropane	620	3-Chlorophenol	230
Phenols		Ethylacrylate	130	1,2-Dichloropropane	180	4-Chlorophenol	270
Phenol	2100	Butylacrylate	150	1,2,3-Trichloropropane	0.6	2,3-Dichlorophneol	58
o-Cresol	890	Acetonitrile	28000	1-Chlorobutane	110	3,5-Dichlorophenol	14
p-Cresol	91	Acrylonitrile	90	1-Chloropentane	150	2,3,4-Trichlorophenol	8
2,3-Dimethylphenol	71	Carbon disulfide	340	Bromomethane	4	2,3,5,6-Tetrachlorophenol	0.1
4-Ethyphenol	240	2-Aminosulfide	6	Bromodichloromethane	2	Pentachlorophenol	0.04
Alcohols		4-Aminophenol	25	1,1,2-Trichlorotrifluoroethane	4	2,2-Dichloroethanol	18
Methanol	22000	2-Nitrophenol	12	**Halogenated Alkanes**		2,2,2-Trichloroethanol	0.3
Ethanol	43000	3-Nitrophenol	18	1,1-Dichloroethane	8	3-Chloro-1,2-propanediol	630
1-Propanol	34000	4-Nitrophenol	4	1,2-Dichloroethane	19	2-Chloropropionic Acid	0.01
1-Butanol	11000	2,4-Dinitrophenol	0.01	t-1,2-Dichloroethane	48	Trichloroacetic Acid	‹0.001

2.3. Treatment of Organic Solvents in Anaerobic Processes

Anaerobic processes have been widely used over the past decades for the treatment of especially high-strength industrial wastewaters and municipal wastewater at mesophilic temperatures due to several advantages of the processes over aerobic ones. Anaerobic treatment systems have been applied for complex wastewaters (Lettinga et al., 1997), to low-strength wastewaters (Kato et al., 1997), and to wastes and wastewaters under low and high temperature conditions (Rebac et al., 1995; van Lier, 1996). In recent years, the processes have also been reported to be an option for treatment of wastewaters containing organic solvents such as chemical synthesis-based pharmaceutical (Enright et al., 2005; Mohan et al., 2001; Henry et al., 1996; Terzis, 1994). These complex wastes can present difficulties for biological treatment systems due to temporal changes in manufacturing processes that result in heterogeneous wastewater composition. Although the wastewaters may contain diverse refractory organic materials, biological treatment is still a viable choice for treatment due to cost and technical advantages (Oz et al., 2003; Enright et al., 2005; Myabhate et al., 1988). The most commonly used solvents are methanol, ethanol, acetone, and isopropanol. Benzene, chloroform, methylene chloride, toluene, methanol, ethylene glycol, methyl isobuthyl ketone, xylene are also used as solvents (EPA, 1997). Organic solvents—though common in these wastewater types—may have toxic effect on biological treatment systems (Inanc et al., 2002). Anaerobic systems used for wastewaters containing organic solvents include membrane reactors (Livingston, 1994), continuously-stirred tank reactors (Terzis, 1994; Oz et al., 2003), upflow (Seif et al., 1992; Ince et al., 2002), hybrid reactors (Oktem et al., 2008) and fluidised bed reactors (Stronach, 1987). Henry et al.,(1996) investigated the performance of anaerobic hybrid reactors treating an organic solvent-containing synthetic wastewater-namely tert-butanol, isopropanol, isobutanol, sec-butanol and ethyl acetate- evaluated under various wastewater volumetric loading rates and influent compositional changes. Enright et al., 2005 studied low-temperature or psychrophilic anaerobic digestion of pharmaceutical-like, solvent-contaminated wastewater to assess the feasibility of the method for this wastewater category to find out toxicity thresholds of key trophic groups within candidate biomass samples assessed against solvents (propanol, methanol and acetone) prevalent in the wastewaters. There are also some studies investigating the effects of toluene and ethyl acetate on pure or binary cultures (Alagappan and Cowan, 2003; Alagappan and Cowan,

2001; Rogers et al., 2000; Ficker et al., 1999). Chloroform (CF) has been reported as an important hazardous contaminant in groundwater impacting drinking water supplies (Williams et al., 2002). In a study, batch serum bottle assays were conducted to determine the effect of exposure to and biotransformation of chloroform (CF) on unacclimated, acetoclastic, methanogenic bacteria and has been found that methanogenesis was negatively impacted both during and following exposure of methanogens to CF (Weather and Parkin, 2000). Yang and Speece (1986) studied the effect of chloroform on mixed, methanogenic cultures enriched on acetate and reported that the time to recover from exposure to CF increased with the duration of chloroform exposure. Chloroform can be degraded anaerobically to CO_2 and dichloromethane by methanogenic enrichment cultures and pure methanogenic cultures (Mikesell and Boyd, 1990) and also by non-methanogenic anaerobic cultures (Egli et al., 1990). Chloroform degradation in methanogenic cultures has been stated to be stimulated by methanol addition (Mikesell and Boyd, 1990), chloroform remains extremely inhibitory to methanogenesis (Yang and Speece, 1986). Bhattacharya and Parkin (1988) studied fate and kinetic effects of slug and continuous additions of formaldehyde and methylene chloride to acetate and propionate enrichment systems in anaerobic chemostats and reported that much higher concentrations of formaldehyde and methylene chloride could be tolerated when added continuously and both toxicants affected the acetate-utilizing methanogens more than the propionate-utilizers under similar conditions. Enright et al., (2005) investigated IC_{50} values for methanol, propanol and acetate in different anaerobic sludge types, including granular and non-granular sludges from different industries. LC_{50} concentrations have been found to be 950 mM for methanol, 1350 mM for a sludge treating alcohol distillery wastewater and 400 mM for a non-granular sludge from a cattle manure.

The implementation of anaerobic technology to waste streams containing organic solvents is still under investigation stage, mainly because of the lack of knowledge on effect of specific wastewater constituents on anaerobic biological sludge activity and microbial composition. Efficiency of treatment systems can be limited by the potential toxicity of some of the chemicals/organic solvents present in wastewater streams, which may not be readily metabolized by the microbial population in the bioreactors. There are many studies about degradation of organic solvents in anaerobic processes in the literature; however, there is a lack of data regarding inhibition studies. The point is that organic

solvent concentrations in wastewaters are generally above the limit dose that can be degraded by microorganisms and that causes serious problems in the systems. Therefore, evaluating inhibitory effects of organic solvents in these systems keeps an important place in terms of better reactor performance. In the scope of this thesis, effects of selected organic solvents including methanol, toluene and isopropanol on anaerobic systems have been investigated.

2.3.1. Methanol Degradation in Anaerobic Processes

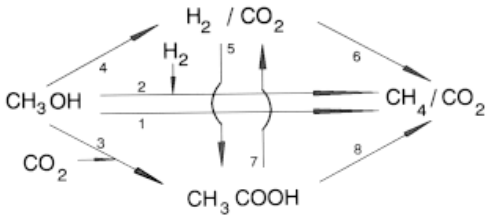
Methanol is one of the main pollutants in several industrial waste streams including pharmaceutical wastewaters, evaporator condensates from the pulp and paper industry, coal-gasification plants, potato-starch producing factories, landfill leachates and as a constituent of wastewater generated in the petrochemical industry (Berube and Hall, 2000; Minami et al., 1991; Paulo et al., 2001; Yamaguchi et al., 2001). Methanol is also an important intermediate during anaerobic mineralization of several compounds like formaldehyde (Gonzalez-Gil et al., 1999a) and methyl esters (Liu and Suflita, 1994). Methanol, also known as methyl alcohol, carbinol, wood alcohol, wood naphtha or wood spirits, is a chemical compound with chemical formula CH_3OH. It is the simplest alcohol, and is a light, volatile, colourless, flammable, poisonous liquid with a distinctive odor that is somewhat milder and sweeter than ethanol. At room temperature it is a polar liquid and is used as an antifreeze, solvent, fuel, and as a denaturant for ethyl alcohol. Anaerobic treatment of methanol-containing wastewater is an attractive option for the wastewaters and has been investigated by several researchers using a variety of bioreactor types under anaerobic conditions (Bérubé and Hall, 2000; Minami et al., 1991; Paulo et al., 2001; Yamaguchi et al., 2001; Florencio et al., 1994; Fukuzaki and Nishio, 1997; Oz et al., 2003).

Table 2.5. Basic properties of methanol

Molecular formula	CH_3OH
Molar mass	32.04 g/mol
Density	0.7918 g/cm³, liquid
Melting point	–97 °C (176 K)
Boiling point	64.7 °C (337.8 K)
Solubility in water	fully miscible

Although methanol is a simple C1 compound, methanol degradation route under anaerobic conditions follow a complex way in which methanogens, sulfate reducing bacteria and homoacetogens interact cooperatively or competitively at substrate level (Weijma and Stams, 2001). In mixed cultures methanol potentially supports a complex food chain as shown in Figure 2.4. It has been reported that direct methanogenesis from methanol seems the predominant route under mesophilic conditions both in the absence and the presence of sulfate. Under anaerobic conditions, methanol is converted to methane via several pathways including direct conversion to methane and carbondioxide by methylothrophic methanogens such as *Methanosarcina* species (Nishio et al., 1992), indirect conversion to acetate by acetogens (Van der Meijden et al., 1984) coupled to acetoclastic methanogenesis (Huser et al., 1982) or indirect conversion to H_2 and CO_2 (Cord-Ruwisch et al., 1988) coupled to hydrogenotrophic methanogenesis (Whitman et al., 1982). It can also be oxidised to H_2 and CO_2. In mesophilic methanogenic bioreactors, it has been reported that methanol was mainly consumed by *Methanosarcina* species (Florencio et al., 1994). High-rate methanol conversion to methane has been reported in properly designed and operated bioreactors under mesophilic (30-40°C) conditions (Weijma and Stams, 2001). However, recent research with a thermophilic methanogenic bioreactor suggested that a major part of the methanol was degraded by syntrophic consortia (Paulo et al.,2003; Weijma and Stams, 2001). The degradation route of methanol

and its final fate in an anaerobic environment depend on specific environmental conditions and the history of the anaerobic consortium. However, acetogenesis of methanol to acetate is also an important fate under mesophilic conditions, especially when methylotrophic methanogenesis is disturbed (Florencio et al., 1994; Lettinga et al., 1981). A syntrophic route via the intermediates H_2 and CO_2 followed by hydrogenotrophic methanogenesis does not appear to be an important route during methanol degradation under mesophilic conditions (Florencio et al., 1994; Gonzalez-Gil et al., 1999). By using 13 C-labelled substrates and specific inhibitors it was shown that in an anaerobic methanol-fed thermophilic bioreactor, about 50% of methanol, at a concentration of 37 mM in the anaerobic reactor, was directly converted to methane by methylotrophic methanogens, and about 50% via the intermediates H_2/CO_2 and acetate (Paulo et al., 2001; Paulo et al., 2003).



Reactions	
1. $4\ CH_3OH$	$\rightarrow 3\ CH_4 + HCO_3^- + H^+ + H_2O$
2. $CH_3OH + H_2$	$\rightarrow CH_4 + H_2O$
3. $4\ CH_3OH + 2\ HCO_3^-$	$\rightarrow 3\ CH_3COO^- + H^+ + 4\ H_2O$
4. $CH_3OH + 2\ H_2O$	$\rightarrow 3\ H_2 + HCO_3^- + H^+$
5. $2\ HCO_3^- + 4\ H_2 + H^+$	$\rightarrow CH_3COO^- + 4\ H_2O$
6. $HCO_3^- + 4\ H_2 + H^+$	$\rightarrow CH_4 + 3\ H_2O$
7. $CH_3COO^- + 4\ H_2O$	$\rightarrow 2\ HCO_3^- + 4\ H_2 + H^+$
8. $CH_3COO^- + H_2O$	$\rightarrow CH_4 + HCO_3^-$

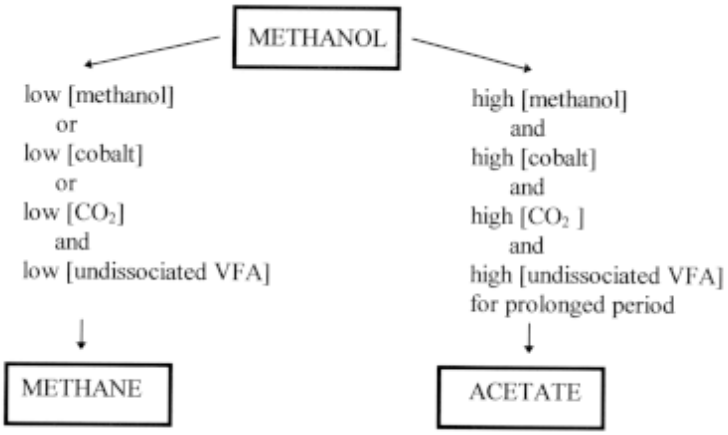


Figure 2.4. Reported reactions involved in the anaerobic degradation of methanol (Florencia et al., 1997).

The important groups that play key roles in the degradation of methanol in the anaerobic reactors are explained in detail as follows:

Methanogens: All methanol-utilizing methanogens isolated from anaerobic digesters are *Methanosarcina* relatives. *Methanosarcina* spp. converts methanol to methyl-coenzyme M by methyltransferases which are enzymes that have a cobalt-containing corrinoid as catalytic group (Vogels et al., 1988). In the presence of hydrogen methyl-coenzyme M is completely converted to methane. However when methanol is the sole substrate, part of the methanol has to be oxidized to CO_2 to provide reducing equivalents for reduction of methanol to methane. This oxidation of the methyl-group likely proceeds via a reversed pathway which methanogens use to reduce CO_2 to methane. In this pathway the methanogenic C1-carrier tetrahydromethanopterin is involved.

Sulfate reducing bacteria: Only a few mesophilic sulfate reducing bacteria (SRB) are reported to grow on methanol, but acetate is always needed as carbon source (Braun and Stolp, 1985; Nanninga and Gottschal, 1986). Growth rates of mesophilic SRB on methanol are very low compared to that of methanogens and acetogens.

Homoacetogens: Methanol is an excellent homoacetogenic substrate. The mesophiles *Acetobacterium woodii*, *Eubacterium limosum*, *Butyribacterium methylotrophicum* and the thermophiles *Moorella thermoautotrophicum* and *M. thermoaceticum* show very fast growth on methanol. For growth of homoacetogens on methanol, bicarbonate must be present as electron acceptor. Bicarbonate is inevitably present when methylotropic methanogens or SRB are also active.

Mixed culture: In mixed cultures methanogens, homoacetogens and SRB compete for methanol. In addition, SRB and methanogens may also compete for hydrogen and acetate, the product of methanol catabolism by homoacetogens.

Table 2.6. Selected methanol-utilizing methanogens, SRB and homoacetogens and some growth kinetic properties and physiological characteristics. (Florencio, 1994; Weijma, 2000; Driessen et al., 2000)

Organism	T_{opt}°C	pH_{opt}	μ_{max} day^{-1}	yield	Ac	H_2/ CO_2	formate
Methanogens							
Methanosarcina acetivorans	35-40		3.2		+	+	-
Methanosarcina barkeri strain MS	30-40	7.0	2.35	3.5a	+	+	-
Methanosarcina mazei	37-40	6.0-7.0	3.24		+	+	-
Thermophilic *Methanosarcina* species	50-58	6.5-7.0			+	+	-
Sulfate reducers							
Desulfovibrio carbinolicus	35a		0.22		-	+	+
Desulfotomaculum kuznetsovii	60-65		0.72		+	+	-
Acetogens							
Acetobacterium woodii	30	7.5		5.3-8.2	-	+	+
Butyribacterium methylotrophicum	39	7.5	1.85	8.2b	-	+	+
Eubacterium limosum	39	7.2	2.38	7.1b	-	+	
Moorella thermoautotrophicum	56-60	5.8	1.8	6-9c	-	+	+
Moorella thermoaceticum	55-60		1.85		-	+	+

[a] cultivation temperature; [b] g dry cell/mol methanol; [c] g dry cell/mol acetate
Topt: optimum growth temperature; pHopt: optimum growth pH; Ac: acetate.

Competition for methanol: Florencio (1994) studied the competition between methanogens and homoacetogens for methanol in mesophilic UASB reactors in detail. The *K*s value of methanogens for methanol is 0.25 mM, while that of the homoacetogens is much higher (16 mM). This shows that at low concentrations methanol is mainly used by methanogens. The opposite is not necessarily true because substantial homoacetogenesis from methanol only occurs when in addition to a high methanol concentration, also sufficient bicarbonate and cobalt is available. The digestion process even may completely collapse when acetate accumulation leads to further reduction of methanogenesis due to toxicity of undissociated acetic acid towards methanogens.

2.3.2. Toluene Degradation in Anaerobic Processes

Toluene which is a relatively water-soluble aromatic hydrocarbon is used as a solvent in the production of paints, thinners, adhesives, inks and many pharmaceutical products. Toluene concentrations in industrial wastewaters have been reported to be approximately between 7–753 mg/L depending on the manufacture type (De Witt, 1999). Several treatment methods including chemical oxidation and combustion, activated carbon

adsorption, and biological stabilization may be used for the conversion of toluene to a non-toxic substance. Aerobic biological treatment methods have been also previously reported. However, partially or substantially volatilization of the compounds to air due to agitation and aeration of the wastewaters limits its use. Therefore, anaerobic digestion can be preferred to aerobic treatment for the treatment of volatile compounds, such as toluene. Losses to the atmosphere due to agitation and aeration of the wastewaters are avoided in anaerobic digestion. Although there are valuable studies investigating the effects of some aromatic hydrocarbons such as benzene, toluene, ethyl acetate on pure or binary cultures, little attempt has been made to assess effects of particular solvents on anaerobic wastewater treatment reactor sludges in terms of qualitative and quantitative measures of methanogenic species and their activities (Meckenstock et al., 2004; Alagappan and Cowan, 2003; Hwang et al., 2003; Alagappan and Cowan, 2001; Rogers et al., 2000). Treatment of toluene-containing waste streams has been studied by using a variety of anaerobic bioreactor types including anaerobic horizontal-flow anaerobic immobilized biomass (Cattony et al., 2005), expanded granular sludge bed reactor (Enright et al., 2007a) and completely stirred tank reactor (CSTR) (Oz et al., 2003).

Table 2.7. Basic properties of toluene

Molecular Formula	C_7H_8 ($C_6H_5CH_3$)
Molecular weight	92.14 g/mol
Boiling point	110.62°C
Vapor pressure	28.5 Torr at 20°C
Solubility in water	0.47 g/L (20-25°C)
Density	0.8669 g/mL (7.234 lb/gal) at 20°C

While much is known about aerobic toluene degradation pathways and the many aerobic species that mineralize toluene, comparatively little is known about anaerobic degradation of toluene. Toluene degradation occurs under all of the major anaerobic electron-accepting conditions, including nitrate-reducing (Fries et al., 1994), sulfate-reducing (Beller et al., 1996), iron(III)-reducing (Lovley et al., 1990), and methanogenic (Edwards and Grbic-Galic, 1994; Ficker et al., 1999) conditions, and pure cultures of

nitrate-reducing, sulfate-reducing, and iron-reducing bacteria that degrade toluene have been isolated. In contrast, toluene degradation to methane and CO_2 requires more than one species because of the limited substrate range of methanogenic bacteria. It is thought that fermentative or acetogenic bacteria first transform toluene to methanogenic precursors, such as acetate and hydrogen; methanogenic bacteria then convert these substrates to methane and CO_2. Since transformation of toluene to acetate and hydrogen is energetically favorable only when the concentrations of hydrogen and acetate are kept low by the activity of methanogenic bacteria, toluene degradation is necessarily dependent on syntrophic relationships between species in a consortium. Anaerobic toluene degradation under methanogenic conditions was first reported in 1986. Several lines of evidence suggest that the activation of toluene via benzylsuccinate synthase is the first step of anaerobic toluene mineralization, and subsequent steps in the mineralization pathway have been proposed based on biochemical and genetic studies (Beller and Sporrmann, 1998).

Research on the anaerobic biodegradation of monoaromatic hydrocarbons, like toluene, and detailed biochemical analysis has been hampered by difficulties associated with studying anaerobic microorganisms such as low growth rates and need for specialized equipment and low substrate concentrations (Edwards et al., 1994). Strict anaerobes found in sulfate-reducing and methanogenic cultures are inhibited by high substrate concentrations (for toluene, typically less than 400 μM), and thus only low amounts of substrate can be used to sustain growth. As a result of these low substrate concentrations, together with the low rates of growth and degradation, and possibly the relatively small amount of energy available from the reaction (Edwards and Grbic-Galic, 1994; Edwards et al., 1992), intermediates in the catabolic pathways do not appear to accumulate and have proven very difficult to detect. Radioactive tracing and isotope trapping are very effective techniques for determining metabolic pathways, especially when the concentrations are very low, because these low concentrations can be overcome by using a radioactive substrate with high specific activity (Edwards et al., 1994). Labeled substrates also provide an indisputable link between the substrate and any labeled products detected. It has been reported that toluene degradation by this methanogenic culture proceeded via methyl hydroxylation to benzyl alcohol, followed by further oxidation steps to benzaldehyde and benzoate, with perhaps a parallel pathway via ring hydroxylation to *p*-cresol (Edwards et al., 1994). The proposed compounds as intermediates in toluene degradation in the study

namely benzoic acid, benzyl alcohol, benzaldehyde, and *p*-cresol, have previously been implicated in anaerobic toluene degradation (Evans et al., 1992; Grbic-Galic and Vogel, 1987; Livley and Lonergan, 1990; Vogel and Grbic-Galic, 1986).

2.3.3. Isopropanol Degradation in Anaerobic Processes

Isopropyl alcohol (IPA) whose production worldwide exceeds 1 million tonnes per annum through its many industrial applications including rubber, cosmetics, textiles, pharmaceuticals, and fine chemicals industries is the most widely used volatile organic compound. Basic properties of isopropanol are given in Table 2.8. The anaerobic degradation of 2-propanol in anoxic paddy soil was studied with soil cultures and a 2-propanol-utilizing methanogen. Acetone has been reported to be the first and the major intermediate involved in the methanogenic degradation of 2-propanol. Analyses with a methanogenesis inhibitor, bacteria antibiotics, and the addition of H_2 to the gas phase revealed that 2-propanol oxidation to acetone directly occurred using 2-propanol-utilizing methanogens, but not with H_2-producing syntrophic bacteria, for which the removal of acetone is required for complete 2-propanol oxidation. The 2-propanol-utilizing strain IIE1,which is phylogenetically closely related to *Methanoculleus palmolei,* was isolated from paddy soil, and the potential role of the strain in 2-propanol degradation was investigated. 2-propanol is one of the representative fermentation intermediates in anaerobic environments. This was the first report on the anaerobic 2-propanol degradation process. 2-propanol is used as a hydrogen donor for methanogenesis in the first step of anaerobic degradation in anoxic paddy soil. It is then mineralized to methane and carbondioxide via acetone followed by acetate production. At least three organisms are concerned in the anaerobic 2-propanol degradation in anoxic paddy soil: 2-propanol-utilizing methanogens, acetone-degrading bacteria, and acetotrophic methanogens. Previous studies have shown that alcohols, such as isopropanol can be oxidised by hydrogenophilic methanogens to acetone during growth on H_2/CO_2 (Widdel, 1986; Widdel et al., 1988). Homoacetogenic bacteria capable of metabolising isopropanol to acetate and higher fatty acids have also been reported (Eichler and Schink, 1984). Co-metabolism with glucose of the compound has been reported in a mesophilic anaerobic study (Fox and Ketha, 1996). However, the studies on effects of the solvents on anaerobic reactors have been limited.

Table 2.8. Basic properties of isopropanol

Molecular Formula	C_3H_8O
Molecular weight	60.10 g/mol
Boiling point	82.3 °C, 355 K, 180 °F
Melting point	−89 °C, 184 K, -128 °F
Solubility in water	miscible

2.4. Anaerobic Bioreactor Configurations

Various anaerobic treatment processes shown in Figure 2.5. are generally classified as low and high rate systems.

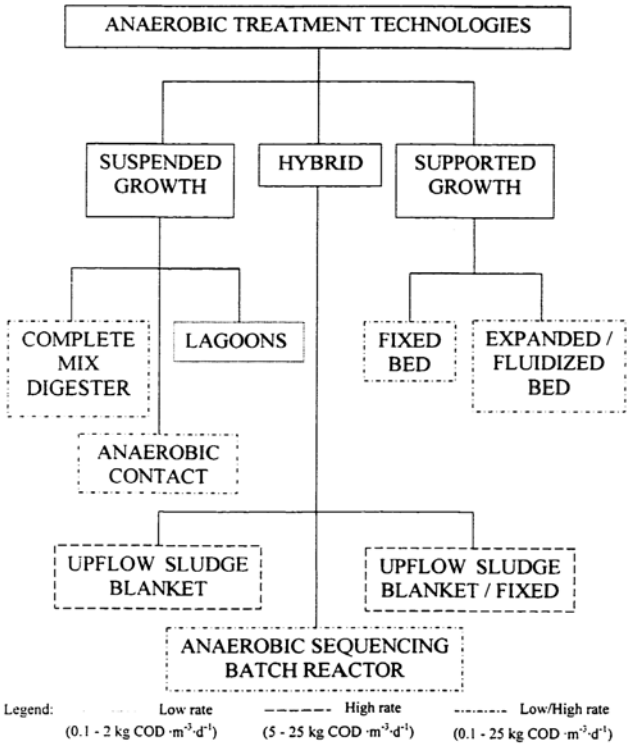


Figure 2.5. Anaerobic treatment process classification (Lalman, 2000)

2.4.1. Suspended Growth Anaerobic Reactor Designs

2.4.1.1. Completely Mixed Digester. Completely mixed digesters are among the earliest configurations of anaerobic digestion and it involves the application of the conventional flow-through tank without any biomass recycle. Waste streams with high concentrations of particulates and very high concentrations of soluble biodegradable organic materials could be applied to these systems. With the chracateristics of the design, the average retention time of anaerobic microorganisms in the reactor (SRT) is almost equal to hydraulic retention time (HRT). Because of the slow growth of methanogens, process stability can be limited by the short SRTs and large reactor volumes are required to maintain necessary SRTs. With the relatively low biomass concentrations and short operating SRTs, loading rates are typically low (1-10 kg COD/m^3/day). Proper mixing conditions provide uniform conditions such as substrate concentrations, temperature and pH throughout the reactor and minimize dead volume accumulation and flow channeling.

2.4.1.2. Anaerobic Contact Processes. Very long hydraulic retention times (HRT) of completely mixed digesters, as it is equal to the solid retention time (SRT) in the system, cause more difficult operation, higher reactor volumes. This disadvantage is overcomed in anaerobic contact process by separating and recycling biomass back to the anaerobic reactor, which is still completely mixed, but includes an additional settling tank and a sludge-recycling unit. In this way, SRT can be controlled independently from the HRT by changing the amount of sludge recycle. Therefore, high treatment efficiency can be achieved by using short HRTs and smaller digesters due to the longer SRTs obtained with sludge recycle. Organic loading rates of 0.5 to 10 kg COD/m^3.day can be applied to the reactor with HRTs of range between 0.5 and 5 days.

2.4.1.3. Upflow Anaerobic Sludge Blanket (UASB) Reactor. The upflow anaerobic sludge blanket reactor (UASB) reactor is characterized by an anaerobic granular sludge with a notably high metabolic activity and good biosolids settleability. The UASB system has found wide application in the treatment of industrial wastewaters, particularly those produced in agriculturally based industries, sugar, potato processing,

slaughterhouse, meat packing, paper mill, food and yeast industries (Lin and Yang, 1991). Also the UASB system has been shown to be a feasible method for treatment of alcohol distillery effluents, COD removal efficiencies in the range of 65%-95% can be achieved depending largely on the kind of raw material used and on the process conditions applied (Driessen et al., 1994). In the UASB process, the waste to be treated is introduced in the bottom of the reactor. The wastewater flows upward through a sludge blanket composed of biologically formed granules. This granular sludge consists of anaerobic microorganisms, which are still visible as granules after settling and is considered as a major form for immobilization of microorganisms. Similar to biofilms, granular sludge provides minimized mass transfer limits, optimal micro-environment and protection for microorganisms such as methanogens and synthrophic bacteria. Treatment occurs as the wastewater comes in contact with the granules. The gases produced under anaerobic conditions (mainly CO_2 and CH_4) cause internal circulation, which helps in the formation and maintanance of the biological granules. Some of the gas produced within the sludge blanket becomes attached to the biological granules. The free gas and the particles with the attached gas rise to the top of the reactor. The particles that rise to the surface strike the bottom of the degassing baffles, which cause the attached gas bubbles to be released. The degassed granules typically drop back to the surface of the sludge blanket. The free gas and gas released from the granules is captured in the gas collection domes located in the top of the reactor. Liquid containing some residual solids and biological granules passes into a settling chamber, where the residual solids are separated from the liquid. The separated solids fall back through the baffle system to the top of the sludge blanket. To keep the sludge blanket in suspension, upflow velocities in the range of 0.6-0.9 m/h have been used (Tchobanoglous and Burton, 1991).

2.4.1.4. Anaerobic sequencing batch reactors (ASBR). The term sequencing batch reactor (SBR) has been defined as a discontinuous wastewater treatment technology, where the volume of the reactor tank is variable in time (Irvine and Ketchum, 1989). The efficieny of SBRs have been demonstrated for nutrient removal (Keller et al., 1997), the control of filamentous bacteria (Wanner, 1992) and the removal of specific organic compounds present in industrial wastewaters (Buitro´n et al., 2001). The SBR can also allow the selection of microorganisms capable of tolerating toxic compounds as well as variable

environments (oxic, anoxic) that favor the nutrient removal. The difference between the ideal batch reactor and the sequencing batch reactor process is that only a predetermined volume of a sequencing batch reactor is emptied at the end of reaction, whereas the entire volume of the ideal batch reactor is emptied at the end of reaction.

A new innovative high-rate methanogenic reactor process called the Anaerobic Sequencing Batch Reactor (ASBR) (U.S. Patent No. 5, 185,079) has been developed by Dague et al., 1992. After several initial studies was carried out by Dague et al.,(1992), the reactors have been widely studied for treatment of different industrial wastewaters as an alternative to continous systems due to their improved retention of biological sludge, process control, absence of primary or secondary settling, high organic matter removal efficiency with smaller reactor volumes (Irvine et al., 1997). A typical cycle in an ASBR is composed of four steps: feed, reaction, settling and liquid withdrawal (Dague et al., 1992). Figure 2.6 shows sequential process of the ASBR.

The optimization of ASBR operation can decrease the overall cycle time. Reaction time depends on the intrinsic kinetics of substrate consumption by the biomass and on the mass transfer rates that must be enhanced by mixing (Bosma et al., 1997). The biomass settling may be the determining step of the cycle time since it is directly related to the formation of self-immobilized biomass as granules with good settleability. Factors affecting performance of ASBRs have been reported as geometric reactor characteristics (Sung and Dague, 1995), agitation (Brito et al., 1997), ratio between substrate and biomass concentrations (Sung and Dague, 1995; Timur and Ozturk, 1999), and initial feeding strategy (Ratusznei et al., 2003). Agitation is one of the most important factors affecting ASBR performance and is used during the reaction step in order to improve the mass transfer rates, increasing the overall organic matter uptake rates. Mixing can be supplied by recirculating the biogas produced during the anaerobic digestion (Sung and Dague, 1995) and liquid recycle or mechanical agitation (Brito et al., 1997). Feeding strategy has also been reported as an important factor in ASBRs. Longer fill cycles has been found to be beneficial, especially for rapidly acidified substrates (Bagley and Brodkorb, 1999). However, the view should be investigated for the tretment of complex wastewaters in the reactors. The variable substrate concentration in the reactor during the sequencing operation results in a variable food-to-microorganism (F/M) ratio in the reactor: high

substrate concentrations during and immediately after feeding (high F/M ratio) and low substrate concentrations at the end of the react step (low F/M ratio). The feast and famine conditions can be considered as an advantage for obtaining high removal efficiency. High F/M ratio ensure high substrate utilization rate providing high driving forces for metabolic activity, in accordance with Monod kinetics (Dague et al., 1998). The low F/M ratio occurring at the end of the react step through the end of the decant step is known to stimulate bioflocculation, granulation, efficient biomass settling (Sung and Dague, 1995) and a long SRT.

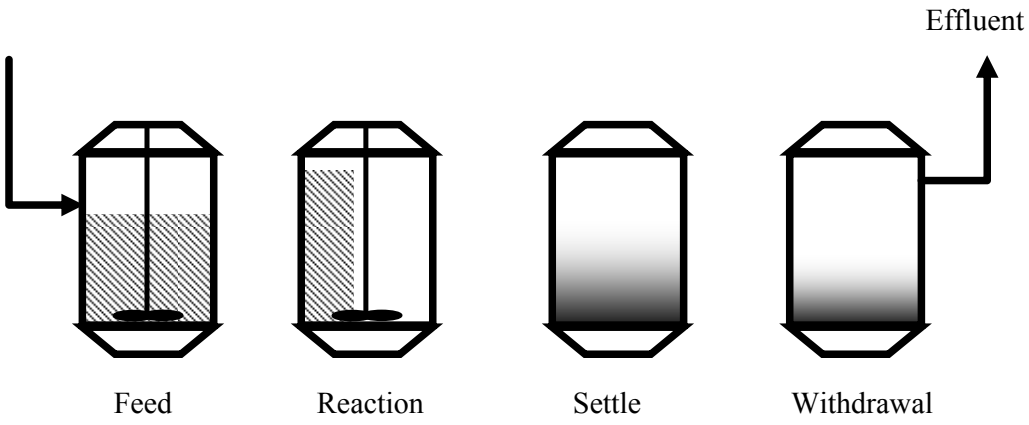


Figure 2.6. Sequential Process of the ASBR

2.4.2. Attached Growth Anaerobic Reactor Designs

2.4.2.1. Fixed Bed Processes. Fixed bed processes contain a flooded bed of inert filter medium which is used for the development of high biomass concentrations required for efficient anaerobic treatment of wastewaters. While wastewater is passing through the medium, soluble organic compounds in the feed diffuse in surfaces of the attached biomass where the organics are converted to intermediate and final products namely methane and carbondioxide. Fixed bed processes can be used for almost all types of industrial wastewaters with low (COD<1000 mg/L) to intermediate (COD>20000 mg/L) concentrations.

2.4.2.2. Anaerobic Expanded/Fluidized Bed Processes. An expanded granular sludge bed (EGSB) reactor is a variant of the UASB concept (Kato et al., 1994). The distinguishing feature is that a faster rate of upward-flow velocity is designed for the wastewater passing through the sludge bed. The use of effluent recirculation in a UASB (or a high height/diameter ratio) results in the EGSB reactor (Seghezzo et al., 1998). The higher upflow liquid velocity keeps the granular sludge bed in an expanded condition (Zoutberg and Frankin, 1996).The increased flux permits partial expansion (fluidization) of the granular sludge bed, improving wastewater-sludge contact as well as enhancing segregation of small inactive suspended particle from the sludge bed. The increased flow velocity is either accomplished by utilizing tall reactors, or by incorporating an effluent recycle (or both). The EGSB design is appropriate for low strength soluble wastewaters (less than 1 to 2 g soluble COD/L) or for wastewaters that contain inert or poorly biodegradable suspended particles which should not be allowed to accumulate in the sludge bed.

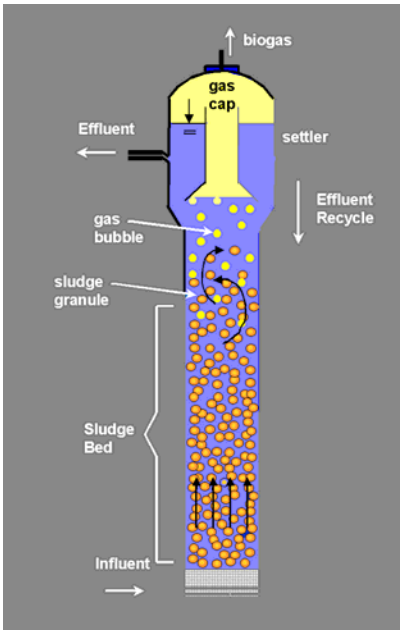


Figure 2.7. EGSB Reactor Configuration

In fluidized bed systems, the biomass is attached to the surface of small particles having low specific gravity particles that are kept in suspension by the upward velocity of the flow of the feed and recycle. The particles which are generally in 0.45-0.7 mm

diameter and made of materials such as porous alumina, high-density plastic beads and quartzite sand provide a very large specific surface for biological growth as a thin film. Therefore, high biomass concentrations that are not subject to diffusional limitations can be developed on the surface of the particles. Biomass retains longer in the reactor because particles increase the settling velocity of the attached biofilm.

2.4.3. Hybrid Anaerobic Reactor Designs

Hybrid systems have simple design and require no special gas or sludge separation device. Hybrid upflow anaerobic sludge blanket (UASB) reactor is among the contemporary design which combines the advantages of UASB and anaerobic filter (AF) concepts together. While UASB reactors are limited by the settling properties of the granular sludge, anaerobic filters are restricted with channeling and plugging due to the accumulation of suspended biomass in the bottom.

2.4.4. Two-Phase Anaerobic Digestion Concept

Anaerobic biodegradation of organic matter was carried out by different groups of microorganisms which can show variations with respect to physiology, nutritional requirements, growth, metabolic characteristics and sensitivity to environmental conditions (Ince et al., 1997). Since the typical design practice keeps these different groups of microorganisms together within the same chemical and physical environment, the process can easily be adversely affected in case of imbalance in the activities of the acid and methane formers. In two-phase systems, the acid and methane fermentation phases are separated by employing two reactor systems in series. Therefore, environmental conditions can be optimized for each microbial community- acid bacteria and methane-forming Archaea. Organic loading and recycle requirements could be controlled individually to enhance overall process efficiency in the systems. Proper selection of the hydraulic retention time (HRT) in the acid reactor tends to preclude the development of significant populations of methanogens, encouraging volatile fatty acid production. Higher loading and shorter HRT favor enrichment of the acid formers, and preclude the establishment of the methane producers (Ghosh et al., 1985). Effluent of the acid reactor then fed to

methane reactor, where the HRT and recycle may be adjusted to provide for optimum growth of the methanogens. The advantages of the two-phase anaerobic digestion process can be outlined as increased stability, optimization of the reactors, increased overall COD and VS reduction efficiencies, higher organic loading rates and increased specific activity of methanogens leading to an increase in methane production (Bhattacharya et al., 1996). Two-phase high-rate systems achieved a higher specific COD degradation capacity than that of single-phase systems, due to increased acetogenic and.methanogenic activity of the sludge. Besides, two-phase operation reduces fluctuations in wastewater strength and composition, which are not desired for reactor start-up. Despite these systems may establish proper conditions for the phased microorganisms, it also requires more extensive care and proper operating conditions in each reactor in order provide the contunity in the preceding reactions. Bhattacharya et al., (1996) also summarized the speculated disadvantages of phase separation. These disadvantages included hydrogen build-up in the acid-phase reactor during the acid formation to levels inhibitory to acid forming bacteria and elimination of possible interdependent nutritional requirements of both acid and methane formers. Comparison of conventional single-phase process and two-phase process are outlined in Table 2.9 (Weiland and Rozzi, 1991).

Table 2.9. Comparison of single-phase and two-phase systems (Weiland and Rozzi, 1991).

Single-phase	Two-phase
Advantages	
Low investment costs	Faster start-up of high-rate systems
Simple operation and control	Better SS conversion
Increased process stability	Enhanced process efficiency
Disadvantages	
Longer start-up period	Higher investment costs
Low process stability (souring)	Eventually need of pH control
Sensitive to load fluctuations	
Non-optimized microbial conditions	

2.5. Methanogenic and Non-Methanogenic Activity and Importance in Anaerobic Biotechnology

Activity can be defined as the specific rate of substrate consumption and indicates the substrate-dependent methane production rate per unit mass of volatile solid biomass. Activity measurements of an anaerobic sludge can be considered in two different ways; an overall measurement, which gives information about the whole degradative activity, and an activity measurement of each basic stage of the process. Overall measurement of methanogenic activity allows the selection of an anaerobic sludge as inoculum while an individual activity provide information on potential unbalanced situations among the different microbial species and relative significance of the steps of the anaerobic processes (Soto et al., 1993). Gas production is mostly preferred parameter for monitoring the activity of the sludge as a whole, especially when the test material is complex and difficult to accurately measure (Rozzi and Remigi, 2004). Measurement of the depletion of the concentration of the test substrate and the accumulation of some intermediates such as short chain organic acids, the formation of hydrogen, etc. can also be employed in parallel to gas measurements.

Determination of the toxic/inhibitory effect of a substance or wastewater on an anaerobic sludge activity is another application of the tests. N-substituted aromatics which are important priority pollutants entering the environment primarily through anthropogenic activities associated with the industrial production of dyes, pesticides, and pharmaceuticals have been examined in terms of the structure-toxicity relationships and effect on acetoclastic methanogenic activity in a study and the nature and the degree of the aromatic substitution were observed to have a profound effect on the toxicity of the test compound (Donlon et al., 1995).

2.5.1. Methanogenic Activity

Knowledge about microbial diversity and activity of the seed biomass is needed for a successful start-up, since, as a general application, seed biomass is taken from another anaerobic reactor which is not adapted to the new wastewater. Furthermore, the performance of an anaerobic treatment system for a wide range of wastewaters depends on

presence of a sufficient quantity of active methanogens in the anaerobic reactors (Ince et al., 1994; 1995; Morgan, 1991). Methanogenic activity are characterized by slow growth rates compared with acidogens and great sensitivity to inhibitor compounds and, therefore, methanogenesis is defined as a rate-limiting step in the whole anaerobic digestion process due to the slow growth rate of the methanogens. (Malina et al., 1992; Noike et al., 1985; Speece, 1983). Table 2.10 summarizes activity tests carried out using different substrates. Formerly, activity measurements have been proposed as a tool to monitor the microbial composition of methanogenic environments using H_2, formate, acetate and propionate as test substrates (Dolfing and Bloemen, 1985). Especially, acetoclastic methanogenic activity tests have been frequently used in evaluating of a biomass in terms of activity since a high proportion of methane is produced through acetoclastic pathway. Approximately two-thirds or more of methane formed during anaerobic degradation of complex substrate results from acetate in anaerobic digesters (Gujer and Zehnder, 1983; Fukuzaki et al., 1990; Zinder, 1993) and, therefore, understanding the factors affecting the methanogenic activity of acetoclastic methanogens is important. Since the stability of the anaerobic reactors is dependent on the step, acetoclastic methanogenic activity is of great importance in anaerobic bioreactors when both starting-up and operating anaerobic reactors. Specific Methanogenic Activity (SMA) tests can be used as a control parameter and a means of determining the optimum operating conditions and potential loading capacity of anaerobic reactors (Ince et al., 1995). It can also be used to characterize biomass prior to its use as an inoculum for new anaerobic reactors, to detect changes in biomass activity during operation, or to assess the occurrence of toxic conditions.

At present, the methane production rate measured in anaerobic digesters is taken as an indication of the biological potential of the methanogenic bacteria present in the sludges and defined as actual methane production (AMP) capacity. Although the AMP indicates the methanogenic activity under the prevailing operating conditions, it would be important to establish a measure of the potential methanogenic capacity (maximum) displayed under ideal operating conditions. Maintaining an AMP/PMP ratio of 0.6-0.7 have been reported to be important to achieve high operating stability and COD removal efficiency during start-up and steady-state operations (Ince et al., 1994; 1995). Comparisons of maximum and actual methanogenic capacities could then be used as control and optimization of digester performance effectively (Ince et al., 1995).

Table 2.10. Activity tests carried out using different substrates

Substrate	**SMA**	**Wastewater/Reactor Type**	**Reference**
Acetate	440 $mLCH_4$/gTVS/d	Glucose/Lab-scale CSTR	Oz et al., 2004
Acetate	389 $mLCH_4$/gVSS/d	Alcohol Distillery/UASB	Dogan et al., 2005
Acetate (200 mgCOD)	1,6 gCH_4COD/gVSS/d	VFA mixture/UASB	Biing-Teo Wong et al., 2007
Butyrate	1.57 gCOD/gVSS/d		Fang et al., 1995
Butyrate	400 $mLCH_4$/gVSS/d		Ianotti and Fischer, 1983
Butyrate (2000 mg/L)	385 mL CH_4/gVSS/d (1.01 gCH_4COD/gVSS/d)		Sun-Kee Han et al., 2004
Propionate (2000 mg/L)	225 $mLCH_4$/gVSS/d		Sun-Kee Han et al., 2004
Propionate (200 mg COD)	0,5 gCH_4COD/gVSS/d	VFA mixture /UASB	Biing-Teo Wong et al., 2007
VFA mixture	1.01 gCH_4COD/gVSS/d	Brewery / EGSB	Simsek, 2007
VFA mixture	1.03 g CH_4COD/gVSS/d		Sun-Kee Han et al., 2004
VFA mixture (32:28:40 acetate/ propionate/butyrate in a COD ratio)	2.3 g CH_4COD/VSS/d	VFA mixture/UASB	Biing-Teo Wong et al., 2007

2.5.2. Non-methanogenic Activity

In conventional anaerobic treatment systems, all phases such as hydrolysis, acidogenesis and methanogenesis take place in one reactor. Some studies have been carried out to investigate the possible adverse effects of highly persistent organics and heavy metals in methanogenic activity, but little study has been reported on effect of the compounds on non-methanogenic activity. In anaerobic digestion processes, hydrolysis phase can be considered as limiting step where a substantial portion of the waste stream contains complex organic compounds. If the test material is particulate, the hydrolytic step is generally the rate limiting one (Sanders, 2001). The rate of hydrolysis is also a function

of pH, temperature, concentration of hydrolysing biomass and type of particulate organic matter (Pavlostathis and Giraldo-Gomez, 1991). The complex wastes must firstly be degraded or hydrolyzed into units as a first step to be taken up by the microbial cell and produce simpler substrates for the succeeding steps in the degradation sequence (Stronach et al., 1986). Therefore, the hydrolytic bacteria and their enzymes are of paramount importance for whole anaerobic degradation process. Therefore, determination of hydrolytic activity of an anaerobic sludge has been stated to be important in terms of selection of the most suitable equipment or control of the process conditions. Hydrolysis may also be monitored by measuring the individual components, i.e. proteins, carbohydrates and lipids.

Acidogenic step is not considered as limiting one since acidogens are less sensitive to toxicant concentrations than methanogens. However, determination of acidogenic activity provides information on biomass development and dynamic behaviour of anaerobic processes. (Soto et al., 1992). For this purpose, glucose which is considered as the main intermediate in the pathway of anaerobic digestion of carbonhydrate complex organics is generally used. The specific acidogenic activity of mixed cultures from an anaerobic reactor have been reported to be usually higher than the 50% of those of pure cultures (Soto et al., 1993).

2.6. Characterization of Microbial Communities using Molecular Tools

Microbial community responsible for the biochemical conversions in the environment is often considered as a black box in the past due to the limitations of available methods for characterisation of microorganisms by pure culture (Amann et al., 1995). Pure culture studies are necessary to obtain an insight into the physiology, biochemistry and genetics of isolated microorganisms. However, it is a well-known fact that only a small fraction of all microorganisms in nature can be isolated with culture-dependent techniques (Giovannoni et al., 1990). An additional problem in microbial ecology studies is the difficulty of species identification and classification based on morphological features which does not reflect evolulutionary relationship between microorganisms. In order to determine the role of microbial diversity in natural or engineered systems, the questions on microbial population including ‘Who is there? How

many of them? Where are they located? What are they doing? How do populations respond to changing environmental conditions? What is the relationship between diversity and community stability?' has to be answered. Therefore, methods that provided more reliable information on microbial diversity and function were needed, which did not involve the cultivation of microorganisms (Muyzer et al., 1996; Head et al., 1998). The advent of molecular techniques to analyze nucleic acids (DNA and RNA), increased our understanding of the composition, dynamics and interactions within microbial ecosystems at a genetic level. Molecular phylogeny which employs nucleic acid sequences to document the history of evolution has provided a new basis for the direct identification and quantification of microorganisms (Olsen and Woese, 1993). Nucleic acids are the ultimate biomarkers and hereditary molecules probably because of their essential role in protein synthesis, making them one of the earliest evolutionary functions in all cellular life-forms (Woese, 1987). Microorganisms can be detected, identified and enumareted by the analysis of genes.

In particular, the 16S rRNAs, and the genes that encode them, are ideal biomarkers since (i) they are present in all living organisms except viruses, (ii) they have conserved as well as variable sequence regions (Figure 2.8.) produced from, on the whole, "selectively neutral" mutational changes, making rRNA's molecular chronometers or evolutionary clocks (Woese, 1987, Ludwig and Schleifer, 1994) and differential sequence variations in the rRNAs, allow phylogenetic determinations at almost any taxonomic level (from domain to species or sub-species (Amann et al., 1995; Head et al., 1998). Therefore it is possible to design general and specific primers and probes, (iii) they are fairly large molecule (≈1500 nucleotides) including sufficient sequence information for the study of evolution to a reasonably high resolution (species level) (Woese, 1987), (ıv) they are very abundant in most cells (10^3 to 10^5 copies) which facilitates detection (Amann et al., 1995). The rRNA is highly conserved in nucleotide sequence as well as in secondary structure since its function remains same through years of evolution. It has many variable regions in which random changes occur time to time. These changes reflect evolutionary relationships of the organisms. Conserved regions functions as binding places for PCR primers or hybridization probes. Even data from this analysis is sufficient to compare statistically significant phylogenetic relations (Olsen et al., 1986). Among the variable regions, V3 region is mostly used in molecular analysis (Neefs et al., 1990; Øvreas et al., 1997). Due to

the reasons listed above, now, it has become common to investigate community diversity using the rRNA gene (rDNA) or the rRNA itself. There is no doubt that the analysis of rRNA gene revolutionized microbial ecology and expanded our knowledge of microbial phylogeny. The rapidly growing rDNA sequence data bank, accessible via the internet (www.ncbi.nlm.nih.gov/entrez) makes it possible to compare sequences from across the world (Dahllöf, 2002). 16S rRNA studies carried out for bacterial evolution created a phylogenetic framework for the identification of microorganisms. This rRNA tree of life consists of 3 domains including *Bacteria*, *Archaea* and *Eukarya* (Figure 2.9).

Although it is obvious that the phylogenetic properties of 16S, as well as the large amount of sequences available offer a considerable advantage, there are also disadvantages (Dahllöf, 2002). For example, the heterogeneity of 16S between multiple copies within one species hampers pattern analysis, and confuses the interpretation of diversity from clone libraries and sequences retrieved from banding patterns (Dahllöf, 2002). The extent of 16S heterogeneity does vary between different regions, but so does resolution (Petri and Imhoff, 2001).

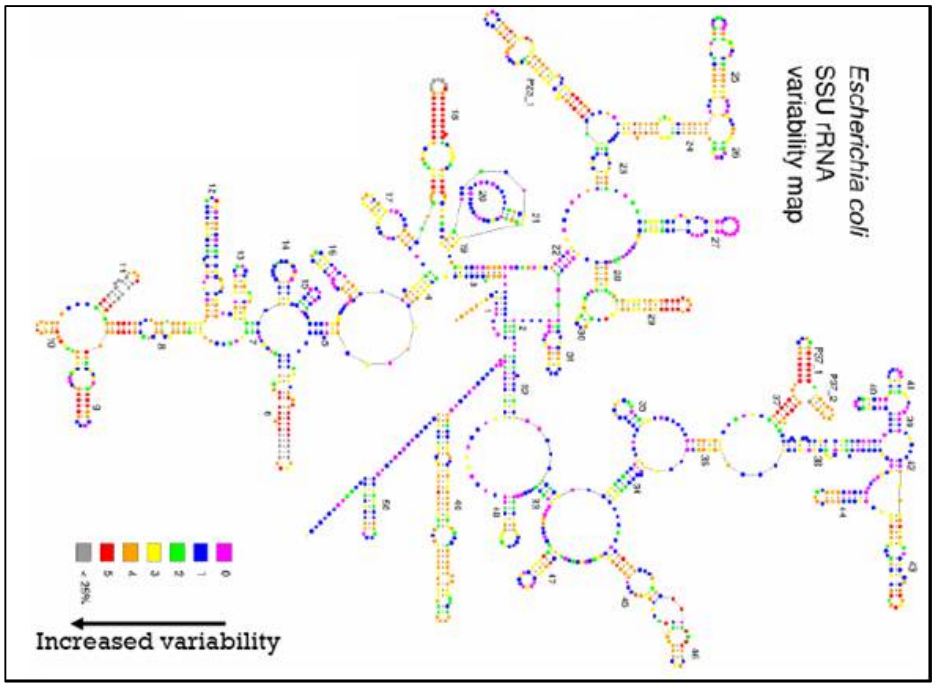


Figure 2.8. Secondary structure of the 16S rRNA of *E. coli*, showing conserved and variable regions (Van de Peer et al., 1996)

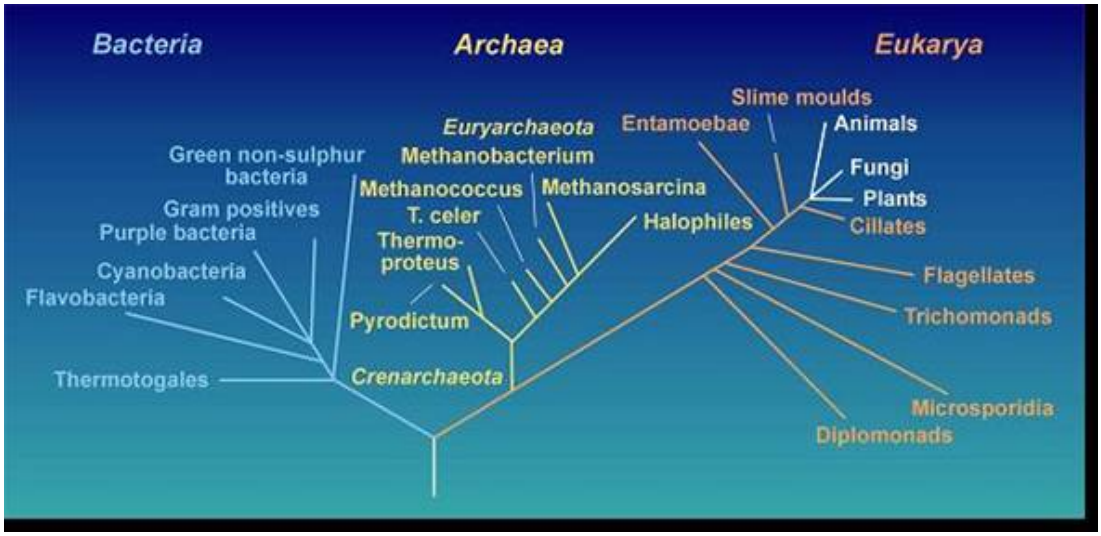


Figure 2.9. The rRNA tree of life (Madigan et al., 2002).

2.6.1. Commonly Used Molecular Tools for the Assessment of Microbial Diversity

In the mid to late 1980's Olsen, Pace and colleagues (1986) used the power of rRNA molecules and their genes for classifying, identifying and quantifying previously uncultured organisms from natural environments by a variety of techniques. This can be recognized as the start of molecular microbial ecology. The application of molecular biological techniques to detect and identify microorganisms by certain molecular markers, such as 16S rRNA or its encoding gene (Olsen et al., 1986; Amann et al., 1995), is now more and more frequently used to explore the microbial diversity and to analyse the structure of microbial communities (Muyzer and Ramsing, 1995). The application of these techniques in microbial ecological studies has even become a discipline on its own, i.e. molecular microbial ecology (Akkermans et al., 1995). Figure 2.10 shows examples of the most commonly used techniques for the analysis of microbial communities and Table 2.11 summarizes the molecular approaches used in microbial ecology. It must be highlighted that in recent years, the study of genes other than rRNA (e.g. functional genes that code for particular enzymes), and other novel techniques have become increasingly appealing, since they enable relationships between structure and function of microbial communities to be elucidated (Gray and Head, 1999).

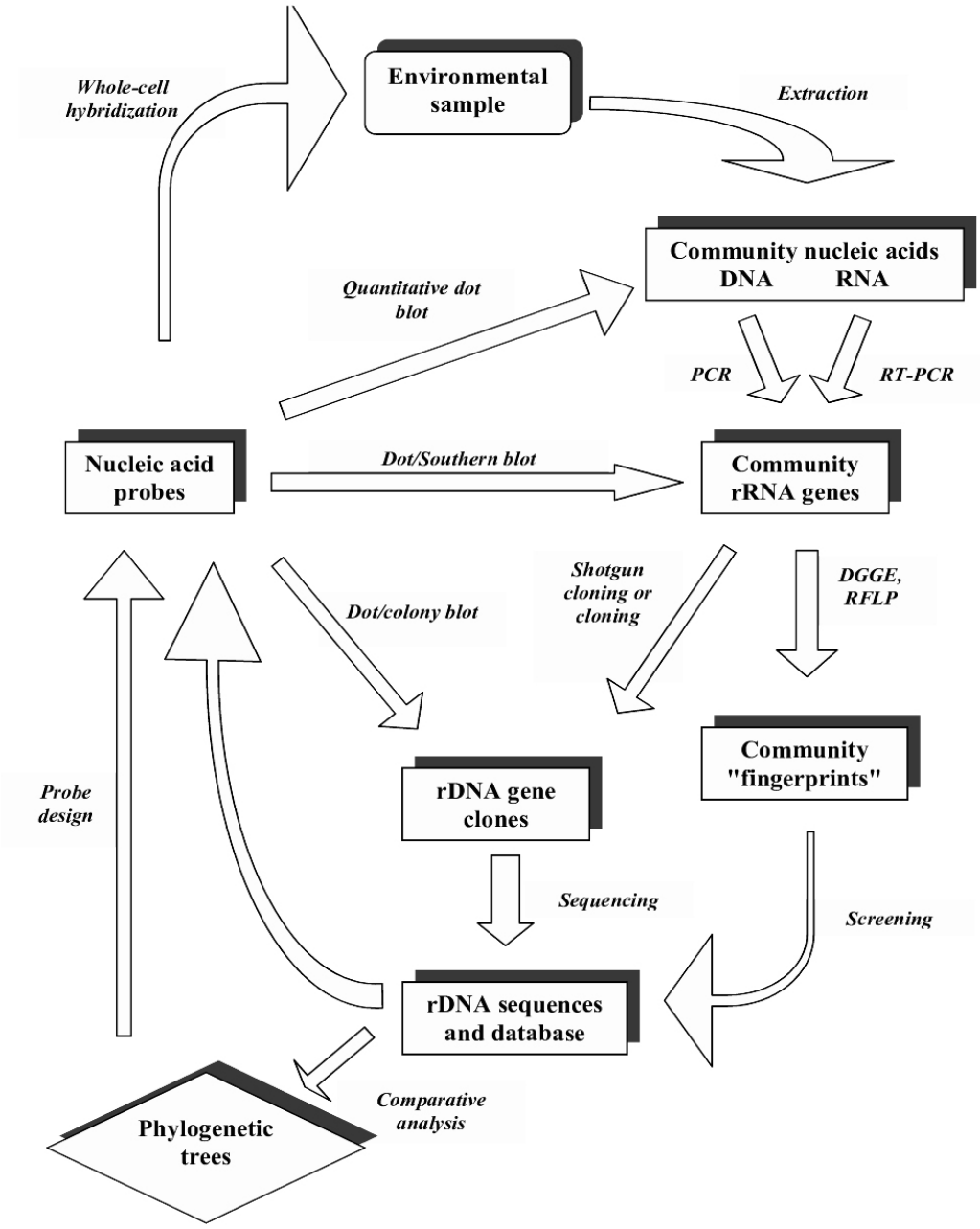


Figure 2.10. Summary of overall methodologies used in the phylogenetic studies (Muyzer et al., 1998).

Table 2.11. Overview of molecular approaches used in microbial ecology (Roest, 2007).

Approach	**Description**	**Remarks**
Cultivation	Study micro-organisms in defined circumstances.	Only a minor fraction of the micro-organisms can be cultivated.
PCR	Specific and sensitive amplification of genetic material (DNA/RNA).	Primers developed from known sequences and can cause bias.
Real-time PCR	Quantification of microorganisms	Sensitive and sensitive quantitative amplification suitable for high-throughput over a wide dynamic range.
Fingerprinting (DGGE/SSCP/ TRFLP etc.)	Rapid overview of diversity. Ideal for comparisons of ecosystems in time or between different samples.	Bias in nucleic acids extraction and PCR. Only dominant populations can be visualised.
Sequencing	Gold standard for sequence retrieval.	Nucleic acids extraction, PCR and cloning can be biased.
FISH	Enumeration of micro-organisms *in situ*. Allows localisation and quantification.	Laborious without automatisation and requires sequence information for probe development. Cell permeabilisation and fixation can cause bias.
In situ isotope tracking (e.g. SIP, MAR-FISH, isotope array)	Combination of cultivation and molecular techniques allowing the functional identification of active micro-organisms.	Not suitable for all environments and crossfeeding might prove difficult to interpretate.

2.6.1.1. Polymerase Chain Reaction (PCR). Polymerase chain reaction (PCR) is used to amplify DNA sequences from environmental samples. The introduction of the PCR further revolutionised microbial ecology, and there are now a number of techniques based on the

nucleic acid sequences of cells which are now widely applied to determine the genetic diversity of microbial communities in the environment (Muyzer et al., 1996; Head et al., 1998). The PCR products can be analyzed by techniques such as DGGE (denaturation gradient gel electrophoresis), TGGE (temperature gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism), or SSCP (single stranded conformation polymorphism), which have the potential to separate the PCR products originating from different DNA sequences representing populations in the original samples. The PCR products can also be cloned and subsequently sequenced to allow identification of population (Hofman-Bang et al., 2003).

The PCR process takes advantage of the double-stranded structure of DNA and the natural process of DNA replication. The PCR reaction contains template DNA, heat-stable *Taq* DNA polymerase (from *Thermus aquaticus*), deoxynucleoside triphosphates (dNTPs), magnesium, buffer, and two primer oligonucleotides. PCR can multiply DNA molecules up to a billion fold in the test tube, yielding large amounts of specific genes for cloning, sequencing or mutagenesis purposes without needing a living cell. PCR makes the use of the enzyme DNA polymerase, which copies DNA molecules. One primer complements a region upstream of the sequence being amplified; the other primer complements a region on the opposite strand downstream of this sequence (Figure 2.11).

PCR process mainly based on three steps: Denaturation, Annealing, and Extension. In the denaturation step, target genetic material is heated to 94°C to denature the template DNA. To replicate DNA using PCR, double-stranded DNA molecule (called the *template*) holded together by strong hydrogen bonds should be separated into single strands. The double helix can be opened by heating the molecule up to a temperature just short of boiling. When heated, the two strands slowly come apart as the hydrogen bonds melt. DNA's sugar-phosphate backbone isn't damaged by heat, so the single strands stay together with the bases still in their original order. In the second step, annealing or hybridization, the reaction temperature is lowered to about 50 °C. The primers match up with their complements on the template strands in a process called ***annealing.*** Cooling allows small, complementary pieces of DNA called *primers* to attach themselves to the the single-stranded DNA template. Primers only attach to the template strand when the match is perfect; if no exact match is found, the next step in the PCR process doesn't occur

because primers are required to start the copying process Then, in the extension step, temperature is increased again to the optimum temperature (72°C mostly) in which Taq polymerase can elongate the chain by adding nucleotides (dNTPs). Taq polymerase starts adding bases — this stage is called *extension* —onto the 3'-ends of the primers by reading the template DNA strand to determine which base belongs next. Meanwhile, on the opposite template stand at the end of the reverse primer, *Taq* rapidly adds complementary bases using the template as a guide. (The newly replicated DNA remains double-stranded throughout this process is going on because the mixture isn't hot enough to melt the newly formed hydrogen bonds between the complementary bases.)

Each cycle takes only 1-3 minutes and repeated 30-40 times to recover enough DNA segment of interest. During the first cycle, the primers anneal to the target sequence and are extended by DNA polymerase beyond the other primer-binding site (Fig. 3.2). In the second cycle, these new strands are used as templates, generating strands running from primer to primer. Thus, the final amplification product contains DNA molecules of a specific length. In theory, 35 cycles of amplification can produce 8,589,934,592 (2^n where n is the cycle number, 33) copies from a single template molecule (the first two cycles do not produce products of a specific length). The resulted product will be run on an agarose gel to monitor efficiency of the PCR. Mostly Ethidium Bromide (EtBr) is used to stain DNA which renders DNA visible under UV light. Although some investigators have detected a single copy of template DNA, PCR reactions never yield the theoretical maximum of product DNA. Because a single molecule of contaminating DNA can be amplified many times, you must take precautions to avoid introducing extraneous DNA. Always perform at least one negative control reaction with no added DNA for each reaction that contains template. A positive control should be used to verify that the DNA polymerase is active and the dNTPs are not degraded

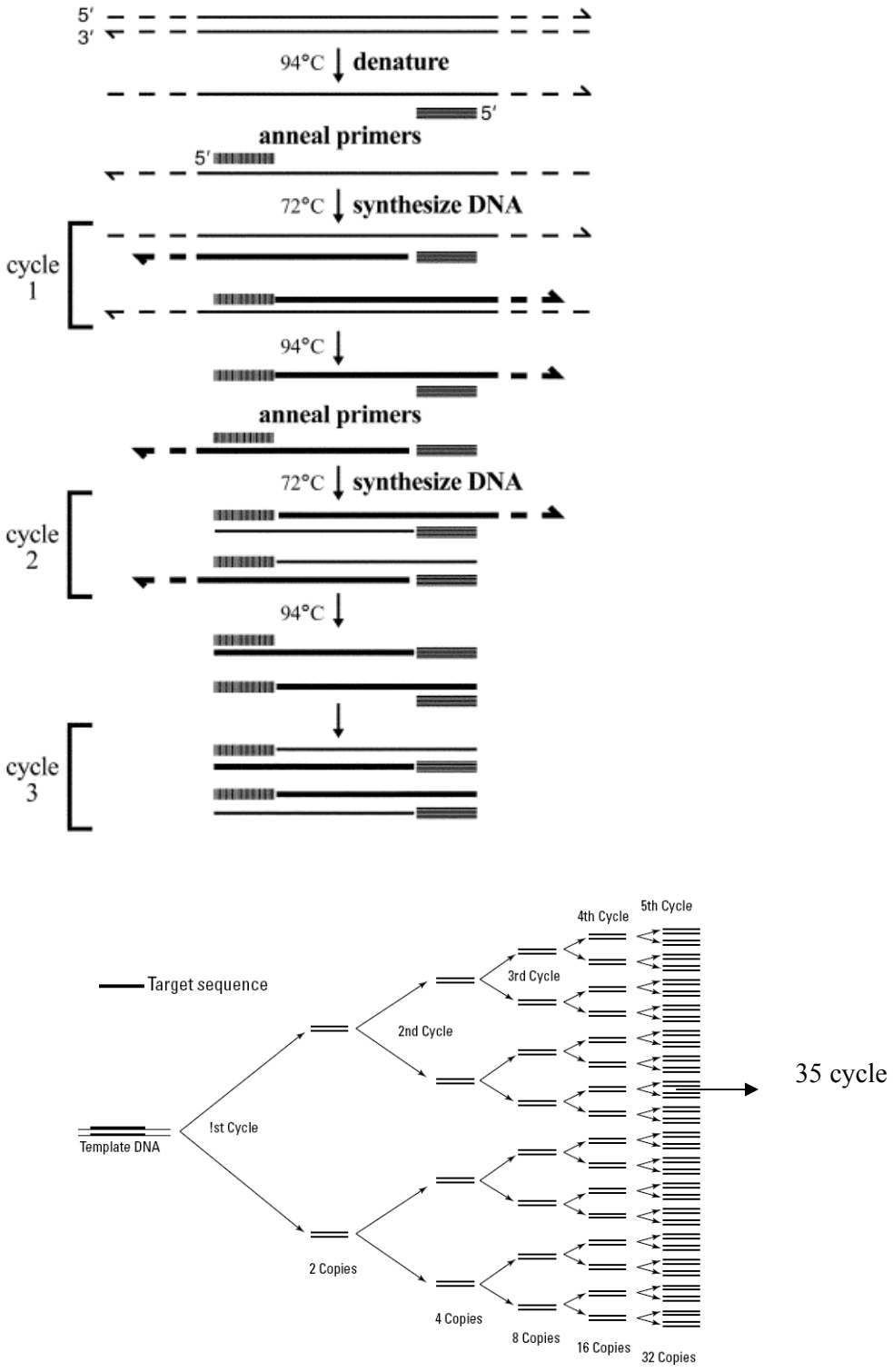


Figure 2.11. Principle of PCR

2.6.1.2. Quantitative Real Time PCR. In contrast to the conventional end-point detection PCR, real-time quantitative PCR (Q-PCR) technology is based on the detection of fluorescence during amplification of target DNA. The technique using a fluorogenic probe is based on the continuous monitoring of changes of fluorescence during PCR at the exponential phase of amplification. The initial amount of target DNA is inversely proportional to the cycle threshold (CT) defined as the moment (or cycle) where the level of fluorescence in the assay is over the baseline fluorescence signal. Q-PCR has better sensitivity and reproducibility than conventional PCR or conventional hybridization techniques (e.g. dot blotting), and can be easily used in studies requiring a large number of samples. The software included in the Q-PCR system can estimate the initial amount of target DNA. This technique allows rapid detection and quantification of various environmental microorganisms and can be used for a large number of samples because of the advantages of PCR (Harms et al., 2003; Yu et al., 2005). Primer and probe sets highly specific for the target microorganisms must be available for successful application of real-time QPCR methods to biological process. The QPCR assay with a TaqMan system is highly specific and sensitive due to the use of three oligonucleotide sequences including two primers, a forward and reverse, and a fluorescent probe complementary to the target DNA (Harms et al., 2003). Although QPCR assays have been increasingly employed in environmental biotechnology (Volkmann et al., 2004), applications of the method to anaerobic processes remain limited due to scarcity of properly designed primer and probe sets for the processes. Group-specific primer and probe sets targeting 16S rRNA genes of various methanogens has been recently reported to be developed for sensitive detection and quantification (Yu et al., 2005). Quantification of rRNA that is isolated directly from the ribosomes may be used to reveal the metabolically most active members of a bacterial community. Assuming that intracellular rRNA level is proportional to the metabolic activity, rRNA/rRNA gene concentration ratios determined by Q-PCR could link ribosomal content to functional activity of a particular population and represent a research avenue to explore.

2.6.1.3. Denaturant Gradient Gel Electrophoresis (DGGE). DGGE is a gel electrophoresis method that separates genes/ DNA fragments of the same size (obtained after PCR of DNA extracted from an environmental sample) that differ in base sequence, at least by one nucleotide into distinct bands on a chemical denaturing gradient polyacrylamide gel and

thus melting at different domains. DGGE is now routinely used to asses the diversity of microbial communities, to monitor their dynamics (Muyzer, 1999; Muyzer and Smalla, 1998) and to screen clone libraries. The DGGE technique has been used to characterize the microbial diversity in different environments such as activated sludge (Curtis and Craine, 1998), sediments (Muyzer and De Wall, 1993), lake water (Ovreas et al., 1997), hot springs (Santegoeds et al., 1996), biofilm (Santegoeds et al., 1998). It provides a quicker, less labor-intensive approach to comparing community composition in many different samples than sequencing of clone libraries but little direct information on population identity (Muyzer and Smalla, 1998). The method can be used to obtain qualitative and semi-quantitative estimations of biodiversity.

Analyzing community diversity. DGGE can be used to determine genetic diversity of a microbial community without identifying individuals. It can be used to compare different communities like two sludge plants (Curtis and Crane, 1996), soil samples (Heuer et al., 1997), bacterial and archaeal communities (Øvreås et al., 1997).

Studying community changes. In some cases microbial ecologist often require to have samples spanning long time periods. As cloning is not suitable to use in this kind of study, DGGE becomes a savior for the scientists. With DGGE different samples taken at different times can be analyzed and compared in one gel. The simultaneous analysis makes it a powerful tool to analyze microbial community changes over time (Santagoeds et al., 1997; Ferris et al., 1997).

Monitoring of enrichment and isolation of bacteria. As it is originally used in complex communities, DGGE can also be used in simple communities. Monitoring enrichment cultures make it possible to determine and analyze conditions of isolation and enrichment (Santagoeds et al., 1996; Ward et al., 1996; Teske et al., 1996; Muyzer, 1997; Bucholz-Cleven et al., 1997).

Comparison of different DNA extraction methods. DGGE can be used to compare efficiency of different DNA extraction protocols (Heuer and Smalla, 1997; Lieasack et al., 1997).

Screening of clone libraries. DGGE is one of the commonly used techniques to screen clone libraries. Rapid and reliable results of DGGE decreases the amount needed to perform clone libraries (Kowalchuk et al., 1997).

DGGE exploits the fact that DNA molecules that have the same length, but differ at least by one nucleotide, can be separated by electrophoresis through a linear gradient of increasing chemical denaturants of urea and formamide (DGGE), In DGGE, double-stranded DNA which is subjected to an increasing denaturant environment will melt in discrete segments, called melting domains. Separation or melting of the two strands of a DNA molecule depends on the hydrogen bonds formed between complementary base pairs (GC-rich domains melt at higher denaturant gradients), and on the attraction between neighboring bases on the same strand (Dorigo et al., 2005). When run on polyacrylamide gel, the mobility of the molecule is retarded when the first melting domain is reached resulting in partial dissociation of the fragment. Complete strand separation is prevented by the presence of a high melting domain, known as GC clamp, which is added to one primer (Dorigo et al., 2005). Differences in melting properties are to a large degree controlled by differences in base sequence. The melting temperature (Tm) of individual domains is sequence specific. When the Tm of the lowest melting domain is reached, the DNA will become partially melted, creating branched molecules which sharply reduce its mobility in a polyacrylamide gel.

There are similar techniques to DGGE like temporal temperature gradient gel electrophoresis (TTGE), RFLP or single strand conformation polymorphism (SSCP) which have been developed to screen clone libraries, to estimate the level of diversity in environmental samples, to follow changes in community structure, to compare diversity and community characteristics in various samples and simply to identify differences between communities (Hofman-Bang et al., 2003; Dahllöf, 2002). However, DGGE is much effective and easier application compared to other mentioned techniques. DGGE and TGGE were firstly introduced to environmental sciences by Muyzer's studies and then became a routine technique to monitor the diversity of microbial communities and their dynamics in complex environments (Muyzer et al., 1993, 1996; 1999; Muyzer and Smalla, 1998). DGGE mainly focus on the number of different bands in order to get an estimate of the community richness. There are also some studies that also take into account the

intensity of each band as providing an estimate of the abundance of each band-population (Nübel et al., 1999).

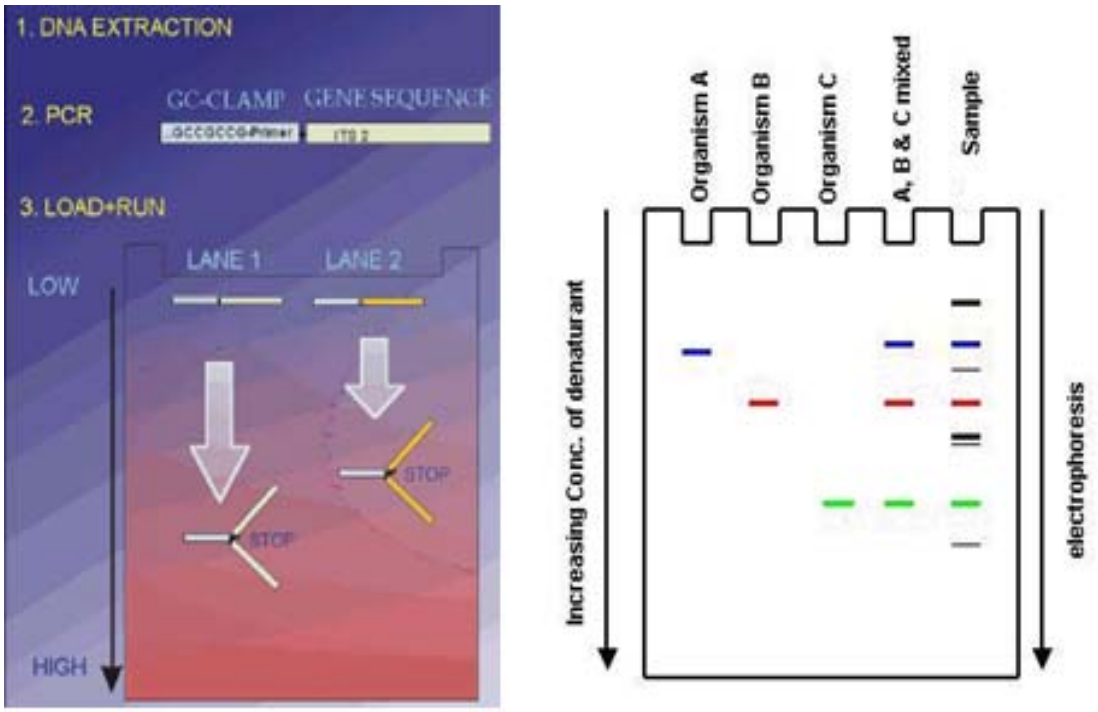


Figure 2.12. Principle of DGGE

The main limitations of DGGE are the choice of the primer set and the optimization of the gel running conditions before technique can be used to screen for sequence polymorphism of a particular gene (Muyzer et al., 1993) and the difficulty of comparing patterns across gels, when these patterns include numerous bands and its limited sensitivity of detection of rare community members (Vallaeys et al., 1997). The DGGE has biases which are mostly related to PCR. DGGE is being negatively affected by biases of PCR like fidelity error of polymerase or chimeric products. Therefore it has been accepted that DGGE is a semi quantitative method since number of bands and intensities may be affected by PCR. Heteroduplex formations (annealing of DNA fragments from different species), multiple bands formed by chimeras or due to resolution of the gel, or different fragments resulting from existence of several rRNA coding regions, may appear on gel causing overestimation (multiple bands on the same lane causes underestimation) of microbial diversity (Curtis and Craine, 1998). In addition, by DGGE only fragments up to

500 base pairs can be separated (Myers et al., 1985); this limits the amount of sequence information used for phylogenetic analysis and probe design (Muyzer and Smalla, 1998). The band excision from gel is a powerful feature of DGGE but it has some difficulties since mostly a band will consist of 150-200bp DNA which is rather short for a phylogenetic analysis. Another problem with excision is distance of two bands. In some cases bands are too close; a proper excision cannot be made. Also during excision step UV may affect DNA and in reamplification it may create ambiguous sequences. Co-migration of bands also will give a poor identification in the sequencing (Felske et al., 1998; Vallaeys et al., 1997). This problem may be overcome by having a clone library screening. It is then combines both techniques' powerful aspects (Giovanni et al., 1990; Ward et al., 1990).

2.6.1.4. Molecular Cloning, Sequencing, Phylogenetic Analysis. Molecular cloning is at the base of most genetic engineering procedures and has greatly facilitated the analysis of any genome. The purpose of molecular cloning is to isolate large quantities of specific genes or chromosomal fragments in pure form (Madigan et al., 2003). It also allows the identification of the members of a community from environmental samples. Cloning can produce large amounts of DNA segments originally isolated from environmental samples. The DNA fragments can be produced after digestion with restriction enzymes of the DNA extracted from a sample (i.e., shotgun cloning), or after PCR or RT-PCR (if RNA is the template) (Hofman-Bang et al., 2003). Analysis of 16S rRNA clone library to assess microbial diversity and populations in natural environments is an important approach (Giovanni et al., 1990). A library is constructed with the DNA from a particular sample. Each clone has the SSU rDNA of one member of the original community. As more clones are sequenced, new taxa arise. In general molecular cloning can be divided into several steps (Madigan et al., 2003);

(1) Isolation and fragmentation of the source DNA.

(2) Joining the DNA fragments to a cloning vector with DNA ligase. The small, independently replicating genetic elements used to replicate genes are known as cloning vectors, and most are derived from plasmids or viruses. Cloning vectors are generally designed to allow recombination of foreign DNA at a restriction site that cuts the vector in a way that does not affect its replication.

(3) Introduction and maintenance in a host organism. The recombinant DNA molecule made in a test tube is introduced into a host organism, for example, by DNA transformation where it can replicate. Transfer of the DNA into the host usually yields a mixture of clones. Some cells contain the desired cloned gene, whereas other cells contain other clones generated by joining the source DNA to the vector. Such a mixture is known as a DNA library or gene library because many different clones can be purified from the mixture, each containing different cloned DNA segments from the source organism. Constructing a gene library by cloning random fragments of a genome is called shotgun cloning.

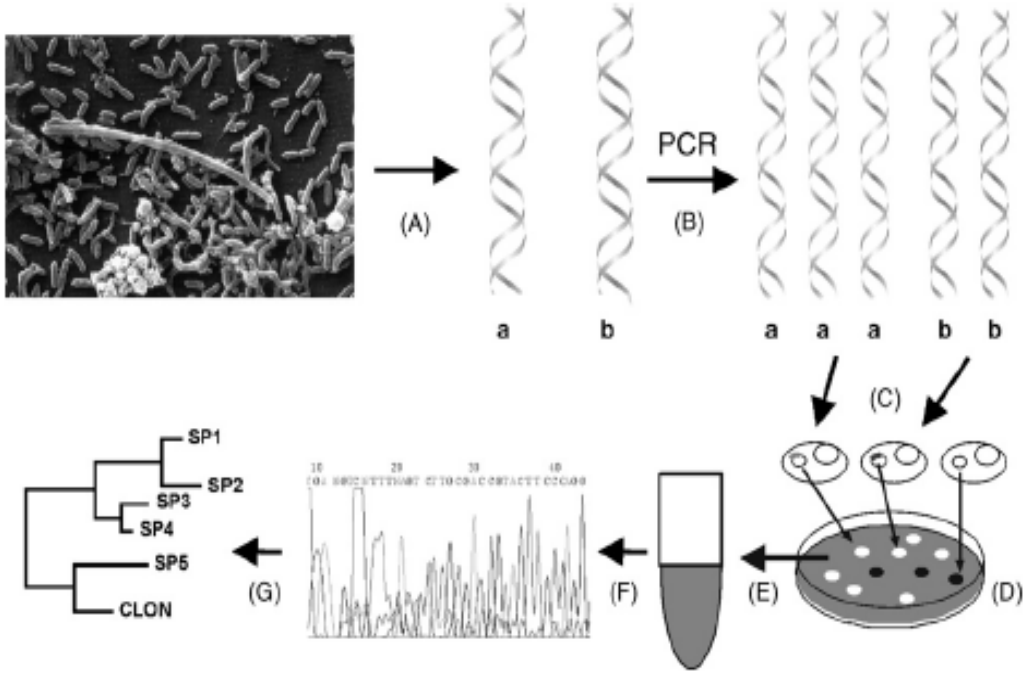


Figure 2.13. Outline of the cloning procedure for studying a microbial community (Sanz and Köchling, 2007). The work cycle is as follows: (A) direct nucleic acid extraction, without the need for previous isolation of microorganisms; (B) amplification of the genes that code for 16S rRNA by polymerase chain reaction (PCR), commonly using universal primers for bacteria or archaea, resulting in a mixture of rDNA copies corresponding to the microorganisms present in the sample; (C) cloning of the PCR products obtained into a suitable high copy number plasmid and transformation of competent E. coli cells with this

vector; (D) selection of transformed clones with an indicator contained in the plasmid (the white colonies in the figure); (E) extraction of plasmid DNA; (F) sequencing of the cloned gene, creating a clone library; (G) determination of the phylogenetic affiliation of the cloned sequence with the help of dedicated computer programs (ARB, Seqlab, PAUP, PHYLIP).

Cloning after PCR is rapid and convenient, but can be biased (Ward et al., 1992; Pace, 1996). The bias can be introduced during the PCR step or during cloning. For instance, the use of rare-cutting restriction enzymes during cloning might also cut amplified rDNA (Amann et al., 1995). Compared to cloning after PCR, shotgun cloning introduces less bias and produces clones of multiple genes at the same time (Pace, 1996). In addition, different rRNA gene fragments may be cloned with different efficiencies. This technique is also time consuming and labor-intensive for the study of the vertical structure of communities in multiple sample analysis.

2.6.1.5. Fluorescent in situ Hybridization (FISH). Whole cell or in situ hybridization is a method to enumerate specific microbial populations and to determine their spatial distribution directly in their natural environment without cultivation. Fluorescence *in situ* hybridisation (FISH) technique that uses fluorescently labelled oligonucleotide probes to detect specific organisms in samples is a valuable tool for the study of microbial dynamics in environmental samples. The use of hybridization *in situ* for counting and identifying organisms was proposed by Olsen et al.,(1986) and rRNA gene fragments was used as phylogenetic stains firstly in 1989 (DeLong et al., 1989). Nowadays, FISH technique is widely used in environmental microbiology studies (De Long, 1992; Raskin et al., 1994; Wagner et al., 2003). Fluorescently-labelled rRNA-targeted oligonucleotide probes were shown to allow the detection of individual cells *in situ* (DeLong et al., 1998). This made whole-cell hybridization with rRNA–targeted probes suitable tool for not only determination of microorganisms but also quantitative analysis in environmental microbiology (Amann et al., 1990). Nowadays it is even possible to detect up to seven microbial groups simultaneously with Rainbow-FISH (Sunamura and Maruyama, 2006). FISH with polynucleotide DNA probes and FISH with oligonucleotide probes targeted to mRNA has also been described (Wagner et al., 1998; DeLong et al., 1999).

Microbial cells are first fixed with appropriate chemical fixatives and then hybridised under appropriate conditions on a glass slide or in solution with oligonucleotide probes. Following hybridization, the sample is washed to remove unbound probe, and the sample is viewed by epifluorescence microscopy. After washing steps, cells showing specific hybridization with the fluorochrome-labelled probe can be identified and enumerated by epifluorescence microscopy or flow cytometry. Before the in-situ hybridization, staining with DAPI (48,6-diamidino-2-phenylindole) allows total cell counts to be determined. It is important to stain with DAPI because before the experiment it must be made sure that there are enough living cells for probe hybridization. After it is seen that there are enough microorganisms to hybridize the probes with, the sample is fixed using either paraformaldehyde or alcohol to permeabilize the cells while maintaining their morphological integrity. The following steps (Figure 7) used in the FISH analysis are separately explained in detail.

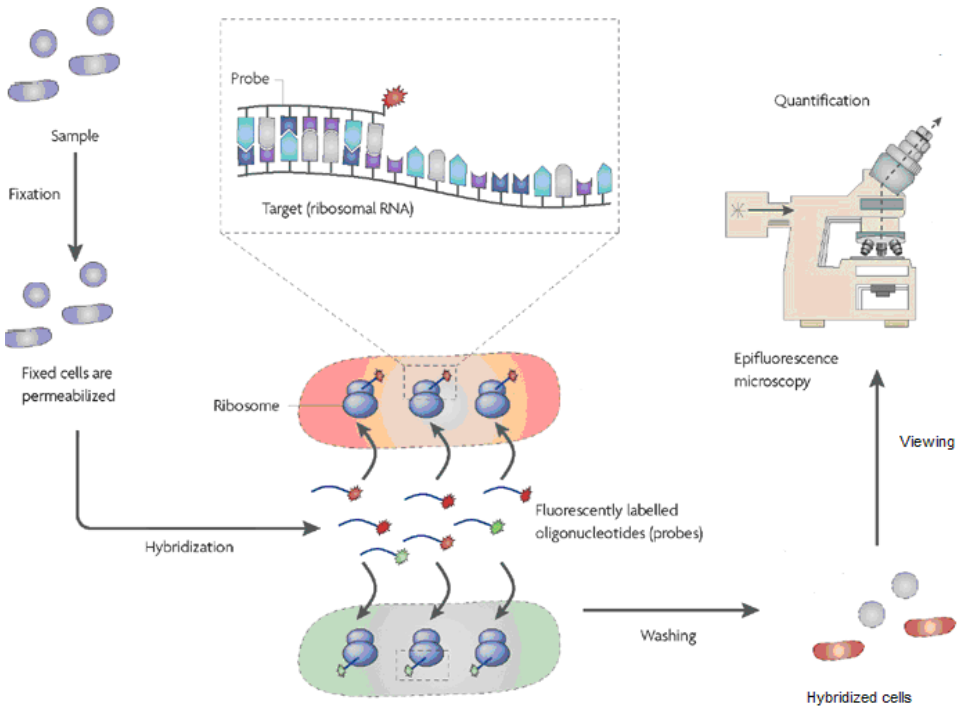


Figure 2.14. Schematic diagram of the steps involved in FISH.

Permeabilization and fixation of the cells. The first step of in-situ hybridization procedure is to make the cell wall and membrane permeable so that the probe can enter the cell and bind to the target rRNA. The terms of permeabilization and fixation which are often used synonymously can be considered as two distinct events. The purpose of the fixation is usually to protect the morphological integrity of the cells. Permeabilization without rupturing the integrity of the cell can be conducted by fixation of the cell.

Commonly used fixatives are alcohols and aldehydes. Fixative solutions permeabilize the cell walls of microorganisms. Alcohols achieve fixation by the denaturation of protein structures causing better permeabilization whereas aldehydes make cell walls rigid by cross-linking these structures. The most commonly used fixative is 4% paraformaldehyde (an aldehyde). This treatment produces successful results for making permeable the most bacterial cells to short oligonucleotide probes. Although, permeabilization is managed, oligonucleotide probes may not permeate all cell types and find 16S rRNA target sequences (Muyzer and Ramsing, 1996). Permeability is a problem often reported in hybridizations with Gram-positive organisms. Paraformaldehyde treatment may, in some cases, be detrimental for hybridizations with Gram-positive organisms. This difference derives from the different characteristics of bacterial cell walls. Generally, the Gram-negative bacteria are more readily permeable than the Gram-positive bacteria.

Hybridization of the probe with the target cells. Hybridization in FISH is the term given to the binding of a fluorescent-labelled probe (oligonucleotide) with a ribosomal ribonucleic acid molecule (rRNA) in a fixed cell, which represents an abundant target in metabolically active cells. The oligonucleotide probes are generally 15–25 nucleotides in length and are labelled covalently at the 5'end with a fluorescent dye. The specificity of the probes ranges from the phylotype to the kingdom, depending on the targeted region on the rRNA (Amann et al., 1995; DeLong et al., 1989). Several hundred rRNA-targeted oligonucleotide probes suitable for FISH have been described, together with a large online database providing an encompassing overview of over 700 published probes and their characteristics (Loy et al., 2003). Such probes can be readily developed and tested to detect lineages of uncultured microbes in environmental samples (Pernthaler et al., 1998; Ravenschlag et al., 2001).

In FISH, a hybridization buffer (HB) is required, containing the appropriate conditions for the hybridization of the probe to its target. Two hybridization buffers are commonly used. One of the HB contains Denhardt's solution (Ficoll, PVP and BSA), which is a cocktail of 'sticky' substances that occupy sites of steric binding, which would otherwise lead to non-specific binding of the probe during hybridization. Hence, this buffer should be used for the analysis of complex microbial communities from environmental samples, such as activated sludge, which contain many organic and inorganic substances that are prone to autofluorescence or interact non-specifically with oligonucleotide probes. The optimal temperature for hybridization must be determined empirically to avoid binding of the probe to rRNA sequences with some mismatches with the probe. A more convenient method for optimizing probe hybridization is by the inclusion of different concentrations of formamide in the hybridization buffer (Manz et al., 1992), with hybridization conducted at a single temperature.

The specificity of the hybridization event is dependent on the presence of the target molecule (and target sequence); the complementarity (i.e. sequence homology) between the probe and its target; and the stringency of the prevailing hybridization conditions. The stringency of hybridization is the condition optimal for specific binding of a probe to its complementary (target) sequence. Temperature, salt concentration and denaturant concentration in the hybridization and washing steps affect the stringency. Thus at low temperatures short oligonucleotide sequences will bind less specifically i.e. to any sequence. As the temperature increases the hybridization complex/hybrid (i.e. probe and target) dissociate and at high temperatures the hybrid 'melts' (dissociates) completely. Hybrids with less sequence complementarity melt more readily, whereas perfect complementarity between a probe and target sequence gives the strongest binding. This principle is used to empirically define the optimal conditions for a probe with its target. The probe is hybridized with a known reference target organism (grown in pure culture) and a known non-target organism (exhibiting the closest possible sequence mismatch with the probe; also grown in pure culture). Different hybridizations are conducted over a range of conditions (usually different denaturant concentrations (formamide) at a set temperature in the hybridization step, and salt concentration in the washing step). The optimal conditions are those at which the target organism shows a good fluorescence signal, whilst the non-target organism shows no, or a weak, fluorescence signal.

Washing off excess and unbound probe. In the washing steps, unbound probes are removed. Temperature, salt concentration and denaturant concentration in the hybridization and washing steps affect the stringency. Stringency of the washing buffer is generally regulated by varying the salt concentration (Lathe, 1985) instead of using formamide.

Viewing the cells. After washing step, the cells can be viewed by means of epifluorescence and confocal laser scanning microscopy or by flow cytometry with fluorescent microscopy.

The main advantage of FISH is that it does not need any DNA or RNA amplification and allows direct visualization of intact cells without cultivation by microscope in the samples. In fact, FISH verifies the presence and in general terms, the cellular activity based on the fact that rRNA is degraded, albeit slowly, in inactive or dead cells of microorganisms in habitat (Wilderer et al., 2002). The signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. All living cells contain ribosomes, therefore thousands of copies of 16S rRNA for the probes to attach to which increases the sensitivity of in-situ hybridization. Ribosomes are the protein production units of the cells. If the numbers and the metabolic activity of the cells are high, the probes will be able to find more targets to attach to. Therefore, results of the in-situ hybridization will give information about the numbers, activities and growth rate of the organisms. FISH technique is also easy, fast technique allowing quantification of specific microbial groups and detection of active microorganisms in the sample (Sanz et al., 2006). Another advantages of FISH technique are (i) it can give highly specific results with specific probes, (ii) in situ growth rates may be estimated by measuring the fluorescence intensity, (iii) It provides an opportunity for using multiple probes labeled by different dyes simultaneously within a single sample. Moreover, by using targeting probes in the order of phyla – order – genera – species, in a few steps complex environments can be characterized.

Limitation of the whole cell hybridization include obtaining effective probe penetration into the cells, background fluorescence of inorganic particles, low signal intensity caused by small number of ribosomes or by inaccessibility of the rRNA for the

oligonucleotide probes. One of the most important limitations of FISH is that all bacterial and archaeal cells may not be permeabilisied using standard fixation protocols (Aman et al., 1995). After the cell is made permeable, it is not for sure that the probe will hybridize with the target rRNA. This is due to the inaccessibility of the target site which could occur because of rRNA being inaccessible due to strong interactions with ribosomal proteins or highly stable secondary structure elements of the rRNA itself (Head et al., 1998). Besides, the use of rRNA targeted oligonucleotide probes, which are covalently mono-labelled with fluorescent dye molecules, limits the use of FISH for identification of prokaryotes with low ribosome content per cell. For detection of a particular microorganism, the rRNA sequence of the microorganims must be known for the design of the probe. Therefore, a priori knowledge of the studied ecosystem and the microorganisms to be detected is necessary, meaning combining with other techniques is obligate. It is not always possible to design a specific probe for a certain group of microorganism, especially if metabolic criteria are applied. Designing a new probe and determination of optimum conditions for hybridization can be a difficult dedication. In addition, quantification of microorganisms can be tedious and subjective (manual counting) or complex (image analysis). The sensitivity of the fluorescently labelled probe is also a problem in in-situ hybridization. It is know n that somewhere between 1-10% of the microbial species have been identified; thus, it is very possible that the probes designed to be specific for only one species can attach to others. As more rRNA sequences become available in sequence databases, the problem of probe specificity has become apparent, and design of diagnostic probes is becoming more difficult (Head et al., 1998).

2.6.1.6. Fluorescence in situ hybridization-microautoradiography (FISH-MAR). Microauto-radiography combined with FISH (FISH-MAR) constitutes an approach to link phylogeny and ecosystem function by in situ association of a particular phylotype to substrate uptake (Lee et al., 1999). An environmental sample is incubated with a radioactive substrate and is fixed and prepared (by filtration on a matrix or by cryosectioning) for FISH analysis (with SSU rRNA probes) and further for microautoradiography. The analysis of the two superimposed images reveals the phylotype of bacteria who have incorporated the radioactive substrate. This approach has been used to study WWTPs to describe the function of newly discovered species and to identify microorganisms responsible for key physiological processes, particularly in enhanced

biological phosphorus removal (EBPR) microbiology (Burow et al., 2007). A full-cycle SSU rRNA approach followed by FISH-MAR was used to investigate the functional Bacteria and Archaea communities of a full-scale mesophilic anaerobic municipal sludge digester (Ariesyady et al., 2007a) to determine major trophic groups (degrading glucose, propionate, butyrate, and acetate) in anaerobic sludge digester.

Restriction analysis based approaches: Molecular techniques mostly make use of specific nucleic acid-modifying enzymes, initially purified and characterised from microorganisms. The thermo-stable Taq DNA polymerase, used for PCR, is a good example. Besides, restriction enzymes are also widely used in the techniques. Restriction enzymes recognize specific DNA sequences and cut in a reproducible way. The combination of PCR and restriction can, for example, be used for enhanced amplification of minor DNA templates (Green and Minz, 2005). Unwanted or dominant DNA templates can be amplified in a first round of PCR, the produced double stranded products cut by restriction enzymes, resulting in the digested template no longer being available for PCR amplification (Green and Minz, 2005).

2.6.1.7. Amplified Ribosomal DNA Restriction Analysis (ARDRA). In Amplified Ribosomal DNA Restriction Analysis (ARDRA), the ribosomal RNA gene is amplified by PCR, and digested into specific fragments by restriction enzymes (usually with 4-bp recognition sites), then incubated with restriction enzymes, The fragments are separated by high resolution gel electrophoresis, resulting in specific patterns from different sequences. ARDRA can be used for rapid comparison of rRNA genes (Moyer et al., 1994). The typical analysis of restriction digests for isolates or clones is performed on agarose gels, while for community analysis the potentially large number of fragments can be resolved by using polyacrylamide gels to produce a community-specific pattern (Martinez-Murcia et al., 1995), but new high resolution matrices are nowadays available as well.

2.6.1.8. Terminal Restriction Fragment Length Polymorphism (T-RFLP). Terminal Restriction Fragment Length Polymorphism (T-RFLP) is a molecular biology technique in microbial ecology for profiling of microbial communities based on the position of a restriction site closest to a labeled end of an amplified gene. T-RFLP is another derived fingerprinting technique and makes use of restriction enzymes as well, but only terminal

restriction fragments (T-RF) are detected and used for qualitative and quantitative analysis (Liu et al., 1997).

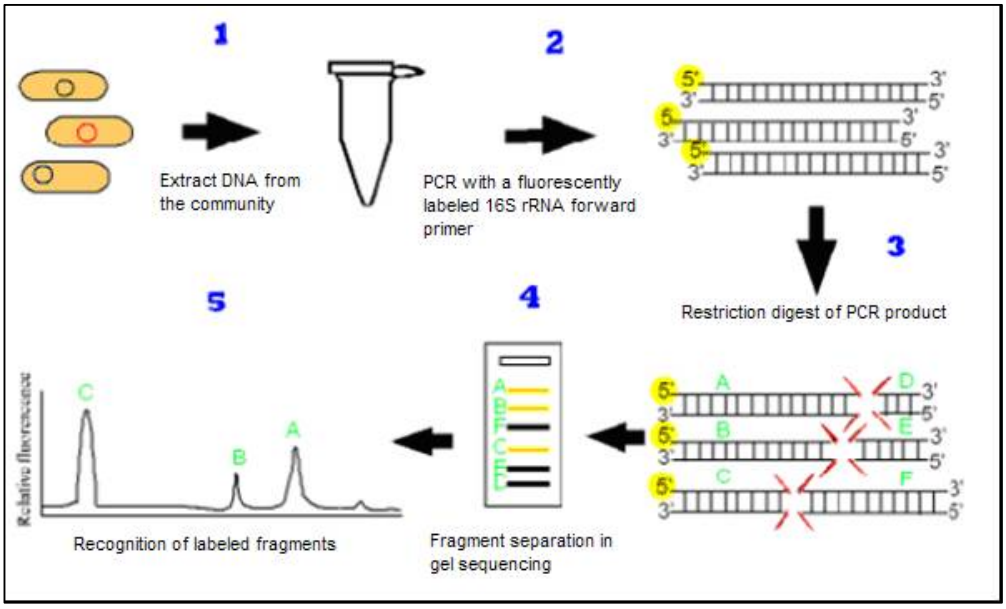


Figure 2.15. Principle of T-RFLP

The method is carried out in a series of steps that combine PCR, restriction enzyme digestion and gel electrophoresis. Like most other community analysis methods, T-RFLP is also based on PCR amplification of a target gene. DNA extracted from a sample is subjected to PCR using primers homologous to conserved regions in a target gene. The amplification is performed with one or both the primers having their 5' end labeled with a fluorescent molecule. The amplified DNA fragments (amplicons) are then subjected to a restriction reaction, normally using a four-cutter restriction enzyme. Following the reaction, the digested amplicons is separated using either capillary or polyacrylamide gel electrophoresis in a DNA sequencer with a fluorescence detector so that only the fluorescently labeled terminal restriction fragments (TRFs) are visualized. The result of a T-RFLP profiling is a graph called electropherogram. In an electropherogram the X-axis marks the sizes of the fragments while the Y-axis marks the fluorescence intensity of each fragment. Because the excised mixture of amplicons is analyzed in a sequencer, only the terminal fragments (i.e the labeled end or ends of the amplicon) are read while all other fragments are ignored. Thus, T-RFLP is differed from ARDRA and RFLP in which all

restriction fragments are visualized. T-RFLP profiles can be satisfactorily quantified by automated electrophoresis systems, making them amenable to analyses using various statistical methods, such as similarity indices, hierarchical clustering algorithms, ordination methods, and selforganizing maps (5). While qualitative analyses of T-RFLP profiles only take into account the presence/absence of fragments, quantitative analyses can be achieved by considering peak height or peak area as a measure of fragment abundance. Like with DGGE, however, data obtained by T-RFLP should be cautiously interpreted, since microbial populations that are not numerically dominant are not represented, because the template DNA's from these populations represent a small fraction of the total community DNA and consequently the species diversity of the microbial community is underestimated (Liu et al., 1997).

2.6.1.9. Single strand conformation polymorphism (SSCP). Single Strand Conformation Polymorphism (SSCP) is a technique for the detection of differences in DNA sequences separated in a polyacrylamide gel based on differences in mobility caused by their secondary structure. By the use of a variable part of the 16S rRNA gene (for example the V3 region) from an environmental sample, each SSCP peak corresponds to a distinct microbial sequence, indicating the presence of a microbial strain or species retrieved from the sample (Leclerc et al., 2001; Lee et al., 1996). In general, SSCP has the same limitations as DGGE. Some single-stranded DNA fragments can form more than one stable conformation and therefore one sequence may be represented by multiple bands (Tiedje et al., 1999). However, SSCP does not require a GC-clamp or the construction of gradient gels and is therefore potentially more simple and straightforward than DGGE. SSCP fingerprinting patterns correspond to a representation of the whole microbial community, when not only the number of visible bands or peaks are considered, but when the whole picture, including background is analysed (Loisel et al., 2006).

2.6.1.10. Dot-blot hybridisation. A disadvantage of PCR based approaches is that they can have amplification biases. Quantification of a certain 16S rRNA sequence type relative to the total 16S rRNA content of a given sample can be obtained by dot-blot hybridization of a directly isolated nucleic acid mixture with universal and specific oligonucleotide probes (Amann et al., 1995, Raskin et al., 1994a). For this purpose, total RNA is isolated from a sample, immobilised on a membrane and hybridised with labelled oligonucleotide probes.

The relative abundance can be calculated as a ratio of the amount of specific probe bound to a given sample to the amount of hybridised universal probe (Amann et al., 1995). When radioactively labelled oligonucleotide probes were used in a dot-blot assay, rRNA sequences with a relatively low abundance between 0.1 and 1% could be quantified (Amann et al., 1995). Although quantification is very accurate, these data of relative rRNA abundance can not be directly translated into cell numbers, since cells of different species have different ribosome contents ranging roughly between 103 and 105 ribosomes per cell (Amann et al., 1995). However, because PCR or other amplification procedures are not involved, the quantification is very accurate, and many oligonucleotide probes have been developed, validated and successfully used in the past 15 years. Recently, many of those probes have been collected in an interactive web-based database, ProbeBase (http://www.microbialecology.net/probebase/) (Loy et al., 2003). Essentially all types of samples can be used for quantitative dot-blot hybridisation, which makes it the method of choice in those systems which are difficult for FISH (e.g. patchy environments) (Amann and Ludwig, 2000).

2.6.1.11. Stable Isotope Probing (SIP). Stable Isotope Probing (SIP) is one of the many emerging inquiry tools used by environmental microbiologists. SIP (Radajewski et al., 2000) relies on the DNA or rRNA labelling of growing bacteria with a stable isotope (e.g. 13C) that has been incorporated in their genome or rRNAs, respectively. SIP combined with community fingerprinting and cloning/sequencing analyses can identify helps to discover the microorganisms responsible for catalyzing biogeochemical reactions in the different environments. It allows investigators to follow the flow of atoms in isotopically enriched molecules through complex microbial communities into metabolically active microorganisms. As important examples, *Syntrophobacter*, *Pelotomacula* and *Smithella* spp. have been identified as responsible for syntrophic propionate oxidation in flooded rice field soil with *Methanobacterium* and *Methanosarcina* spp. and also of yet uncultivated rice cluster- I-Archaea (Lueders et al., 2004). Recently, Manefield et al.,(2002a) have introduced rRNA-based SIP (RNA-SIP) as demonstrated by analysing phenol-consuming populations in an aerobic bioreactor since rRNA is a much more sensitive biomarker (Molin and Givskov, 1999) showing the activity of cells is linked directly to turnover and synthesis of rRNA. Factors influencing the maximum attainable enrichment include the operative anabolic pathway, the substrate for assimilation, the duration of label addition,

the relative abundance of naturally occurring unlabeled substrate, and the rate of DNA synthesize of the microbial populations involved. Therefore, SIP studies need to be performed carefully in order to eliminate artifactual results that might be caused by incubation conditions that favor ecologically irrelevant populations (e.g. by adding substrates in forms and concentrations that mimic classic microbiological media rather than the bioreactor's environment). SIP is less expensive than FISH-MAR and can be used to identify substrate degraders and to select the most appropriate substrates for FISH-MAR experiments (Collins et al., 2006).

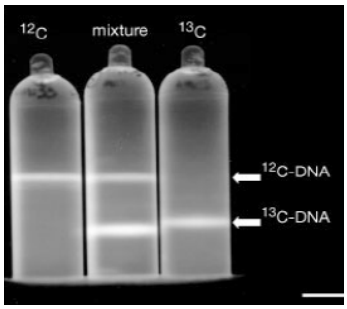


Figure 2.16. Principle of SIP (Radajewski et al., 2000)

2.6.1.12. Phylogenetic micro-arrays. DNA micro-arrays (also called DNA chips, gene chips or biochips) typically consist of thousands of immobilised DNA fragments (PCR product, oligonucleotides or other DNA fragments) present on a surface, such as coated glass slide or membrane (Ye et al., 2001). The microarray experiment output consists of a list of hybridisation events, indicating the presence or the relative abundance of specific DNA or RNA sequences present in the sample. Micro-arrays are already widely used for the detection of transcriptional profiles (expression arrays) or the similarities and differences of genetic contents among different micro-organisms, and they can be used to subtype (fingerprint strains relative to the reference strain) bacterial isolates and for the identification of new and diagnostic genetic markers (Call et al., 2003) and for the mutation detection and the search of polymorphisms. DNA micro-arrays are promising for the quantification of microbial genes and therefore highly suited for molecular ecology studies (Palmer et al., 2006). However, the detection and identification of high numbers of different microbes, especially from complex microbial communities in environmental samples with micro-arrays is still very challenging.

3. PROBLEM DEFINITION AND AIM

Anaerobic treatment of waste streams containing organic solvents is still at an investigative stage. These kinds of wastes may have a detrimental effect on anaerobic biological treatment systems in terms of the composition of the microbial population and methanogenic/non-methanogenic activity in the bioreactors. There is a lack of literature on treatment of organic solvents in anaerobic reactors. Defining effects of organic solvents on both microbial community structure and activity changes and the strategies which may increase the biodegradation capacity can lead to improvements in the understanding of interactions in the bioreactors treating wastewaters containing organic solvents, thereby obtaining better reactor performance.

There is a lack of research in the area of the characterization of microbial community structure changes during the anaerobic treatment of the organic solvents in parallel to activity measurements. Elucidating the ecology of these bacterial consortia is critical in bioreactors. However, there is no such a study on microbial community structure changes and quantitative methanogenic composition during anaerobic treatment of organic solvents in ASBRs. A better understanding of the relationships between the dominant microorganisms in the mixed cultures and how they relate to the degradation of key components in influent waste streams is particularly important for treatment of inhibitory compounds that require a consortium of syntrophic microorganisms. The study aims to bridge the gaps between the understanding and manipulation of the factors affecting microbial diversity together with activity of the anaerobic biomass treating selected organic solvents including isopropanol, toluene and methanol. Based on this, the objectives of the research are outlined below as to investigate:

- the importance of seed sludge quality in terms of methanogenic activity and microbial diversity for the performance of anaerobic reactors.

- the IC_{50} concentrations of the selected solvents including toluene, methanol and isopropanol on acetoclastic methanogenic activity of the anaerobic sludge.

- the microbial community dynamics in anaerobic batch reactors-single phase and two-phase including acid and methane reactor.

- the effects of the selected solvents/solvent mixtures on microbial community and methanogenic activity of the unacclimated sludges taken from anaerobic batch reactors operated at different phases including single phase and two-phase.

- microbial population dynamics associated with anaerobic solvent treatment including toluene, methanol and isopropanol in anaerobic sequencing batch reactors by using molecular methods such as DGGE and FISH to determine mixed culture interactions.

4. MATERIALS AND METHODS

4.1. Bioreactor Studies

In the scope of this study a variety of anaerobic reactor configurations were employed. The characteristics of the reactors and related studies are summarized in Table 4.1 and explained in detail below.

4.1.1. Lab-scale anaerobic sequencing batch reactor (stock reactor)

Anaerobic sequencing batch reactors (Figure 4.1) with a working volume of 8 L were used throughout the study for mixed culture studies. The lab-scale reactors used in this study were made of plexiglass and located in a water bath. The temperature inside the reactors was adjusted to 35 ± 2°C by an external, thermostatically controlled water bath. There were three ports which are used to withdraw samples and one gas outlet positioned on the top of the reactor. A serum cap was set into the flanged top to enable a sample to be taken for gas analysis. All pumps were Masterflex pumps of Cole Parmer Instrument Co. (Chicago, IL, USA). These reactors were inoculated with a seed sludge taken from an upflow anaerobic sludge blanket (UASB) reactor of an alcohol distillery treatment plant and an EGSB reactor treating a brewery wastewater. The influent was buffered with $NaHCO_3$ and fortified, as described by Shelton and Tiedje (1984), with macro- and micro-nutrients. The cycle length of the ASBR was 24 h and consisted of a feed step (0.5 h), a react step (22 h), a settling step (1 h), and a decant step (0.5 h).

Table 4.1. The properties of the reactors used throughout the study.

Reactor Name	**Reactor Material**	**Active Volume (L)**	**Feed**	**Type of Experimental Study**
Anaerobic stock reactor	Plexiglass	8	Glucose-based synthetic wastewater	Investigation of seed sludge
Anaerobic Sequencing Batch Reactor	Glass	1.6	Glucose-based synthetic wastewater	Single-phase/ Two-phase Anaerobic Digestion
Anaerobic Sequencing Batch Reactor	Plexiglass	1.4	Glucose-based synthetic wastewater Glucose-based synthetic wastewater + toluene Glucose-based synthetic wastewater + methanol Glucose-based synthetic wastewater + propanol	Solvent treatment
SMA Test Reactor	Glass	1.6	Acetoclastic methanogenic activity Indirect methanogenic activity	Methanogenic and Non-Methanogenic Activity Tests

Figure 4.1. Photographic view of anaerobic stock reactor.

4.1.2. Anaerobic sequencing batch reactors

Anaerobic sequencing batch reactors with a working volume of 1.4 L (Figure 4.2) were used throughout the study for mixed culture studies. Mixing was performed by a magnetic stirrer system that was placed underneath the water bath. Timers were used to control reactor operation. All pumps were Masterflex pumps of Cole Parmer Instrument Co. (Chicago, IL, USA). A stirrer with 150 rpm was restored to provide the mixing of substrate and biomass in the reactor. Gas production was recorded using a miligas counter (RITTER MGC-1). The temperature inside the reactor was controlled at 35 ± 2 C using a temperature controlled water bath. The cycle length of the ASBR was 24 h and consisted of a feed step (0.5 h), a react step (22 h), a settling step (1 h), and a decant step (0.5 h).

Figure 4.2. Photographic view of ASBRs

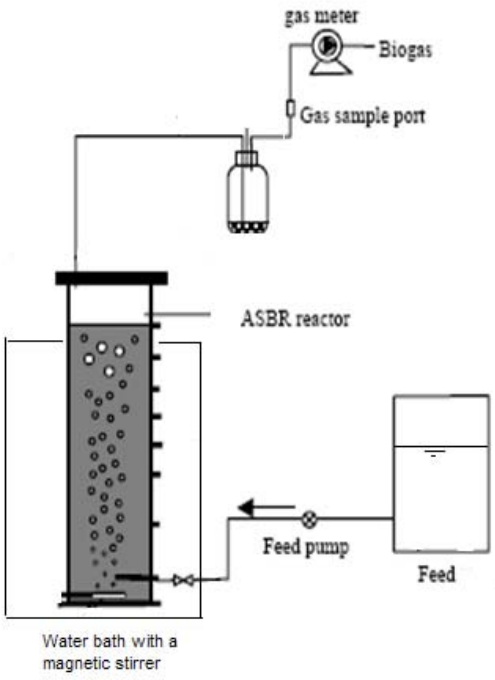


Figure 4.3. Schematic view of an ASBR

4.1.3. Single- phase and two-phase anaerobic sequencing batch reactors

Three laboratory-scale glass reactors with an active volume of 1.6 L each were used as anaerobic batch reactors. R1 was used as single-phase reactor, R2A for acidogenic phase and R2M for methanogenic phase (Figure 4.4). The three reactors were placed in a water bath to control the temperature at 35±2°C. Mixing was provided by magnetic stirrer which run at a speed of 90 rpm. The pH of the single-phase reactor and methanogenic reactor of two-phase was maintained between 6.8-7.2. pH of the acidogenic reactor of the two–phase system was maintained between 5.0-5.5.

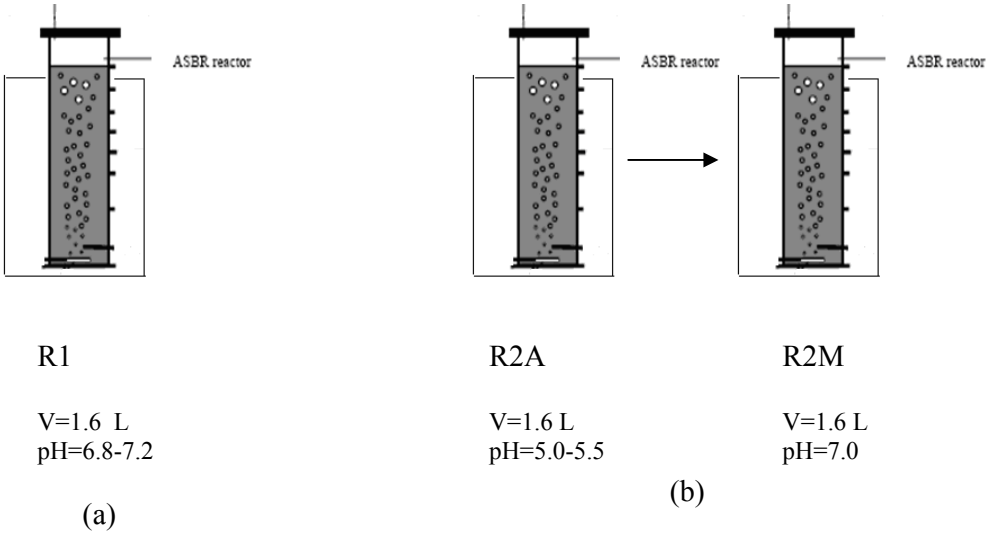


Figure 4.4. Schematic view of single-phase reactor (R1) system (a) and two-phase reactor (b) system (Acid reactor (R2A), Methane Reactor (R2M))

4.2. Seed Sludge

Seed 1 was obtained from a full-scale UASB reactor (with a volume of 143 m^3) being the first stage of a two-stage anaerobic-aerobic biological treatment plant at a local alcohol distillery. The temperature, pH and alkalinity in the UASB reactor were maintained within the ranges of 35-37°C, 6.4-7.5 and 1300-1500 mg$CaCO_3$/L respectively. Nutrients (nitrogen and phosphorus as $(NH_2)_2CO$ and KH_2PO_4, respectively) were mixed with the

effluent to give a COD:N:P ratio of 400:5:1 during anaerobic treatment. Total solid (TS) and total volatile solid (TVS) concentrations of the granular sludge was 154000-159000 mg/L and 142000-145000 mg/L, respectively.

Seed 2 was taken from a full-scale EGSB reactor (2280 m^3) used in the anaerobic stage of a two stage anaerobic-aerobic biological treatment system at a brewery. Volumetric loading rate of the EGSB reactor was 15 kgCOD m^3/d. The temperature, pH and alkalinity in the reactor were maintained within the ranges of 35-37°C, 6.4-7.5 and 1000-3000 mg$CaCO_3$ /L respectively. Total solid (TS) and total volatile solid (TVS) concentrations of the anaerobic granular sludge were 149000-169000 and 139000-158000 mg/L, respectively. The sludge was taken in May 2007 and used for seed sludge screening study. In August 2007, sludge was taken from the same treatment plant for the studies carried out selected solvents. Total solid (TS) and total volatile solid (TVS) concentration of the anaerobic granular sludge was 80000 mg/L and 70000 mg/L, respectively.

4.3. Wastewater Characteristics

A synthetic wastewater made up mainly of glucose as a sole carbon and energy source was used for ASBRs. The synthetic wastewater was buffered with $NaHCO_3$ and fortified with macro- and micro- nutrients, as described by Borja et al., 2001. Nutrients (nitrogen and phosphorus as $(NH_2)_2CO$ and KH_2PO_4, respectively) were added to the nutrient balance in the feed solution according to the C: N: P ratio of 400:5:1. The feed medium was prepared to 1 liter using $FeCl_2.4H_2O$, 2000 mg/L; $CoCl_2.6H_2O$, 2000 mg/L; $MnCl_2$, 318 mg/L; $CuCl_2$, 24 mg/L; $ZnCl_2$, 50 mg/L; H_3BO_3, 50 mg/L; $(NH_4)Mo_7O_{24}.4H_2O$, 90 mg/L; Na_2SeO_3, 68 mg/L; $NiCl_2.6H_2O$, 50 mg/L; EDTA, 1000 mg/L, HCl %36, 1 mL/L and resazurine 500 mg/L. 5 mL nutrient solution was added to per 1 L synthetic wastewater.

4.4. Analytical Methods

All biochemical analyses (Table 4.2) were carried out according to American Public Health Association APHA, 1995.

Table 4.2. Analysis carried out during the operation of anaerobic reactors.

PARAMETER	METHOD	INSTRUMENT
pH	4500-H B Method Electrometric (APHA, AWWA-WPCF-1995)	ORION SA 520 pH meter
COD	5220 D Method Closed Reflux, Colorimetric (APHA, AWWA-WPCF-1995)	HACH COD digester ; HACH DR/3 Spectrophotometer.
VFA	Gas Chromatograph	Gas Chromatograph HP 5890
Alkalinity	2320 B Method Titration (APHA, AWWA-WPCF-1995)	
TKN	4500 E Method Titration (APHA, AWWA-WPCF-1995)	Gerhardt Vapodest Digester Apparatus
Ammonia-N	4500 E Method Titration (APHA, AWWA-WPCF-1995)	Gerhardt Vapodest Disstillation Apparatus
Orthophosphate	4500-P E Method Ascorbic Acid (APHA, AWWA-WPCF-1995)	HACH DR/3 Spectrophotometer
SS/VSS	2540 Method (APHA, AWWA-WPCF-1995)	-
TS/TVS	2540 Method (APHA, AWWA-WPCF-1995)	-
Biogas	Milligas counter	Ritter (digital counter)
CH_4, CO_2	Gas Chromatograph	Gas Chromatograph HP 6850
Activity	Gas Measurement	Specific Methanogenic Activity Test

4.5. Methanogenic / Non-methanogenic Activity Tests

4.5.1. Methanogenic activity test

In this study, a fully computerized specific methanogenic activity (SMA) test unit originally developed by Monteggia (1991) and modified by Ince (1995) was used to determine acetoclastic methanogenic activity. The SMA test unit consisted of eight 2 L digestion flasks which are placed into a water bath to control the temperature stability. Mixing is provided by magnetic stirrers, which run at a speed of 90 rpm. Gas measurement system contains pressure sensors, miniature valves and tubing for interconnection between the anaerobic reactor and the other units. This system has eight selenoid valves. The valve which has 3 ports is controlled with a pressure measurement device which is set to a pressure value of 100 kpa. As the pressure inside the system reached a set value, the control system sent an electrical signal to a control interface that activated the three-way selenoid valve, simultaneously closing the second port (to maintain the pressure inside the reactor) and opened the third port to the atmosphere. This made the connection of bulb to the atmosphere, releasing the excess gas accumulated during the build-up in pressure. The selenoid valve was set so that the two normally open ports (1 and 2) communicate with the pressure measurement device. When the third port was closed, the pressure in the system increased progressively. The valve was deactivated after an interval of time (3 s for the complete release of the gases) and a new cycle was initiated. The test unit can simultaneously monitor the gas production of the eight independent digesters. The device used for calibration of the eight digesters with their respective gas flow meters will be carried out by using a very sensitive Health Care Pump.

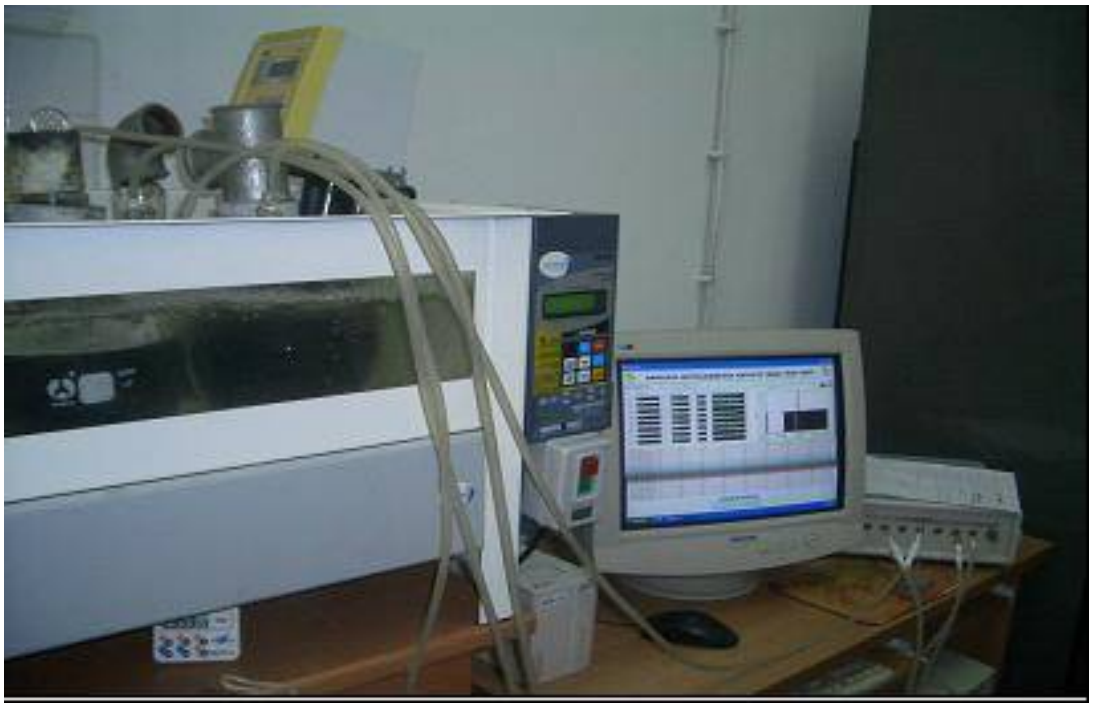


Figure 4.5. Photographic view of SMA test unit.

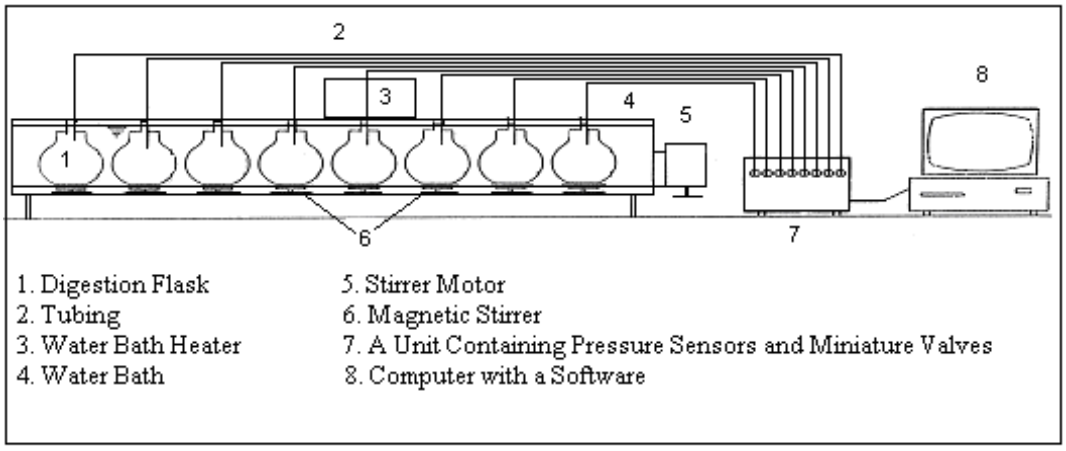


Figure 4.6. Schematic diagram of SMA test unit.

4.5.1.1. Experimental procedure of specific methanogenic activity test.

The procedure for the application of the SMA test and operation of the SMA unit is as follows:

1. Before starting the experiment (preferably 12 hour in advance), volatile suspended solids content (VSS) of the sludge is determined.
2. The seed sludges used for activity tests were diluted to 2000 mgVSS/L for SMA tests as described in the laboratory routine. The concentration of volatile suspended solid (VSS) in the reactors is brought about 2000 mg/L by diluting sludge sample with a mineral stock solution given in Table 4.2.
3. Sample sludge and mineral stock solution are taken to the digester flask.
4. The pH of the reactors is adjusted to 7.0.
5. Reactors are flushed with nitrogen gas with a pressure about 5-10 PSI for about 10 minutes to maintain anaerobic conditions in the reactor. The taps of the reactors are closed immediately after flushing and all connections of the SMA test unit are greased in order to prevent air leakage.
6. Water level in manometer is adjusted by using respirometer.
7. Temperature of the reactors content is maintained at 35±0.5°C by heating water bath with the heater in the unit.
8. The test sample is acclimatized the test sample for 12-16 hours. Gas production during the acclimation could be neglected.
9. Acetate solution with a pre-determined concentration is added to the reactor.
10. Mixing system is opened and data collection system is started. In the data collection system, biogas production is saved automatically for every hour.
11. Methane concentration is determined at regular intervals by taking 1 mL gas sample.
12. When the biogas production levels decrease to very low amounts, meaning that acetoclastic methanogenic activity is about to end, the system is stopped and collected data is used to calculate specific methanogenic activity.

Table 4.3. A mineral stock solution for SMA test (Valcke and Verstraete, 1983)

Chemical	**Final Concentration (mg/L)**
KH_2PO_4	2500
K_2HPO_4	1000
NH_4CI	1000
$MgCI_2$	100
$Na_2S.7H_2O$	100
Yeast extract	200

4.5.1.2. Substrate for methanogenic activity test. Acetate, propionate and butyrate concentrations between 1000-4000 mg/L have been tested for SMA tests. Optimum concentrations were found to be 2000 mg/L for acetate; 3000 mg/L for propionate; 4000 mg/L for butyrate, respectively. In addition, a VFA mixture (2000 mg/L acetate, 500 mg/L propionate, 500 mg/L butyrate) was used as substrate for determination of total methanogenic activity (Soto et al., 1993).

4.5.1.3. Calculation of specific methanogenic activity. The potential methane production is calculated by the formula expressed below:

$$SMA\ (mLCH_4/gVSS/d) = (A \times B \times C \times 24) / (D \times E) \quad (4.1)$$

A: Biogas production per hour, mL/hour
B: Methane content of biogas produced (%CH_4)
C: Valve factor
D: Active volume of the SMA test reactor (L)
E: Concentration of biomass in SMA test reactor (mgVSS/L)

4.5.2. Non-methanogenic activity test

4.5.2.1. Experimental procedure for non-methanogenic activity test. Non-methanogenic activity test procedure given by Soto et al., 1993, and Hutnan et al., 1999 was used. The

VSS of the sludge sample to be analyzed must be determined before the test is started (preferably 12 hour in advance). The concentration of VSS in the SMA reactors is brought about 2000 mg/L by diluting sludge sample with a mineral stock solution (Ince et al., 1995). pH of the reactors was adjusted to 7.0. Temperature of the reactor content was maintained at 35±0.5°C by heating water bath. The reactors were acclimatized for 12-16 hours. After the incubation period, substrate was added to the SMA reactors. For COD measurements 20 ml sample was taken from the reactors for every three hours.

4.5.2.2. Substrate for non-methanogenic activity test. For determination of the maximum acidogenic activity and hydrolytic activity, 1000-4000 mg/L of glucose and 1000-4000 mg/L sucrose concentrations were tested respectively.

4.5.2.3. Calculation of specific methanogenic activity. Acidogenic and hydrolytic steps were analyzed via COD removal rate (Hutnan et al., 1999). Calculations and activity expressions were presented in the work by Soto et al., (1993). The activity (Ac) is usually expressed as g COD per VSS per day and calculated from the substrate consumption rate (e. g. hydrolytic and acidogenetic phases).

$$Acs = -1/p \; dp(COD)/dt \qquad (4.2)$$

Acs = activity of the sludge, (mgCOD/mgVSS/d)
t: time (d)
ρ: density of the sludge

4.6. Molecular Tools for Identification of Microbial Community

4.6.1. Sample collection and preparation

Samples were collected from lab scale anaerobic reactors. For FISH studies, sludge samples were transferred into sterile containers with the addition of absolute ethanol (1:1 (v/v)) for FISH studies and fixed immediately, stored at -20ºC. For DNA-based molecular methods, the sludge samples were extracted and stored at -20ºC.

4.6.2. Fluorescent in situ Hybridization (FISH)

4.6.2.1. Short term fixation. Granular sludge samples were transferred into sterile containers with the addition of absolute ethanol (1:1 (v/v)). Samples were stored at -20°C and fixed within a week.

4.6.2.2. Standard paraformaldehyde (PFA) fixation. 500 µl of granular sludge-ethanol mix (1:1 (v/v)) was washed once with phosphate-buffered saline (PBS) [130 mM NaCl, 10 mM sodium phosphate, pH 7.2] and resuspended in 0.25 ml of PBS. 0.75 ml of freshly prepared 4% PFA in PBS (pH 7.2) was added to the suspension and incubated for at least 3 hours, or overnight, at 4°C. After fixation, cells were washed once with PBS, resuspended in 1.5 ml of PBS-absolute ethanol (1:1, v/v) and stored at -20°C.

4.6.2.3. Hybridization. 200 µL of the fixed samples were washed once with PBS and dehydrated at room temperature in increasing concentrations of ethanol (50, 80 and 100%). Dehydrated samples were resuspended in 40 µL of hybridization buffer (0.9M NaCl, 2 mg/mL Ficoll, 2 mg/mL Bovine Serum Albumen, 2 mg/mL polyvinyl pyrrolidone, 5 mM EDTA, pH 8.0, 25 mM NaH_2PO_4, pH 7.0, 0.1% SDS, 5-35% deionised formamide) and prehybridized at the intended hybridisation temperature for 20 minutes. After prehybridisation, 2 µL of probe (50 ng/µL) was added and incubated at the optimal hybridisation temperature for the given probe for at least 4 hours or overnight. Following hybridization, the cells were washed twice in a wash buffer containing 20 mM Tris-HCl (pH 7.2), 0.01% SDS, 0-5 mM EDTA and between 0.9 M and 56 mM NaCl according to the formula of Lathe (26) for 15 min at the optimal washing temperature before a final wash in MilliQ water. The cells were resuspended in 200 µL of MilliQ water, and a 10 µL aliquot was placed on a gelatin-coated slide and air dried. One drop of Citifluor antifadent (Citifluor Ltd.) was added to the sample, and a coverslip was applied to the preparation and sealed with nail polish before epifluorescence microscopy.

16S rRNA-targeted oligonucleotide probes used in this study and their target microbial groups nucleotide sequences are listed in Table 3.3. Optimal hybridization

conditions for each probe are also given in Table 4.5. All probes were made, labelled, and obtained commercially (Qiagen Corp.). For each sample hybridization, two negative controls were prepared; one of these controls was used to assess non-specific binding (containing the non-sense probe Non338 or ANTIEUB (S-D-Bact-0338-a-S-18; Manz et al., 1992, which is reverse to the Bact338 probe (also known as EUB338). This probe does not hybridise with any known bacteria). and the other (lacking a probe) was used to monitor autofluorescence. In addition to negative controls, one positive control was prepared to assess success of cell permeabilization and rRNA content of the cells (with universal probe UNIV1392). Whole microbial community in the sludge samples was also stained using DAPI to visualize intact cells in the samples.

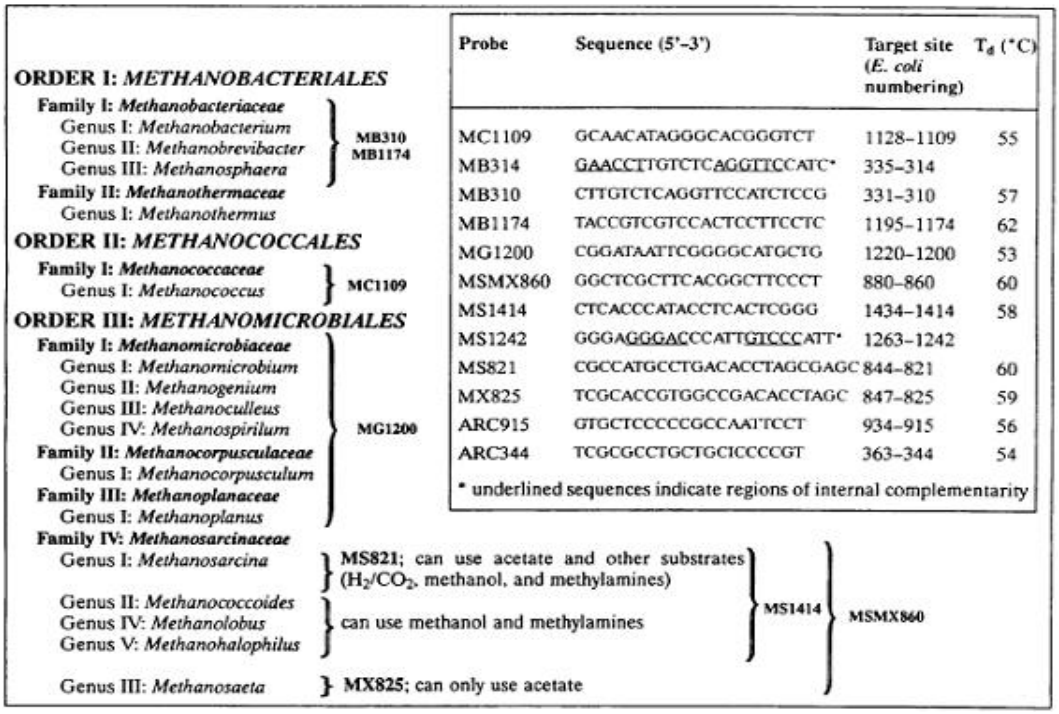
Table 4.4. 16S rRNA-targeted oligonucleotide probes used in this study

Probe	**Target Group**	**Prob dizilimi (5'-3')**	**Labelling (5')**	**Reference**
MC1109	*Methanococcales*	GCAACATAGGGCACGGGTCT	CY3	Raskin et al., 1994
MB310	*Methanobacteriales*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin et al., 1994
MG1200	*Methanogenium* relatives	CGGATAATTCGGGGCATGCTG	CY3	Raskin et al., 1994
MS1414	*Methanosarcina* + relatives	CTCACCCATACCTCACTCGGG	CY3	Raskin et al., 1994
MS821	*Methanosarcina*	CGCCATGCCTGACACCTAGGCCAGC	CY3	Raskin et al., 1994
MX825	*Methanosaeta*	TCGCACCGTGGCCGACACCTAGC	TAMRA	Raskin et al., 1994
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	CY3	Stahl et al., 1988
UNIV1392	Virtualy all known organisms	ACGGGCGGTGTGTAC	TAMRA	Pace et al., 1986
NON338	Non sense probe	ACTCCTACGGCAGGCAGC	TAMRA	Manz et al., 1992
EUBMIX	*Bacteria*	GCTGCCTCCCGTAGGAGT	Fluorescein	Amann et al.,1990

Table 4.5. Optimum hybridization conditions for oligonucleotide probes (Kolukirik, 2004)

Probe	Formamide concentration (%)	Hybridization temperature (°C)	Washing temperature (°C)	NaCl Concentration (mM)
MC1109	20	46	48	225
MB310	20	46	48	225
MG1200	30	46	48	112
MS1414	35	46	48	84
MS821	20	46	48	225
MX825	20	46	48	225
ARC915	35	46	48	84
EUB338	10	46	46	450
UNIV1392	10	37	37	450

Table 4.6. Classification of methanogens in relationship to the oligonucleotide probes used in this study.



4.6.2.4. DAPI Staining. The total cells present in the samples were determined by counting 42,62-diaminephenylindol (DAPI) stained cells. 200 µL fixed samples were put into the eppendorph tubes and centrifuged at 13000 rpm for 3 minutes. After the centrifugation, 500 µL 1XPBS was added to tubes and resuspended by syringe. Then, the mixture was centrifuged at 13000 rpm for 3 minutes again. Following centrifugation, supernatant was put out without destroying the pellet. 500 µL 1XPBS was added to the tube and resuspended secondly. The supernatant was put out and 500 µL MQ water was added to tubes for the dilution. After the suspension, 20-30 µL samples were taken on each well and dried in the incubator. The slides were dehydrated in the ethanol series (50%, 80%, 100%) for 3 minute at each concentration. After the dehydration, 49 µL 1XPBS, then 1 µL DAPI stain was added on each well. The slides were kept in the dark at room temperature for 30 minutes. After that, slides were washing into two washing buffer (40 mL 1XPBS) for 7 minutes in each of them. Finally, slides were put in two 40 mL MQ water for 1 minute in each of them. Slides were dried in incubator and covered with lamel by enamel.

Sludge samples were initially stained by DAPI before hybridization to observe intact cell concentration. For each sample hybridization, two negative controls were used; one of these controls was used to assess nonspecific binding (with Non338 probe), and the other (lacking a probe) was used to monitor autofluorescence. In addition to negative controls, one positive control was used to assess success of cell permeabilization and rRNA content of the cells (with universal probe UNIV1392). Whole microbial community in the sludge samples was also stained using DAPI stain to visualize intact cells in the samples. 10 random fields of views were used for each quantification study. As mentioned above, before hybridization, DAPI staining was applied to the sludge samples to indicate intact cell concentration. 1/40 dilution factor and 5 µLsample volume were decided in accordance with the DAPI staining applied to the seed sludge from EGSB reactor. These dilution factors were decided to be optimum and applied to the FISH analyses that are done during the study. DAPI results of the seed sludge are shown in Appendix B.

4.6.2.5. Visualisation. Slides were examined under Olympus BX 50 Epifluorescence Microscope equipped with a 100 W high-pressure mercury lamp, U-MWIB and U-MWG filter cubes. Images were captured using a Spot RT charged coupled device (CCD) camera having special software supplied by the camera manufacturer (Diagnostic Instruments Ltd., UK). The images were processed and analyzed using Image-Pro Plus version 5.1 image analysis software (Media Cybernetics, U.S.A.). Different fluorochromes are excited and emitted at different wavelengths. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used in this study are given in Table 3.5.

Table 4.7. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used in this study

Fluorochrome	**Color of Fluorescence**	**Maximum excitation wavelength (nm)**	**Maximum emission wavelength (nm)**	**Filter cube used**
CY3	Red	550	565	U-MWG
DAPI	Blue	365	397	U-MWIB

4.6.2.6. Quantification. Quantification of microoorganisms in the sludge samples collected during three different periods was conducted by using version 5.1 of Image-Pro Plus image analysis software. Quantification involves counts of total microorganisms with DAPI staining and counts of specific methanogenic groups with other oligonucleotide probes using FISH.

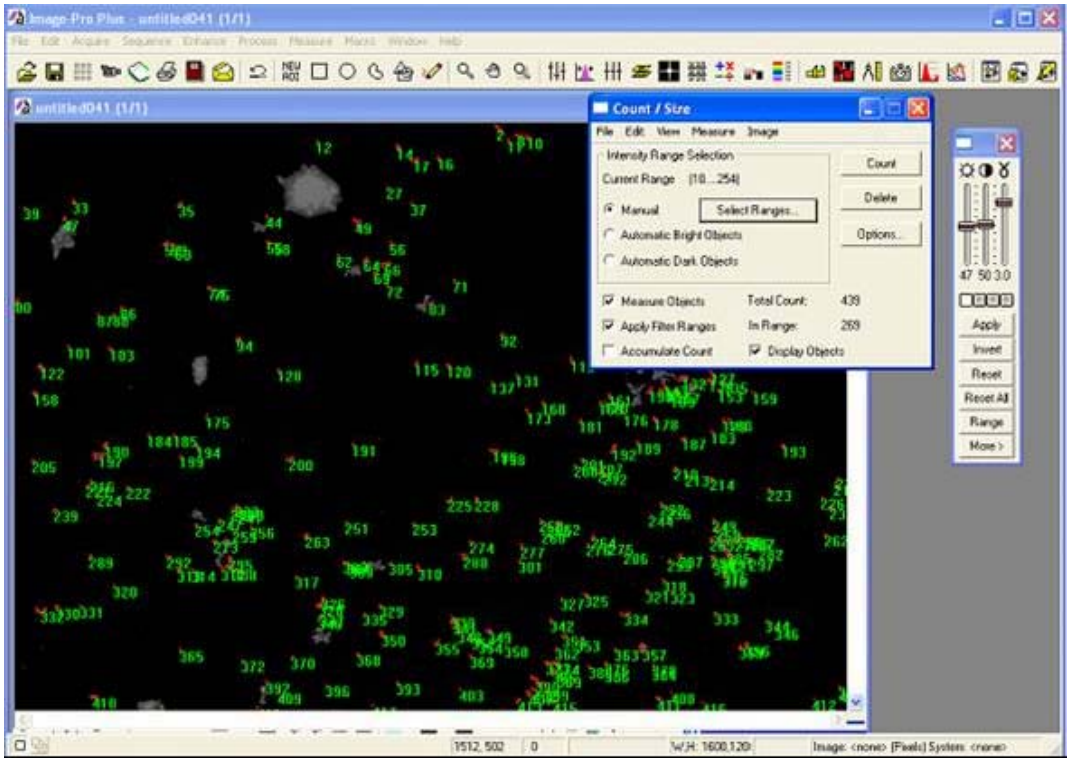


Figure 4.7. Capture image of Image Pro Plus 6.3.

4.6.3. DNA-based molecular analysis

4.6.3.1. Genomic DNA Extraction. DNA was extracted from 0.5 g sample by using Fast DNA Spin Kit for Soil (Q-Biogene, Bio 101 Thermo Electron Corporation, Belgium) and a Ribolyser (Fast PrepTM FP120 Bio 101 Thermo Electron Corporation, Belgium) according to the manufacturers' instructions. The methodology of Genomic DNA extraction of by Fast DNA Spin Kit for Soil was as follows: Approximately 0.5 g sludge was added up to lysing matrix tubes provided by the kit. The tube contains mixture of ceramic and silica particles to lyse all microorganisms in sample. Then lysing matrix tubes

were spinned in Ribolyser (Fast PrepTM FP120 Bio 101 Thermo Electron Corporation) for 30 seconds at speed of 5.5 m/s. The tubes were then centrifuged at 14000Xg for 30 seconds. After centrifugation supernatants were transferred to clean 1.5 mL eppendorf tubes and added 250 µl PPS reagent. To mix the composition tubes were shaked by hands for 30 seconds. After mixing the tubes centrifuged again at 14000Xg for 5 minutes to pellet the precipitate. Supernatants were transferred to 2 mL eppendorf tubes and 1 mL of Binding Matrix Suspension was added to supernatant. Tubes were inverted by hand for 2 minutes to allow binding of DNA to matrix. To settle the silica matrix tubes were incubated 3 minutes at room temperature. 500 µL of supernatant was removed carefully without disturbing settled silica matrix. Then the binding matrix was resuspended in the remaining supernatant. All mixture was filtered and filter was placed to a new tube. Filter was washed by 500 µL SEWS-M wash solution. After washing, filter was dried by centrifugation at 14000Xg for 2 minutes. Filter was removed to a new tube and 50 µL DES (DNase/Pyrogen free water) was added. The filter with DES was vortexed and then centrifuged at 14000Xg for 1 minute. Application-ready DNA was obtained in the tube. 1/100 diluted genomic DNA was run on the %1 (w/v) agarose gel, prestained with Ethidium Bromide (EtBr) in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA, pH 8) at 7 V/cm. Gel was visualized by using a gel documentation system, Chemi-Smart 3000 (Vilber Lourmat, France).

4.6.3.2. Polymerase Chain Reaction (PCR). Amplification of 16S rDNA gene sequences was performed by PCR using archaeal and bacterial specific primers. Primers used in this study are given in Table 4.8. Bact8f-Bact1541r and Arch07f-Arch1384r primers were used for the amplification 16S rDNA of bacteria and archaea respectively. Extracted GDNAs were used as a template for these primers. Arch07f-Arch1384r PCR product was further used as a template for PCR using Arch344f-Univ522r. Bact8f-Bact1541r and Arch07f-Arch1384r amplification products were also used for cloning and sequencing analysis. Bact341fGC-Bact534r and Arch344f-Univ522r primers were used to amplify approximately 200 bp region of 16S rDNA of bacteria and archaea. Extracted GDNAs were used as a template for PCRs using Bact341fGC-Bact534r primers and Arch07f-Arch1384r PCR products were used as a template for amplification using Arch344f-Univ522r primers. The PCR products were used in DGGE analysis. PCR reactions were performed in a 50 µL (total volume) mixture containing 0.2 µM forward primer, 0.2 µM

reverse primer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1U of Taq polymerase enzyme and the buffer supplied with the enzyme (Fermentas Life Sciences), and 1 µL of template. Amplification was performed with a thermal cycler. (TECHNE-TC 512) PCR programs in thermal cycler were given in Table 4.8. Products of all reactions were screened for the amplification of correct band size. All PCR products were run on the %1 (w/v) agarose gel prestained with Ethidium Bromide (EtBr) in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA; pH 8). Gels were visualized by using a gel documentation system, Chemi-Smart 3000 (Vilber Lourmat, France).

Table 4.8. Bacterial and archaeal oligonucleotide primers used for PCR amplification

Primer	Target	Experimental Stage	Annealing (°C)	Position[1]	Reference
Bact341f_GC[2]	Bacterial 16S rDNA	DGGE	55	341-357	Muyzer et al., 1993
Bact534r				534-518	
Bact8f		Cloning		8-27	Edwards et al., 1988
Bact1541r				1541-1522	
Bact342f		Sequencing		342-361	
Arch07f	Archaeal 16S rDNA	First round of nested PCR - Cloning	52	07-24	Lueders et al., 2004
Arch1384r				1384-1368	
Arch344f_GC[2]		DGGE	53	344-358	Raskin et al., 1994
Arch855r				855-836	Shinzato et al.,1998
Univ522r				522-504	Amann et al., 1995
M13f	β-galactosidase	Clone screening	54	–	Schrenk et al., 2003
M13r					

[1]*Escherichia coli* numbering.
[2]5′-GC clamp on Arch344f and Bact341f (GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGG).

Table 4.9. PCR conditions used in the study

Primers	**Denaturation (°C)**	**Time (s)**	**Annealing (°C)**	**Time (s)**	**Elongation (°C)**	**Time (s)**	**#of cycles**
Bact8f-Bact1541r Bact341fGC-Bact534r Bact342f	94	45	55	45	72	60	30
M13f-M13r	94	45	55	45	72	60	30
Arch07f-Arch1384r	94	45	52	45	72	60	35
Arch344-Arch855 Arch344-Univ 522	94	30	53	30	72	60	35

4.6.3.3. Denaturant Gradient Gel Electrophoresis (DGGE). The first step was the assembly of the perpendicular gradient gel sandwich. The thickness of the sandwich was established by using 1 mm spacers between two glass plates which provided by the instrument. Before assembly, glass plates were cleaned carefully to avoid any particle matter which may affect the gel. The position of spacers were checked to avoid any leakage and glass plate sandwich then placed on the casting stand. The next step was preparation of the denaturing gradient gel. For bacterial DGGE, 10% (w/v) acrylamide:bisacrylamide 30% denaturant solution was prepared by mixing 33.3 mL of %30 acrylamide:bisacrylamide with 2 mL 50XTAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid) and 12 mL formamide and 12.6 g Urea. 60% of denaturant concentration was reached by adding 24 mL formamide and 25.2 g urea to 33.3 mL of %30 acrylamide: bisacrylamide and 2 mL 50XTAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid).Both solutions were added distilled water up to 100 mL.

For archaeal DGGE, 10% (w/v) acrylamide:bisacrylamide 40% denaturant solution was prepared by mixing 33.3 mL of 30% acrylamide:bisacrylamide with 2 mL 50xTAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid) and 16 mL formamide and 16.8 g urea. 70% of denaturant concentration was reached by adding 28 mL formamide and 29.4 g urea to 33.3 mL of 30% acrylamide: bisacrylamide and 2 mL 50xTAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid). 100% denaturant solution is defined as 40% (v/v) formamide and 7 M Urea. Both solutions were added distilled water up to final volume of 100 mL.

After solutions were prepared, they were sonicated for 10 minutes and filtered with 0.45 μm filter. The bottles were wrapped with foil paper to avoid sunlight and stored at 40 C for further uses. Into two beakers, 25 mL of 10% (w/v) acrylamide: bisacrylamide solutions containing 30% and 60% (40% and 70% for archaeal samples) denaturants were poured. To both solutions, 100 mL freshly prepared 1% ammonium per sulfate (APS) and 10 mL TEMED was added and immediately solutions were poured U-tube provided by instrument. Gradient maker was operated and gradient formation was established. When the gel sandwich was filled, combs were placed carefully to avoid any bubble formation. The equipments were cleaned immediately with distilled water to prevent any

polymerization in the capillaries. The polymerization was depending directly to the amount of APS and TEMED in solutions; usually took 60-90 minutes at room temp. During polymerization, electrophoresis tank was filled with 1XTAE until marked level and temperature was set to 60°C.

Figure 4.8. DGGE equipment used in the study (Ingeny phorU)

Sample loading step was started with preparation of samples. 4 µL of loading dye was mixed with 7 µL of PCR product to be run. The comb was removed and polymerized gel sandwiches in the core were inserted into the preheated tank. The wells were washed with 1XTAE buffer to avoid any early denaturation due to presence of denaturants in wells. The samples were carefully loaded into the wells. The DGGE was conducted at a constant voltage of 200 V at 60°C for 360 minutes in 1XTAE containing electrophoresis tank.

The last step was staining and visualizing gels. The core was taken from the tank and gel sandwiches were separated from it. Glass plates were disassembled and the direction of gel was marked with a cut on the upper left corner. 30 µL of 1:100000 diluted SYBR Gold DNA staining dye was added to 300 mL 1XTAE washing buffer and gels were incubated for 30 minutes. Gels were distained and washed three times with distilled water to remove background. Again gels were visualized using a gel documentation system, Chemi-Smart 3000 (Vilber Lourmat, France).

4.6.3.4. Analysis of DGGE Gels Using Bionumerics. DGGE gels were analyzed by using Bionumerics 5.0 software (Applied Maths, Kortrijk, Belgium) to determine the

phylogenetic relationship between the samples. Presence-absence data of DGGE fingerprint were created based on similarity matrix obtained using Bionumerics software. Band intensity analysis was done automatically by the software if selected. Bionumerics software also used to create relation analysis tree from DGGE gels photos. Different correlation analysis can be performed with the software). Similarities between tracks were calculated by using the Dice coefficient (S_D) (unweighted data based on band presence or absence) and band-independent, whole-densitometric-curve-based Pearson product-moment correlation coefficients (r) and Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) clustering.

Dice coefficient. For analysis using Dice coefficient a band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at100%. The Bionumerics package used the Dice algorithm with the numbers of common and total bands to generate similarity coefficients between banding patterns. For two banding patterns A and B, the Dice similarity coefficient is calculated as,

$$\text{Similarity}_{\text{Dice}} = 2x\Sigma AB \,/\, [(2x\Sigma AB]+(\Sigma Ab)+(\Sigma aB)] \qquad (4.3)$$

where AB is the number of bands common to both banding patterns, Ab is the number of bands found in banding pattern A but not banding pattern B, and aB is the number of bands found in banding pattern B but not banding pattern A. The Bionumerics program was used to calculate the best optimization and tolerance values for the comparison. This index ranges from 0 (no common bands) to 1 (identical band patterns). Within Bionumerics, optimization refers an adjustment of bands beyond normalization and was necessary when imperfect normalization resulted in residual shifts. Likewise, tolerance refers to the total distance that bands in different lanes differed by before they were determined to be distinct. The default values were used for optimization and tolerance and were 0.5 and 0.7%, respectively. Fuzzy logic was employed when determining similarity scores. Use of fuzzy logic in Bionumerics results in bands being scored as identical only when there was zero distance between them, meaning that with additional distance (in pixels) in band position, the matching score was decreased until the maximal position tolerance was reached, after which the similarity score was zero. This allowed larger

tolerances to be chosen while still obtaining meaningful clustering. Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation coefficient [Häne et al., 1993].

Unweighted Pair-Group Method with Arithmetic Mean (UPGMA): UPGAMA is an example of clustering method and has built on the assumption that tree is additive. Thus, all taxa are equally distant from a root in UPGMA tree which is an unlikely assumption.

Neighbour Joining Method (NJ). NJ is similar to UPGMA in manipulating a distance matrix as reducing it in size at each step, and then reconstructs the tree from that series of matrices. It differs from UPGMA in that it does not construct clusters but directly calculates distances to internal nodes. NJ does not assume that all taxa are equally distant from a root. NJ is like parsimony, a minimum-change method, but it does not guarantee tree with the smallest overall distance.

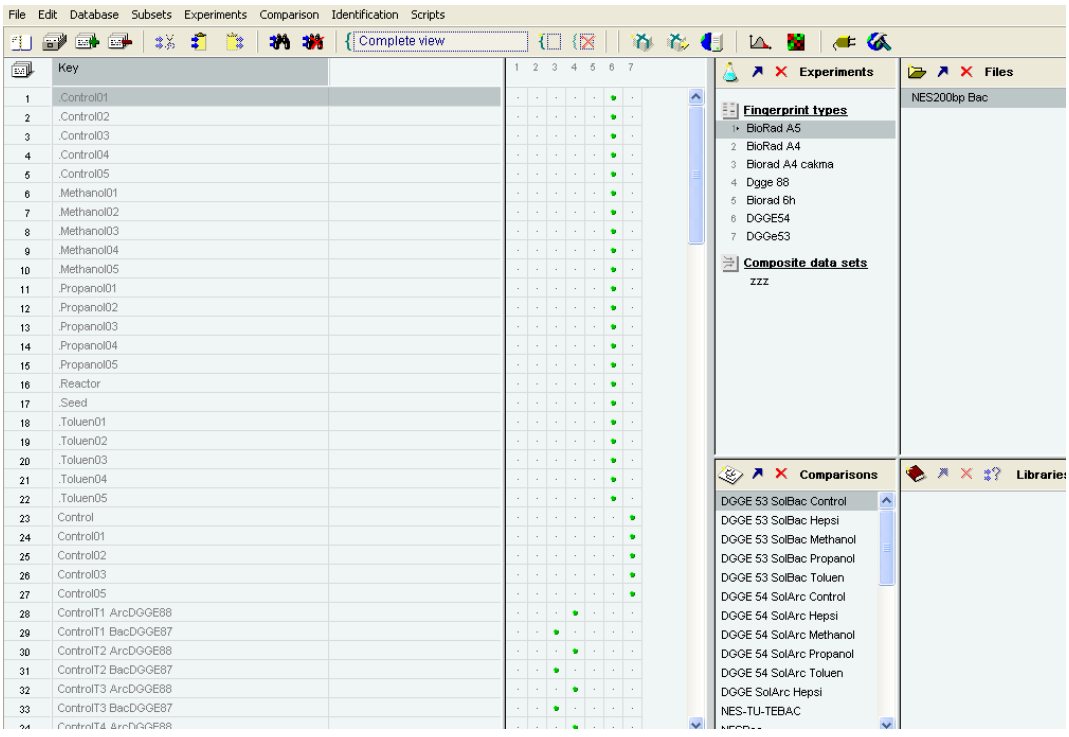


Figure 4.9. Main operations window of Bionumerics software.

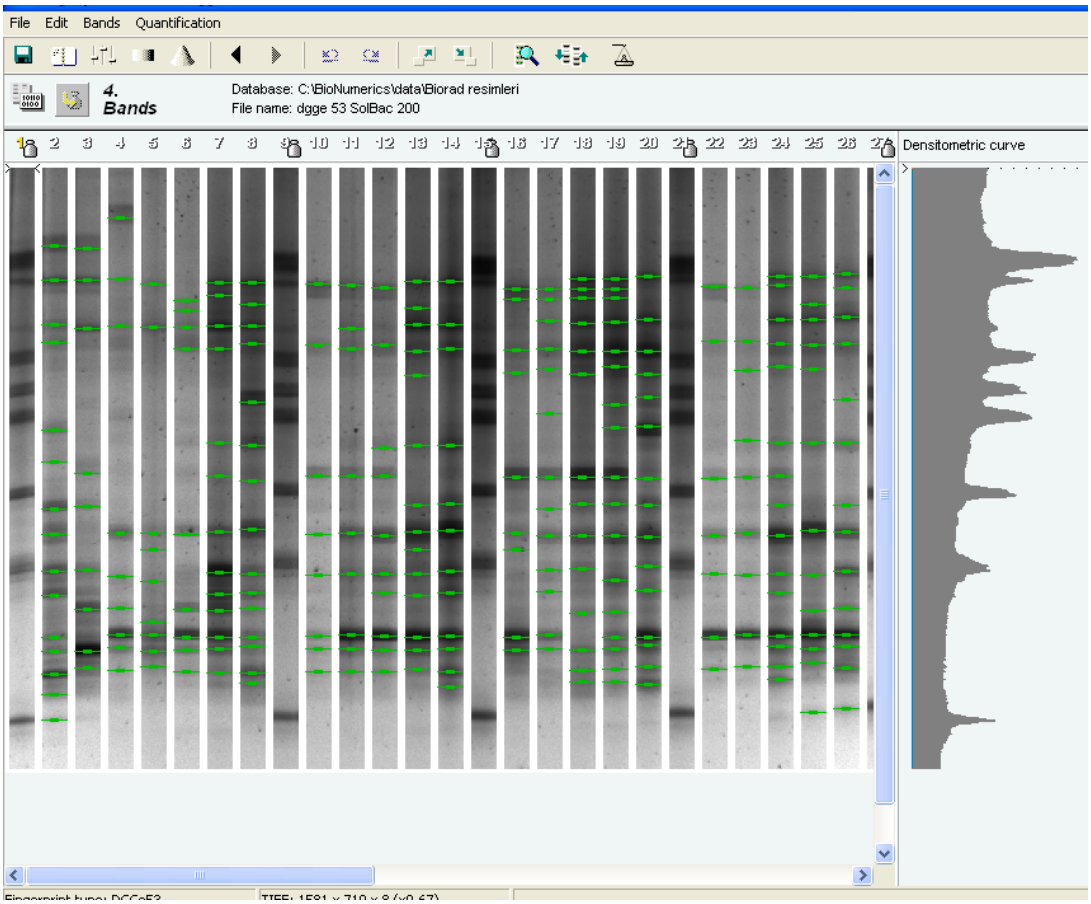


Figure 4.10. Selection of bands in fingerprinting of photos window.

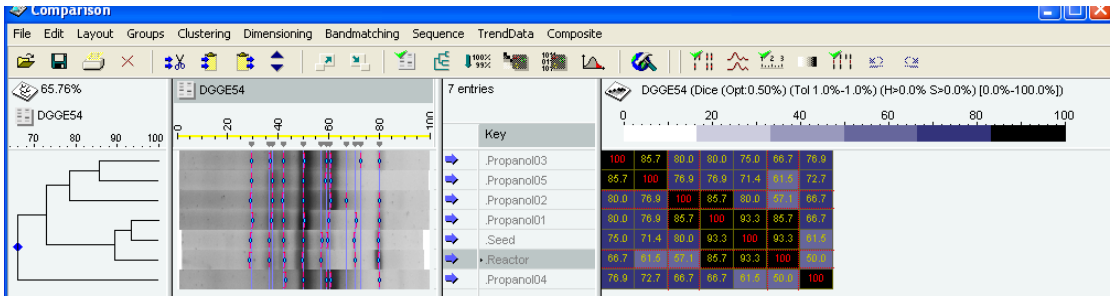


Figure 4.11. Relation analysis window of Bionumerics software.

4.6.3.5. Generation of 16S rDNA Cloning Library. A clone library of 16S rDNA was generated to identify microorganisms present in the sample. Two clone libraries, a bacterial and an archaeal, were generated to analyze microbial communities from Seed sludge sample. Whole bacterial 16S rDNA (ca.1.5 kb) was amplified by using primers Bact8f-Bact1541r, as described above. PCR products were cloned with a TOPO TA cloning kit (Invitrogen Ltd,) and gene libraries were screened by DGGE. Nearly whole archaeal 16S rDNA (ca.1.3 kb) was amplified by using primers Arch07f-Arch1384r, as described above. PCR products were cloned with a TOPO TA Cloning Kit for Sequencing (Invitrogen Ltd,) and gene libraries were screened by DGGE. The procedure of generating 16S rDNA Clone Library was as follows:

The process was started with preparing 6 µL reaction mix by adding 3 µL PCR product, 1 µL salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), 1 µL TOPO vector and 1 µL Sterile Water. The solution was mixed gently and incubated at room temperature (R.T.) for 20 minutes. After incubation reaction mix was placed on ice before its usage in One Shot TOPO transformation step. One shot TOPO transformation was started by thawing one vial of One Shot TOPO reaction mix on ice. After thawing, 2 µL of reaction mix was added to One Shot vial. The solution was mixed gently without pipetting or shaking. The solution was incubated on ice for 30 minutes. After incubation, tube was subjected to a heat shock at 42°C for 30 seconds and transferred immediately to ice and 300 µL of S.O.C. medium at R.T. (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The solution was shaked horizontally for 60 minutes. Three LB plates containing 50 µg/mL Kanamycin were warmed to R.T. 100 µL of solution was spread on plates using glass spreader. The plates were incubated overnight and white colonies were observed after incubation. Colonies were picked from plate and transferred into 200 µL PCR tubes containing 50 µL TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). Colonies were boiled at 95°C for 5 minutes then frozen at -20°C overnight. Thawed solution was used as templates for PCR. The 16S rDNA fragments were isolated from vector by PCR with primers M13f-M13r (M13 Forward 5′-GTA AAA CGA CGG CCA G-3′/ M13 Reverse 5′-CAG GAA ACA GCT ATG AC-3′). From PCR products of reaction with M13f-M13r primers nested PCRs were done to screen the clones in DGGE. Nested PCRs were performed for bacterial and archaeal separately as described above. The

screening of gene library was done with DGGE. The gels were stained with 25 µL EtBr (10 mg/mL) instead of SYBR Gold.

4.6.3.6. Phylogenetic analysis of microbial community. Phylogenetic analysis of microbial community was done by sequencing clones and drawing phylogenetic tree by Treecon software (Treecon for windovs 1.3b. University of Konstanz, Germany). Sequencing PCR was done using dominant clone DNA as template. Reaction was held by mixing 4.7 µL dIwater, 1 µL template, 0.3 µL primer, (10 pM of pC primer [5'-CTA CGG GAG GCA GCA GTG GG-3'] was used for bacterial clones and Arch 344f primer was used for archaeal clones) 2 µL Big Dye Reaction mix and 2 µL Big Dye Reaction buffer. PCR program was as follows:

denaturation at 95°C for 5 minutes,
denaturation at 95°C for 30 seconds
annealing at 55°C or 53°C for 30 seconds
elongation at 60°C for 4 minutes
} 30 cycles

Products of PCR were then purified according to following protocol:

PCR products were mixed with 2 µL 3 M Sodium Acetate (NaAc) and 50 µL of 95% ice cold ethanol. The mixture was incubated for 30 min on ice and centrifuged for 30 min at 14000 rpm. Supernatant was discarded and pellet was resuspended in 250 µL 70% ice cold ethanol and centrifuged for 30 min at 14000 rpm. Supernatant discarded and pellet was dried at 95°C for 5 minutes. Pellet was resuspended in 20 µL formamide and denatured at 95°C for 3 minutes. Then the mixture were loaded to sequencer (A 3130 Sequencer, Abi Prism, USA) and sequenced automatically. Partial 16S rRNA gene sequences were analyzed and manually edited in Chromas software package version 1.45 (http://www.technelysium.com/au/chromas.html). The 16S rDNA sequences were checked for chimerical constructs by using the CHECK-CHIMERA program of the Ribosomal Database Project (Cole et al., 2007). Homology searches of the EMBL and GenBank DNA databases for the 16S rRNA gene sequences were performed with FASTA (Pearson, 1988) provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html) to identify putative close phylogenetic relatives.. Distance analyses using

the Jukes and Cantor (1969) correction and bootstrap resampling (1000 times) were done using the TREECON package (van De Peer and De Wachter, 1997) and trees were generated from distance matrices using the neighbour-joining method (Saitou and Nei, 1987). 16S rRNA gene sequences showing 95% similarity or higher were considered to belong to the same phylotype. Related 16S rRNA gene sequences were placed within tentative taxa (between Phylum and Order) by determining the taxonomic class (using the NCBI taxonomy database) of the closest relative in GenBank of sequences that formed a phylogenetic clade. Sequences that showed no or low (below 70%) relation with known bacterial or archaeal phylogenetic groups were listed as unclassified. The distribution of clone types present in the clone libraries was determined and used to calculate the Shannon-Weaver index $\{H = -\Sigma[n_i \cdot \log(n_i)]$, where n_i is the relative contribution of clone type i to the whole library$\}$. Coverage was calculated as $1 - (n_1/N)$, where n_1 is the number of clone types that was encountered only once in the library and N is the total number of clones analyzed. The Chao1 estimator of species (here, clone type) richness (S_{chao1}) was calculated as; $S_{obs} + n1^2/2n_2$, where S_{obs} is the number of observed different clone types, n_2 is the number of clone types encountered twice in the library and m is n_1/n_2.

The partial 16S rRNA sequences reported in this study were submitted to the EMBL database under accession numbers FM212973-FM212985.

5. RESULTS AND DISCUSSION

In the first section of the results and discussion part (5.1), different anaerobic sludges were screened in terms of methanogenic activity and microbial diversity in order to select most appropriate inoculum sludge for the anaerobic reactors. Successful start-up and operation of anaerobic reactors requires a seed sludge with a well-balanced microbial community including diverse bacterial species and high methanogenic activity. A special emphasis was given to this part of the study since characteristics of the sludge would be a determinative factor during the reactor studies fed with solvents. The candidate seed sludges were taken from a UASB reactor treating alcohol wastewater and a full-scale EGSB reactor treating brewery wastewater (seed sludge taken in May 2007).

In section 5.2, studies including methanogenic activity and microbial diversity for the selected seed sludge were repeated in order to set initial sludge characteristics since the inoculum sludge used throughout the studies as explained in the following sections, was taken from the EGSB reactor in August 2007.

In section 5.3, IC_{50} concentrations of selected solvents including toluene, methanol and isopropanol on acetoclastic methanogenic activity, which represents the most vulnerable step of the anaerobic digestion process to the inhibitory compounds- and microbial community of the anaerobic seed sludge, have been investigated.

In section 5.4, three anaerobic batch reactors-single phase and two-phase including acid and methane reactors were operated with a glucose-based synthetic wastewater. At the end of the operation period, methanogenic activity and microbial diversity of the reactors were determined by SMA and FISH, respectively.

In section 5.5, effects of IC_{50} concentrations of the selected organic solvents on anaerobic sludges taken from single phase and two-phase reactors have been evaluated in order to understand the influence of solvents on the sludge.

In sections 5.6, 5.7 and 5.8, anaerobic sequencing batch reactors were fed with toluene, methanol and isopropanol containing synthetic wastewater, respectively and microbial community dynamics were monitored using DGGE during the operation period. Fluorescence in situ hybridization (FISH) was also applied, to quantitatively assess the abundance of selected microbial groups during the operation period of the reactors fed with organic solvents.

In the last section of results and discussion part (5.9), the molecular results of anaerobic sludges obtained from ASBRs fed with the solvents were compared since the solvents can be found together in some specific wastewaters such as pharmaceutical wastewaters.

5.1. Seed Sludge Screening

In anaerobic reactors, start-up is one of the most critical duration of process operation. It has been stated that poor start-up in anaerobic reactors can lead to ineffective removal of organic matters (Griffin et al., 1998), or a prolonged period of acclimation (Wu et al., 2001). During reactor start-up, biomass should be acclimated to new reactor conditions such as type of wastewater, operating conditions, reactor type in order to establish a proper community structure and to maintain a new equilibrium among different microbial populations. Therefore, start-up periods generally can take a long period of time. The time required for acclimation of microorganisms to new environment can be shorter if adequate inoculum in terms of amount and quantity is available (de Zeeuw, 1984). Therefore, in order to select an inoculum, potential seed sludge should be characterized with respect to its metabolic capacity to anaerobically transform the main intermediates of the anaerobic degredation process. Anaerobic processes can be considered as a network in which a number of fermentative, syntrophic, and methanogenic populations work together as a community to convert organic substrates to methane. Therefore, stable and efficient operation of anaerobic reactors depends primarily on the growth and maintenance of sludge containing all the microbial trophic groups necessary for complete methanogenic degradation of the organic constituents of the wastewater and their methanogenic activity (Grotenhuis et al., 1991; Brito et al., 1997; Ince et al., 1995). Importance of acetoclastic methanogens in the stability and performance of anaerobic reactors has been reported previously (Oz et al., 2003; 2004). A sufficient quantity of active methanogenic populations should be maintained within an anaerobic reactor in order to obtain high performance from anaerobic reactors (Ince et al., 1995; Oz et al., 2004). Before start up, the choice of anaerobic inoculation sludge should be made using to the activity measurements which has a great potential for classifying biomass in terms of methane production potential thereby making a reliable operation possible.

Besides activity measurements, characterization of microbial community structure of anaerobic sludge may help to achieve a more rational reactor start-up. In the start up period, determining the diversity and functioning of the complex microbial communities and defining the links within each key group in biological systems may help to design optimized biological treatment systems thereby ensuring to avoid process failure. With

advanced molecular techniques, microbial groups within a mixed population can be identified at different phylogenetic levels (Amann et al., 1995; Raskin et al., 1994). The techniques also allow the investigation of spatial and temporal community changes within engineered biological systems (Briones et al., 2003). Application of both qualitative and quantitative molecular methods have led to new insights into microbial processes in anaerobic reactors. Since types of methanogenic species and their relative population levels in the bioreactors rely on wastewater characteristics as well as operational/environmental conditions maintained in an anaerobic reactor (Novaes, 1986), more research is needed to link the microbial ecology to reactor performance and activity of reactor sludge. A few number of studies have particularly investigated the start-up of a variety of anaerobic reactors through monitoring of microbial community dynamics using molecular-based methods in an acidogenic anaerobic reactor (Liu et al., 2002), anaerobic digester (Leclerc et al., 2001), a psychrophilic anaerobic digesters treating synthetic industrial wastewaters (Collins et al., 2003), an anaerobic sequencing batch reactor treating swine waste (Angenent et al., 2002) and an anaerobic digesters treating municipal solid waste and biosolids (Griffin et al., 1998). However, activity of methanogens also affects the success of the biodegradation processes and, therefore, methanogenic activity of anaerobic inoculum sludge should be taken into consideration together with determination of microbial community.

This part, therefore, emphasizes the importance of initial seed sludge quality in terms of methanogenic activity and microbial composition on process performance of anaerobic reactors during the start-up. Specifically, denaturant gradient gel electrophoresis (DGGE), cloning, sequencing and phylogenetic analysis were used to compare the community structure of the seed and reactor sludges.

5.1.1. Activity test results of seed sludges and reactor operation

5.1.1.1. Acetoclastic methanogenic activity. Initially, SMA tests were carried out to determine acetoclastic methanogenic activity for Seed1 and Seed2. Acetate is generally employed as the substrate for the assay since it is not only the main intermediate in the anaerobic digestion process but also the major precursor for methanogenesis (Schmidt and Ahring, 1996). In order to determine maximum acetoclastic methanogenic activity, acetate

concentrations in a range of 1000-4000 mg/L were tested. All activity test results obtained at different acetate concentrations are summarized for Seed1 and Seed2 in Figure 5.1 and Figure 5.2, respectively. According to the SMA tests results, potential methane production (PMP) rates of Seed1 was found to be 150 ±13 mLCH_4/gTVS/d (Figure 5.1) at 4000 mg/L acetate concentration whereas this was 457± 24 mLCH_4/gTVS/d (Figure 5.2) at 2000 mg/L acetate concentration. It has been reported that the maximum PMP rate of enriched cultures cultivated on acetate was found to be approximately 1000 mLCH_4/gTVS/d if all biomass (measured as VSS) consists of acetoclastic methanogens (Valcke and Verstraete, 1983). Based on this assumption, Seed1 was, constituted of approximately 15% acetoclastic methanogens whereas this was 46% for Seed2. In the literature, sludges having a PMP rate higher than 300 mL CH_4/gTVS/d have been reported to be a good quality (Ince et al., 1995, 2005). It can, therefore, be interpreted that the UASB granular sludge (Seed1) has low quality (<300 mL CH_4/gTVS/d) when the PMP rates are compared with similar studies reported in literature (Ince et al., 2005).

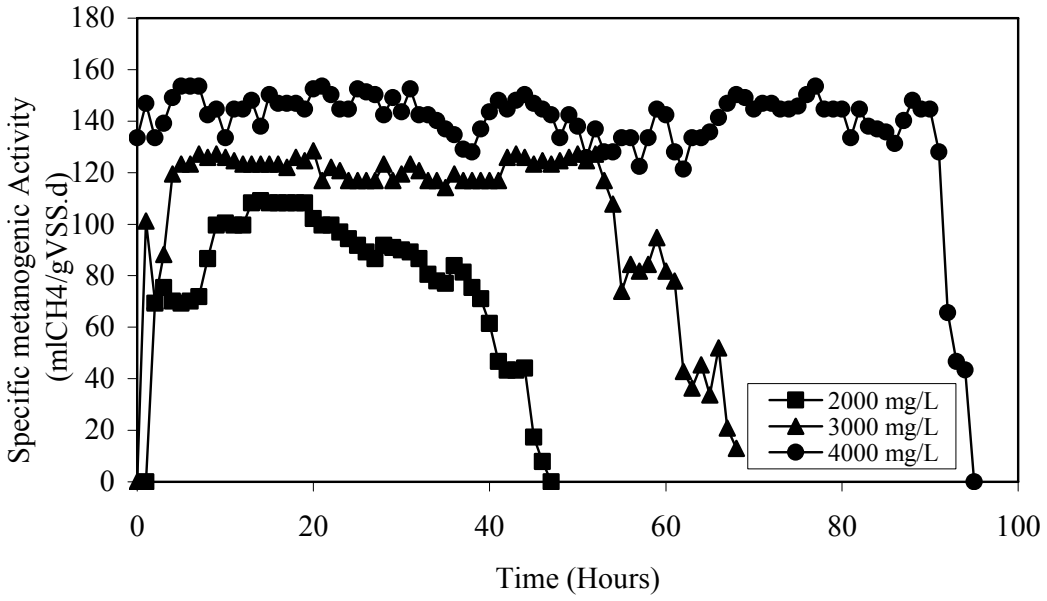


Figure 5.1. SMA test results of Seed1 at different acetate concentrations

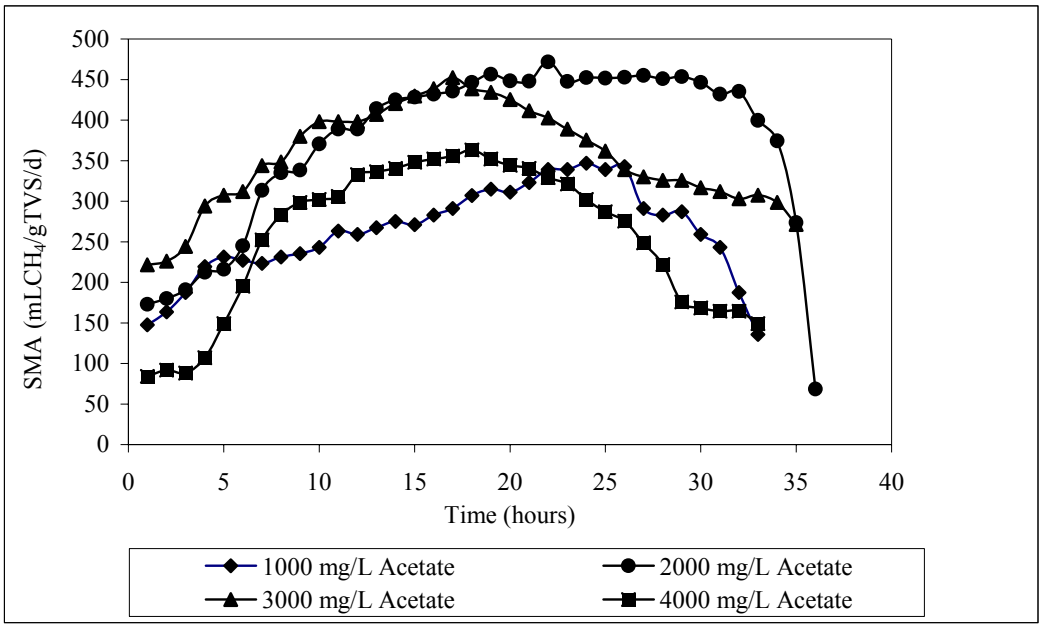


Figure 5.2. SMA test results of Seed2 at different acetate concentrations

Seed1 was inoculated into a lab-scale anaerobic batch reactor (Reactor1) treating a glucose-based synthetic wastewater. Influent COD was increased in a stepwise mode from 1000 mg/L to 4500 mg/L resulting a final S/X ratio of 0.38. The reactor was operated for approximately 40 days. Although a glucose-based synthetic wastewater was used as feed, a COD removal efficiency of over 78.8±4.17% could not be obtained during the study (Figure 5.3) and higher loadings could not have been applied to the reactor. Glucose is a readily degradable, soluble carbohydrate that does not, itself, limit the rate of anaerobic biodegradation (Noike et al., 1985) and, is commonly used as a carbonaceous substrate in many experimental studies (Oz et al., 2003). Glucose was, therefore, used as feed during the start-up period in this study in order to minimize the effect of a complex substrate on microbial diversity.

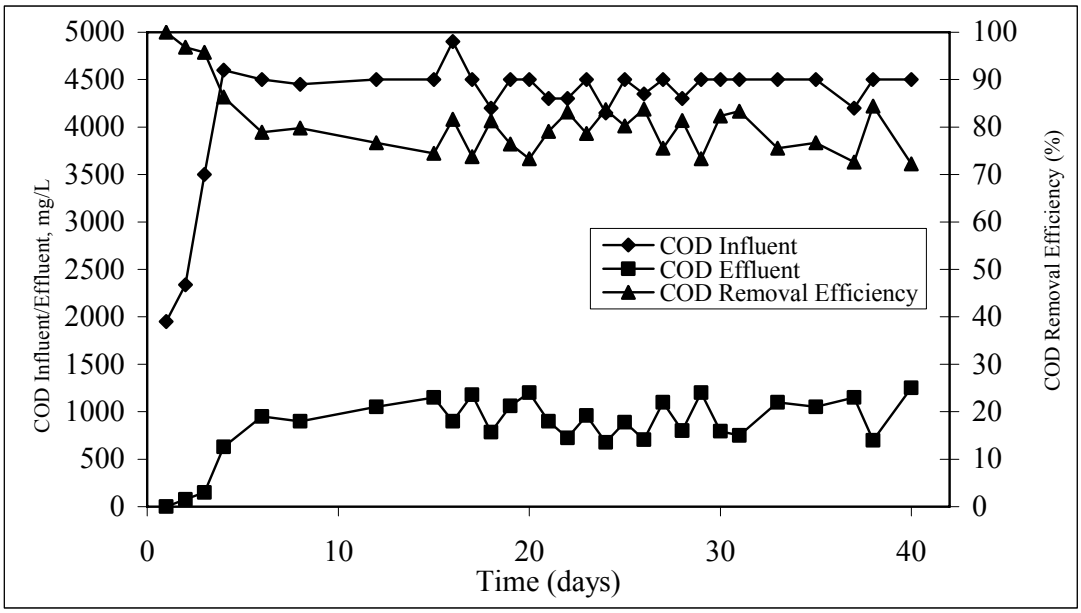


Figure 5.3. Changes in COD removal efficiency of the anaerobic reactor (Reactor1) inoculated with Seed1 with respect to time

It should be mentioned that the seed sludge used in the study has been taken from an EGSB reactor treating brewery wastewater in May 2007. Seed2 with a high SMA value of 457 mLCH_4/gTVS/d has been inoculated to a lab-scale anaerobic batch reactor (Reactor 2) treating synthetic wastewater. Influent COD was increased in a stepwise mode from 1000 mg/L to 7000 mg/L with a glucose-based synthetic wastewater resulting a final S/X ratio of 0.53. A COD removal efficiency of over 95% was obtained during the study. TS concentration in the anaerobic reactor was approximately 20000 mg/L while TVS concentration was 13000 mg/L. Changes in COD removal efficiency of the Reactor 2 is given in Figure 5.4.

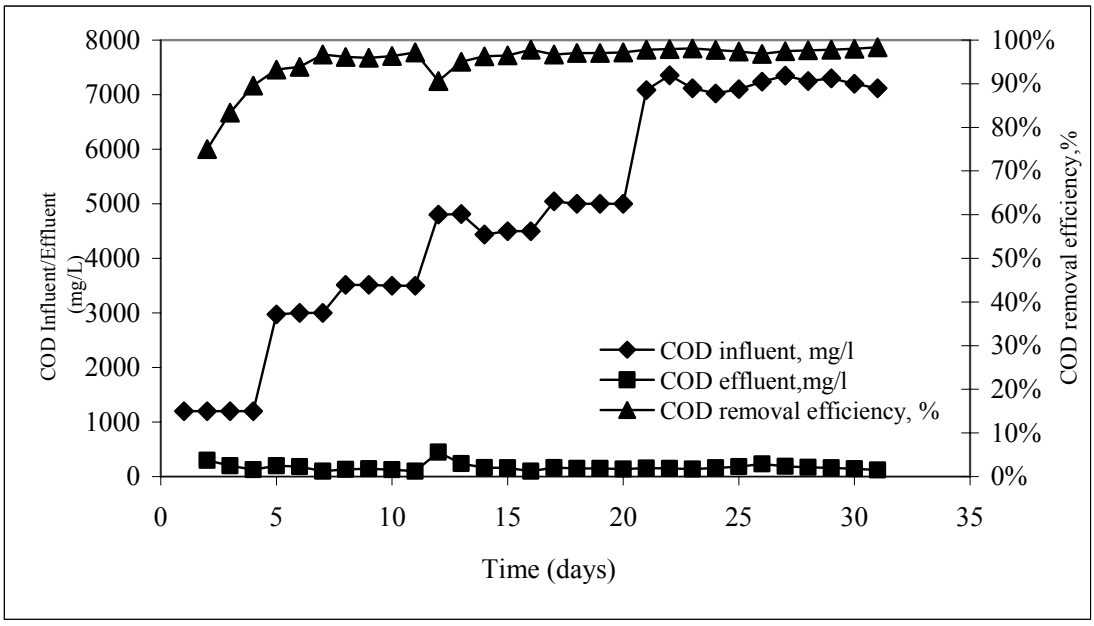


Figure 5.4. Changes in COD removal efficiency of the anaerobic reactor (Reactor2) inoculated with Seed2 with respect to time

Sludge samples from Seed1 and Reactor1 were further analyzed by excision of dominant bands and sequencing whereas samples from Seed2 were analyzed by cloning, sequencing and phylogenetic analysis. More detailed molecular studies were carried out for Seed2 showing a better reactor performance.

5.1.1.2. Indirect methanonegenic activity. In methanogenic systems, a number of fermentative, syntrophic, and methanogenic populations work together as a community to convert organic substrates to methane via the well-recognized anaerobic food chain. The stability and efficiency of the overall anaerobic digestion process depends on the stability of the individual biochemical processes. Short chain fatty acids are important intermediates in the anaerobic degradation process. Propionate and butyrate oxidation to acetate, CO_2, H_2 and formate which are energetically very unfavourable reactions require syntrophic interaction with methanogens or sulphate-reducing bacteria (SRB) to make these oxidations feasible (Schink, 1997). Any significant increase in the concentration of intermediate substrates may inhibit directly other biochemical processes and lead to digester instability (Batstone et al., 2002). Oxidation of propionate and butyrate to acetate,

hydrogen, and bicarbonate which are the main precursors of methanogenesis can only be possible under balanced methanogenic conditions. For thermodynamic reasons propionate and butyrate can be degraded only when acetate and especially hydrogen are effectively eliminated by the methanogens. Therefore, determination of specific methanogenic activity from indirect substrates such as butyrate and propionate can be important to determine how the different biochemical stages in anaerobic digestion are affected during the operation. In this study, propionate and butyrate were used as substrates for the determination of respective trophic methanogenic activities. Activity measurements of certain trophic groups of microorganisms could provide comprehensive information on the relationship between process conditions of anaerobic reactors and microbial consortia (Ahn and Park, 2003; Collins et al., 2006; Pender et al., 2004; Pereira et al., 2002; Rincon et al., 2006; Scully et al., 2005; Syutsubo et al., 2001, Yang et al., 2004).

For Seed 2, indirect methanogenic activity has been also measured for propionate and butyrate. Maximum indirect methanogenic activity has been found to be 430 $mLCH_4$/gTVS/d (at butyrate concentration of 4000 mg/L) and 250 $mLCH_4$/gTVS/d (at 3000 mg/L propionate concentration), respectively. Figure 5.5 and Figure 5.6 gives SMA profiles for indirect methanogenic activity from butyrate and maximum activity values for butyrate, respectively. Figure 5.7 and Figure 5.8 gives SMA profiles for indirect methanogenic activity from propionate and maximum activity values for propionate, respectively. Maximum overall methanogenic activity was found to be 395 $mLCH_4$/gTVS/d using a VFA mixture (2000 mg/L acetate, 500 mg/L butyrate and 500 mg/L propionate). These results showed that besides high acetoclastic methanogenic activity, the sludge (seed 2) has also a high indirect methanogenic activity. In a previous study, specific methanogenic activity was found to be 385 $mLCH_4$/gVSS/d (1.01 gCH_4 COD/gVSS/d) at 2000 mg/L butyrate concentration, by Sun-Kee Han et al. (2004); Ianotti and Fischer (1983) reported that maximum specific methanogenic activity has been found to be approximately 400 $mLCH_4$/gVSS/d for different butyrate concentrations and reported that 10000 mg/L butyrate concentration have a significant inhibitory effect; Sun-Kee Han et al.,(2004) has reported an indirect methanogenic activity of 225 $mLCH_4$/gVSS/d (0.59 gCH_4COD/gVSS.d) at 2000 mg/L propionate concentration. Reported studies in literature showed that over the 3000 mg/L propionate concentrations has vital effect on anaerobic sludges.

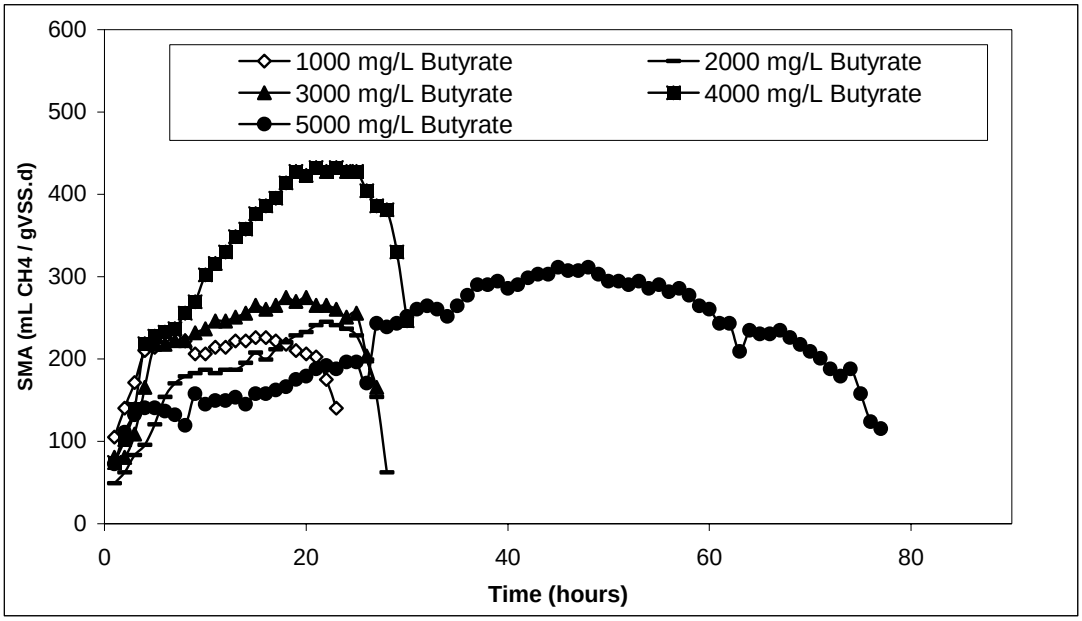


Figure 5.5. SMA test results of Seed2 at different butyrate concentrations.

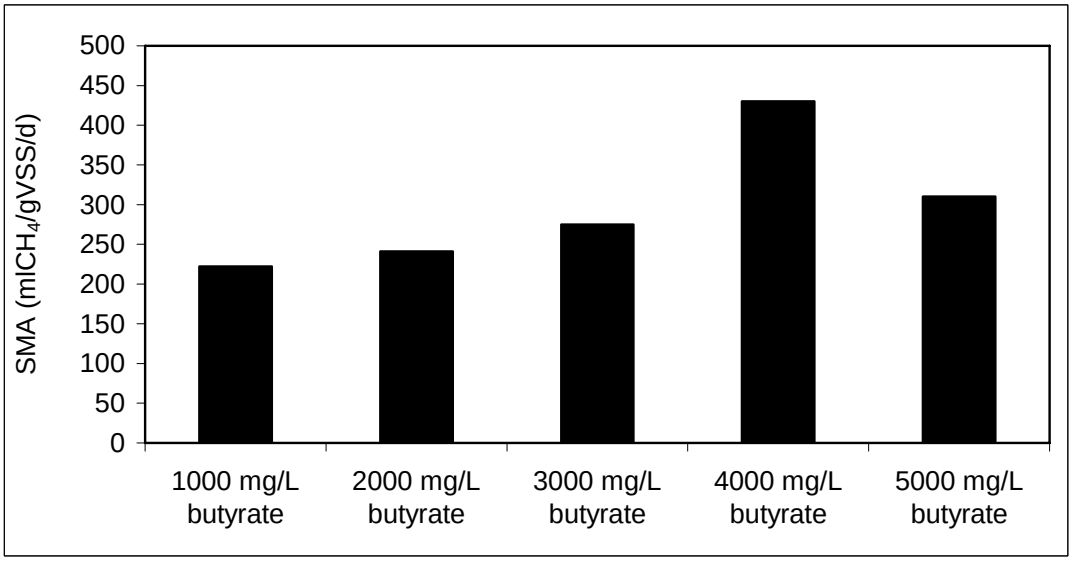


Figure 5.6. Maximum activity test results of Seed2 at different butyrate concentrations.

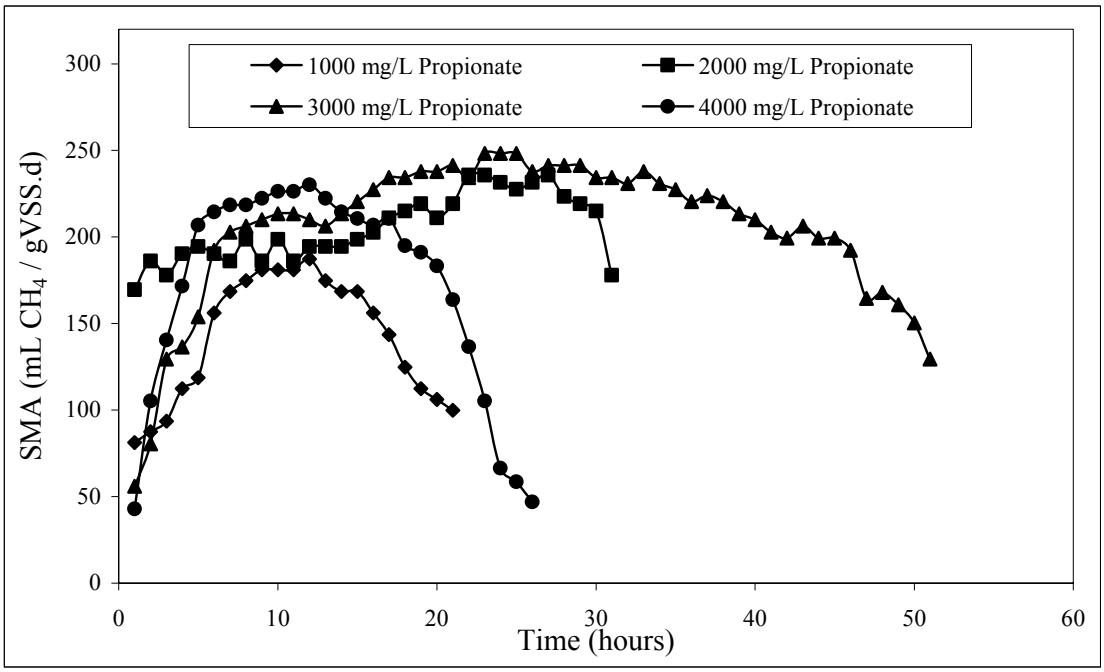


Figure 5.7. SMA test results of Seed2 at different propionate concentrations.

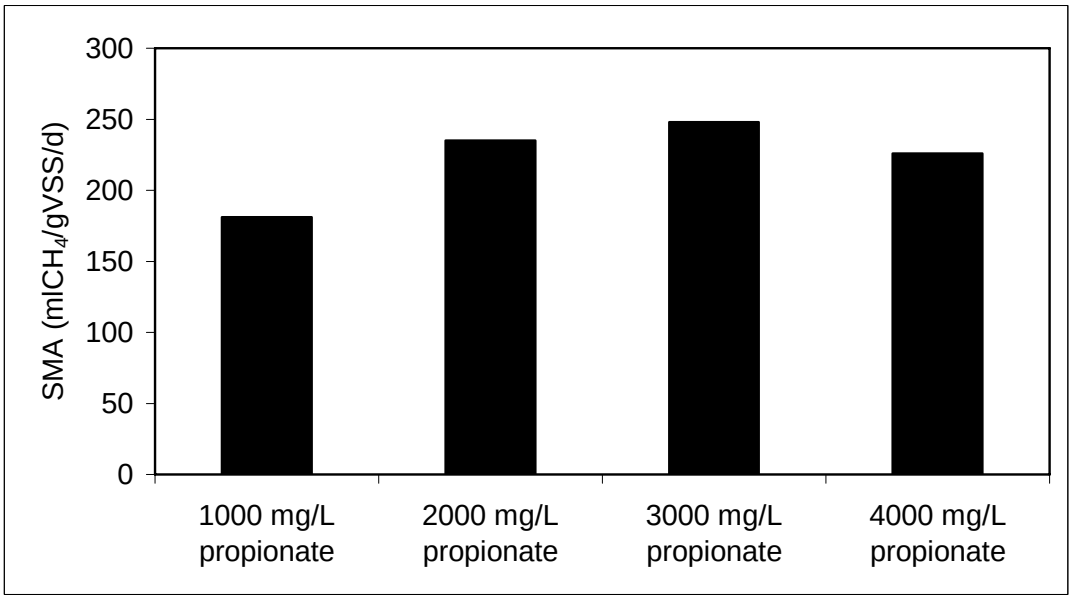
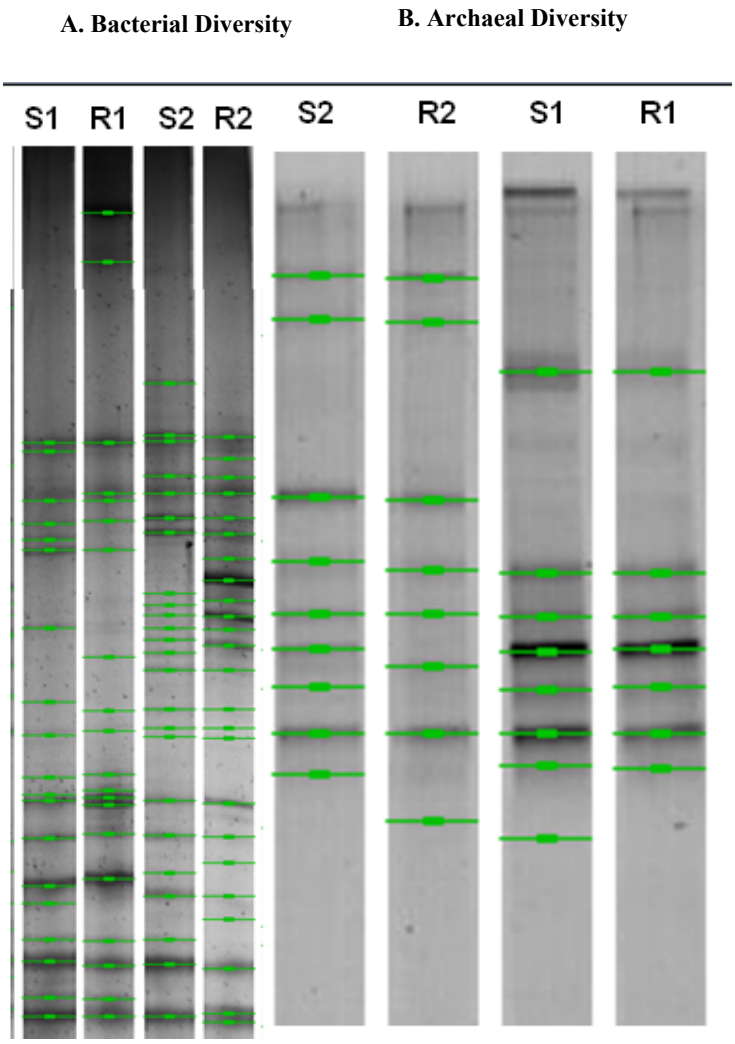


Figure 5.8. Maximum activity test results of Seed2 at different propionate concentrations.

5.1.2. Molecular results of seed and reactor sludges

5.1.2.1. Diversity analysis of sample communities. Sludge samples were analyzed via DGGE to assess relative diversities and to detect differences in the community composition during the operation of the anaerobic batch reactors. In the Seed1, 19 Bacterial and 8 Archaeal bands have been observed whereas the Seed2 indicated much more diverse microbial assemblage (24 bands for Bacteria and 9 for *Archaea*). According to DGGE fingerprinting data, number of bands in the reactor sludges remained nearly same (20 and 24 for bacteria as 7 and 8 for *Archaea* in Reactor1 and Reactor2, respectively). The data obtained from Bionumerics software analysis of DGGE gel photos were used to construct a relation analysis tree (Figure 5.9). Low similarities between DGGE banding patterns of seed and reactor samples are indicative of the changes in the communities. A focused analysis using the Bionumerics software package showed a number of low-intensity bands which differed in the seeds and the reactors samples. These low-intensity bands can be detected by software programs and may be responsible for the differences between the samples.

5.1.2.2. Sequence analysis of Seed1 and Reactor1. Total 16 bands were excised from bacterial DGGE gel of seed1 and reactor1 as 8 bands were excised from archaeal DGGE gel. Bands were amplified and sequenced to assess the identity of community members. After analysis, excised bacterial bands related to species of groups *Proteobacteria*, *Firmicutes*, *Chloroflexi* and *Actinobacteria* (Table 5.1). Literature review of identified bands reveal that the most of the sequences (9/16 bands) are affiliated with fermentative bacteria as many sequences (4/16 bands) relate to sulfate/metal reducing bacteria. Archaeal bands were related to known *Methanosaeta*, *Methanosarcina, Methanomicrobiales* species and four unidentified archaeal clones (Table 5.2). As five Archaeal sequences were related to methanogenic *Archaea* either acetoclastic or hydrogenotrophic; two unidentified Archaeal sequences relate to *Archaea* species isolated from sulfur springs or hydrothermal vents.



*Reactor1. Lab-scale anaerobic reactor inoculated with seed1

* *Reactor2. Lab-scale anaerobic reactor inoculated with seed2

Figure 5.9. DGGE profiles of 16S rRNA genes from anaerobic reactor sludge samples.

A. Diversity of bacterial microorganisms of sludge samples,

B. Diversity of archaeal microorganisms of sludge samples

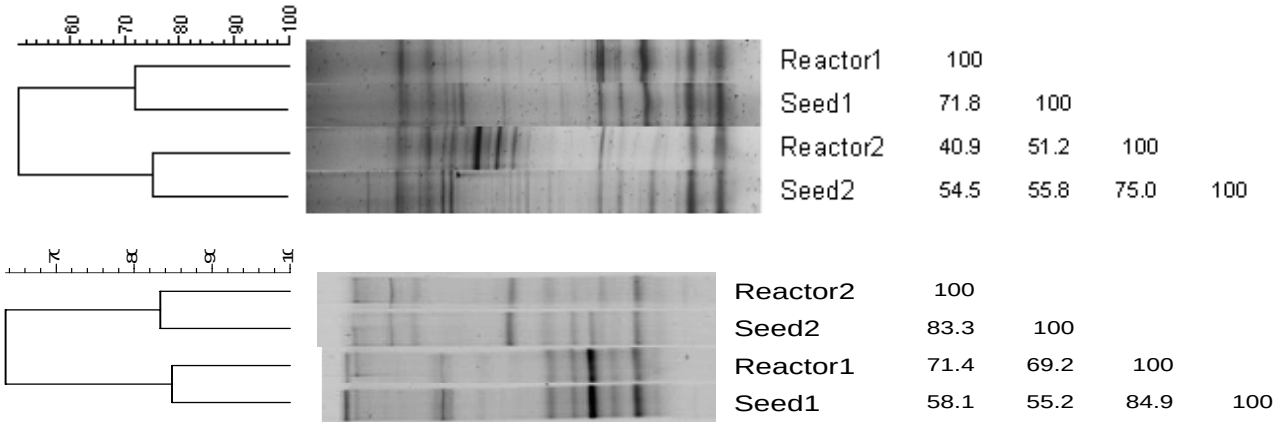


Figure 5. 10. Relation analysis tree of samples by using the Dice coefficient (based on band presence or absence) and UPGMA clustering.

DGGE banding patterns representing the bacteria and archaea seed and reactors. At the left a dendrogram is given, representing the similarity between the patterns according to cluster analysis based on Dice's algorithm and UPMGA clustering.

A. Relation Analysis Tree of bacterial samples of sludge samples

B. Relation Analysis Tree of archaeal samples of sludge samples

Table 5.1. Affiliation of band excision of Seed1 and Reactor1. Presence in the sludge samples was indicated as "X" sign.

Accession number	Closest relative	% similarity	Seed 1	Reactor 1
AY936767	*Thermal spring bacterium 19 isolate 19*	82	X	X
CP000084	*Candidatus Pelagibacter ubique HTCC1062*	86	X	0
CP000612	*Desulfotomaculum reducens MI-1,*	80	X	X
CP000471	*Magnetococcus sp. MC-1*	81	X	0
AB266952	*U. Bacterial clone SwB93fl*	80	X	0
AB243827	*U. Bacterial clone Niigata-16*	83	X	X
CP000698	*Geobacter uraniireducens Rf4*	81	X	X
CP000850	*Salinispora arenicola CNS-205*	82	X	X
BA000016	*Clostridium perfringens str. 13*	85	X	X
CP000688	*Dehalococcoides sp. BAV1*	81	X	X
CR936503	*Lactobacillus sakei strain 23K*	83	0	X
AJ296179	*Trichococcus palustris*	90	X	X
AJ306612	*Trichococcus collinsii 16S r*	87	X	X
DQ205459	*Aeromonas sobria isolate U66*	82	X	X
AE016828	*Coxiella burnetii RSA 493*	81	X	0
BA000016	*Clostridium perfringens str. 13 DNA*	87	X	X

Table 5.2. Affiliation of band excision of Seed1 and Reactor1 (Archaeal). Presence in the sludge samples was indicated as "X" sign.

Accession number	**Closest relative**	**% similarity**	**Seed 1**	**Reactor 1**
AY817738	*Methanosaeta harundinacea strain 8Ac*	88	X	X
CP000562	*Methanoculleus marisnigri JR1*	87	X	X
AF526971	*U. Archeaon clone pEPR206*	82	X	0
AE010299	*Methanosarcina acetivorans C2A*	82	X	X
DQ513419	*methanogenic archaeon CH50*	81	X	X
AY693811	*U. Archeaon clone ARC12*	80	X	0
AB161336	*U. Archaeon ASC36*	88	X	X
AY341273	*U. Archaeon ZAR104*	82	X	0

Clone libraries of Seed2. 16S rRNA bacterial clone library generated 58 clones which grouped under 22 different taxa. The dominant species in the bacterial clone library are identified as two fermentative bacteria, *Spirochaeta stenostrepta* with 12% and *Clostridium botulinum A str. Hall* with 10%. Similarities and phylogenetic affiliation are given in Table 5.3. The identified bacteria in Table 5.3 are grouped according to their phylogeny. 41.5% of the clones are belonging to *Firmicutes*, 27.5% of the clones to *Proteobacteria*, 12% of the clones to *Spirocheates* and 10% of the clones to *Bacteriodetes,* respectively. Main metabolic pathways of clones were found as 60% fermentation, 17% syntrophic sulfate reduction, 14% metal reduction and 9% unidentified.

16S rRNA archaeal clone library from Seed2 generated 60 clones which grouped under 6 different taxa. The dominant species in the archaeal clone library are identified as *Methanosaeata Concilii* with 63% and *Methanoculleus marisnigri JR1* with 23%. Similarities and phylogenetic affiliation are given in Table 5.4.

Sequence statistics and Diversity indices of the Seed2 communities. The overall diversity of archaeal and bacterial communities in the sludge samples were analyzed by comparing three different metrics: the number of clones estimated (richness), the coverage of clone library, and the Shannon–Weaver diversity index (H), which is a statistic that consider species richness and the evenness of species distribution to estimate diversity in a system. H index increases when richness increases. High coverage values indicated a high total microbial diversity was represented by the clone libraries constructed (Table 5.5).

Table 5.3. Identification of clones of bacterial 16S rDNA clone library from Seed2 sample. Presence in sample was indicated as "X" sign under corresponding sample.

Clone	Closest Phylotype[1]	% similarity [1]	Accession number	Closest described relative[2]and Accession number	% similarity[2]	Phylogenetic affinity	Seed2	Reactor2
1	Uncultured clone LS4-168.	98	AB234244	Prolixibacter bellariavorans strain F2 (AY918928)	89	Bacteroidetes	x	0
2	Uncultured clone H03	100	EU136226	Thermoactinomyces sp. JAM-FM1001(AB362275)	85	Firmicutes	x	x
6	Uncultured Firmicutes clone 6	This study	FM212973	Clostridium botulinum F str. Langeland (CP000728)	82	Firmicutes	x	x
7	Uncultured proteobacterium clone 7	This study	FM212974	Syntrophobacter fumaroxidans MPOB (CP000478)	92	Deltaproteobacteria	x	0
11			AB231802	Anaerobic syntrophic bacterium NE23-3	96	Deltaproteobacteria	x	x
19	Uncultured Firmicutes clone 19	This study	FM212975	Catabacter hongkongensis (AY574991)	91	Firmicutes	0	0
21	Uncultured Firmicutes clone 21	This study	FM212976	Clostridium thermocellum ATCC 27405 (CP000568)	86	Firmicutes	x	x
22	Uncultured proteobacterium clone 22	This study	FM212977	Pelobacter carbinolicus DSM 2380 (CP000568)	71	Deltaproteobacteria	0	0
24	Uncultured clone Zplanct13	96	EF602474	Francisella tularensis subsp. holarctica LVS (AM233362)	76	Gammaproteobacteria	x	x
25	Uncultured Firmicutes clone 25	This study	FM212978	Acetanaerobacterium elongatum (AY487928)	73	Firmicutes	0	0
33	Uncultured clone TC76	98	EF644509	Clostridium viride (X81125)	93	Firmicutes	0	0
34	Uncultured clone R1p32	98	AF482435	Syntrophobacter fumaroxidans MPOB (CP000478)	93	Deltaproteobacteria	x	x
35	Uncultured clone R1p32	98	AF482435	Syntrophobacter wolinii (X70906)	92	Deltaproteobacteria	x	x
37	Uncultured clone R6b1	99	AF482447	Thermotoga maritima MSB8 (AE000512)	82	Thermotogae	x	x
46	Uncultured Bacteroidetes clone 46	This study	FM212979	Prolixibacter bellariavorans (AY918928)	86	Bacteroidetes	x	0

[1] Based on FASTA under environmental database
[2] Based on FASTA under prokaryote database

Table 5.3. Identification of clones of bacterial 16S rDNA clone library from Seed2 sample. Presence in sample was indicated as "X" sign under corresponding sample (cont).

Clone	Closest Phylotype[1]	% similarity [1]	Accession number	Closest described relative[2]and Accession number	% similarity[2]	Phylogenetic affinity	Seed2	Reactor2
47	Uncultured clone P1mT_024	97	EF551966	Thermolithobacter ferrireducens (AF282253)	83	Firmicutes	x	0
50	Uncultured Firmicutes clone 50	This study	FM212980	Clostridium thermocellum ATCC 27405 (CP000568)	79	Firmicutes	0	0
54	Uncultured cyanobacterium clone 54	This study	FM212981	Phormidium lumbricale UTCC 476 (AF218375)	75	Cyanobacteria	0	0
63	Uncultured clone S06	100	AB195927	Spirochaeta stenostrepta (M88724)	86	Spirochaetes	x	x
65	Uncultured Firmicutes clone 65	This study	FM212982	Catabacter hongkongensis (AY574991)	90	Firmicutes	0	0
67			AB363973	Bacteroides sp. XDT-1	100	Bacteroidetes	0	0
68	Uncultured clone S06	100	AB195927	Spirochaeta stenostrepta (M88724)	86	Spirochaetes	x	x
70	Uncultured clone ZEBRA_12	96	AY858451	Clostridium botulinum A str. Hall (CP000727)	86	Firmicutes	x	x
84	Uncultured clone MBF16_D.	99	AB290387	Prolixibacter bellariavorans (AY918928)	85	Bacteroidetes	x	x
85	Uncultured Bacteroidetes clone 85	This study	FM212983	Alkaliflexus imshenetskii (AJ784993)	81	Bacteroidetes	x	x
88	Uncultured Firmicutes clone 88	This study	FM212984	Thermoanaerobacter mathranii (Y11279)	73	Firmicutes	x	x
89			X99706	Syntrophobotulus glycolicus	98	Firmicutes	x	x
95	Uncultured clone 31d06	98	EF515641	Anaerolinea thermophila (AB046413)	84	Chloroflexi	0	0

[1] Based on FASTA under environmental database
[2] Based on FASTA under prokaryote database

Table 5.4. Identification of clones of archaeal 16S rDNA clone library from EGSB Seed sludge sample. Presence in sample indicated as "X" sign under corresponding sample.

Clone	Closest Phylotype[1]	% similarity[1]	Closest described relative[2]and Accession number	% similarity[2]	Accession number	Phylogenetic affinity	Seed2	Reactor2
17			Methanosaeta concilii	99	X51423	Methanosaetaceae; Methanosaeta.	X	X
9			Methanosaeta concilii	99	X51423	Archaea; Methanosaetaceae; Methanosaeta.	X	X
92			Methanosaeta concilii	96	X51423	Methanosaetaceae; Methanosaeta.	X	X
80			Methanomethylovorans sp. Z1	98	EF174501	Methanosarcinaceae; Methanomethylovorans	X	X
81			Methanosaeta concilii	100	X51423	Methanosaetaceae; Methanosaeta.	X	X
43			Methanosaeta concilii	96	X51423	Methanosaetaceae; Methanosaeta.	X	X
14	Uncultured archaeon clone 14	This study	Methanoculleus marisnigri JR1	69	FM212985	Methanomicrobiales; Methanomicrobiaceae; Methanoculleus.	X	X
62			Methanosaeta concilii	96	X51423	Methanosaetaceae; Methanosaeta.	X	X

[1] Based on FASTA under environmental database
[2] Based on FASTA under prokaryote database

Table 5.4. Identification of clones of archaeal 16S rDNA clone library from EGSB Seed sludge sample. Presence in sample indicated as "X" sign under corresponding sample (cont.).

Clone	Closest Phylotype[1]	% similarity[1]	Closest described relative[2]and Accession number	% similarity[2]	Accession number	Phylogenetic affinity	Seed2	Reactor2
82			Methanosaeta concilii	99	X51423	Methanosaetaceae; Methanosaeta.	X	X
89			Methanosaeta concilii	100	X51423	Methanosaetaceae; Methanosaeta.	X	X
28			Candidatus Methanoregula boonei 6A8	96	CP000780	Methanomicrobiales; Genera incertae sedis; Candidatus Methanoregula	X	X
21			Methanolinea tarda	95	AB162774	Methanomicrobiales; Genera incertae sedis; Methanolinea.	X	X
25			Methanocorpusculum labreanum Z	99	CP000559	Methanomicrobiales; Methanocorpusculaceae; Methanocorpusculum.	X	X
72			Methanosaeta concilii	96	X51423	Methanosaetaceae; Methanosaeta.	X	X
26			Methanocorpusculum labreanum Z	100	CP000559	Methanomicrobiales; Methanocorpusculaceae; Methanocorpusculum.	X	0

[1] Based on FASTA under environmental database
[2] Based on FASTA under prokaryote database

Table 5.5. Shannon–Weaver diversity indices, Coverage and Chao1 Estimation of species richness for both bacterial and archaeal clones of the Seed2.

	Shannon-Weaver Diversity Index	Coverage	Chao1 Estimator of species richness
Bacterial	3.13	0.86	23
Archaeal	2.4	0.97	15

Bacterial and Archaeal diversity and methanogenic activity were found to be higher in Seed2 compared to Seed1. Both reactors were started up under the same conditions and fed with same synthetic wastewater, however, the Reactor2 showed a superior performance and was loaded with higher loading rates compared to Reactor1. Although the same synthetic wastewater is used, the differences observed in the performances between the two reactors could be attributed to their different initial quality of the sludges'. The population diversity in the bacterial domain was considerably higher than the archaeal domain in both anaerobic inoculums and reactors as it is previously reported (Collins et al., 2003). This can be attributed to availability of much more diverse substrate for the bacterial populations compared to archaeal populations represented mostly by the methanogens in anaerobic bioreactors. The most dominant member of the archaeal cells in Seed2 was the genus *Methanosaeta* (63%) as previously reported a dominant achaeal group in anaerobic granular sludges in the literature (Sekiguchi, 1999; Harmsen et al., 1995). Overall the bacterial community of Seed2 was dominated by *Firmicutes* (42%), *Bacteroidetes* (10%), *Spirochaetes* (12%) and *Delta-Proteobacteria* (24%*)* as Seed1 dominated by *Firmicutes* (6/16 bands) and *Proteobacteria* (5/16 bands). *Firmicutes* are found in various environments and can survive in extreme conditions and have been reported widely in beer, wine, and cider spoilage including a fluidized bed reactor treating vinasses (%46) (Godon et al., 1997), a mesophilic citric acid (57%) (Collins et al., 2003) and methanogenic granules treating brewery wastewaters (Diaz et al., 2006). Actually Seed2 was taken from a full-scale EGSB reactor treating a brewery wastewater. These bacteria can anaerobically utilize glucose, propionate, butyrate, and acetate (Ariesyady et al., 2007) and produce CO_2 and H_2 (Girbal et al., 2005). The microbial community playing role in glucose degradation has a higher diversity represented by at least seven different phylogenetic groups including *Betaproteobacteria*, *Bacteroidetes, Spirochaetes* and

Chloroflexi. Recently, it has been shown that Syntrophobacter group of *Deltaproteobacteria and Betaproteobacteria* was one of the numerically important glucose-degrading bacterial groups in anaerobic sludge digester (Ariesyady, 2007). Members of the genus *Spirochetes* which ferment glucose directly to acetate, along with lesser and variable amounts of ethanol or lactate (Pohlschroeder et al., 1994) depending on the species have been also present in Seed2. Many *Spirochetes* have been reported to be found in many anaerobic environments, but they are generally not numerically dominant (Harwood and Canale-Parola, 1984). This group has been reported to be playing a significant role in the glucose degradation. Although *Spirochetes* were not dominant in the Seed2, the minor group may have a critical functional role in glucose degradation in the Reactor2.

Microbial diversity of the Reactor2 compared to Seed2 did not show a significant change during the operation of the anaerobic reactors. It has been reported that population persistence is of great importance for establishing ecosystem stability (Grimm et al., 1992). It has been also stated that the constant population flux may be partly important in maintaining a positive correlation between biodiversity and ecosystem stability (Briones et al., 2003). Therefore, it is important to define the interactions between different organisms establishing a sustainable well-functioning system from an ecological point of view. If there are multiple pathways towards a product, it can lead to functional redundancy, which is ensured by the presence of a reservoir of species able to perform the same ecological function and stability (Person et al., 1998). In anaerobic reactors, methanogens and syntrophic bacteria are representatives of the driver species which have strong influence upon overall process function. However, a higher degree of population diversity among fermenting bacteria which represents parallel pathways toward methane production would be expected to be important in the system (Briones and Raskin, 2003). In this study, *Methanosaeta* which is known as one of the most important key species in anaerobic reactors is the most dominant methanogenic *Archaea.* Low performance of Reactor1 could be attributed to the composition and lower bacterial diversity of the Seed1. Absence of syntrophic bacteria and presence of sulfate/metal reducing bacteria may affect several metabolic pathways especially acetoclastic methanogenesis performed by mainly *Methanosaeta* group (Isa et al., 1986). Besides presence of functionally important key drivers such as methanogens and syntrophic bacteria in anaerobic reactors, minor bacterial

communities contributing functional redundancy may be of great importance for maintaining stability (Briones and Raskin, 2003). It can be deduced that both dominant and minor populations can be important for sustaining the system stability indicating a well-balanced ecosystem in terms of microbial composition (Fernandez et al., 1999).

Start-up period is generally the most critical step in the operation of anaerobic bioreactors. If an anaerobic reactor has been started up successfully, it is expected to run without much attention as long as operating conditions are maintained in desired ranges (Hobson and Wheatley, 1993). In practice, there are different strategies applied for start up periods. Generally biomass originating from another bioreactor is inoculated to the anaerobic reactor for more rapid reactor start up. However, microbial community and activity of the inoculation sludge may not be appropriate for the new waste type. Certain specific bacteria may not be present in the starting sludge or the existing microbial population can not be adapted to conditions in a new reactor. These factors not only cause prolongation of start up periods but also affect the success of the reactor's performance during the operation. Source of seed, amount of the inoculum, activity of the sludge and initial mode of operation are important factors during startup (Cecchi et al., 1992; Hobson and Wheatley, 1993; Oz et al., 2004). SMA test can be used as a parameter besides classical ones when a decision has to be taken for the use of an inoculum. Especially, acetoclastic methanogenic activity tests have been frequently used in evaluating of a biomass since a high proportion of methane is produced through acetoclastic pathway (Oz et al., 2004; Ince et al., 2005). In this study, the initial test results showed that the acetoclastic capacity of the Seed1 is low compared to the SMA values reported in literature. The reactor inoculated with this sludge performed poorly even at a low loading rate compared to Reactor2. The activity measurements indicated that Reactor1 could not be loaded with higher loading rates. On the other hand, Reactor2 inoculated with a sludge having a much diverse microbial composition and high methanogenic activity showed a better performance and has the capacity to treat higher loading rates. It was reported that maintainance of high methanogenic activity in anaerobic reactors provides safer operation under field conditions, under varied influent flows and variable concentrations of organic matter thereby ensuring high reactor performances (Ince et al., 2005; Jawed and Tare, 1999). It has been stated that anaerobic biodegradability tests even for an easy-to-degrade substrates such as glucose shows a great variability due to the use of different sources of

inoculum sludge (Andrade and Buitrón, 2004). It has been already reported that the acclimation of an anaerobic sludge to a specific substrate may lead to a change in population that may be quite different from that of the inoculum sludge due to several operational and nutritional factors (Anderson et al., 1994; Zhang and Noike, 1994). It can be stated that initial quality of anaerobic sludges at start-up may determine performance of anaerobic reactors during long-term operation.

5.1.2.3. FISH studies. The microbial community structure of the seed sludge (Seed2) was characterized using fluorescent rRNA targeted oligonucleotide probes specific for Bacteria, *Archaea* and phylogenetically defined groups of methanogenic *Archaea* namely *Methanosaeta* (MX825 probe), *Methanosarcina* (MS821 probe), *Methanococcales* (MC1109 probe), *Methanobacteriales* (MB310 probe), *Methanomicrobiales* (MG1200 probe). For each sample hybridization, two negative controls were used; one of these controls was used to assess nonspecific binding (with Non338 probe), and the other (lacking a probe) was used to monitor autofluorescence. In addition to negative controls, one positive control was used to assess success of cell permeabilization and rRNA content of the cells (with universal probe UNIV1392). Whole microbial community in the samples was also stained using DAPI stain to visualize intact cells in the samples for quantification studies.

In Seed2, 86% total microorganisms produced positive signal, that is, 86% microorganisms were metabolically active and hybridized with the UNIV1392 oligonucleotide probes. The relative abundance of Eubacteria was in the range of 35±0.5% whereas that of was 43.2±1.1% for archaeal cells. The results (Table 5.6) showed that the relative abundance of *Archaea* is high. Archaeal population in anaerobic reactors has been shown previously to range from 10 to 90% of the total prokaryotic cells (Angenent et al., 2004; Ficker et al., 1999; Gonzalez-Gil et al., 2001; Harmsen et al., 1996; Hansen et al., 1999; McMahon et al., 2001; Raskin et al., 1994b and 1996; Saiki et al., 2002; Tay et al., 2001). The archaeal subpopulation in the Seed2 consisted of 38.09.2±1.3% of members of the genus *Methanosaeta,* 17.4±0.2% of *Methanosarcina*, 19.8±0.5% of *Methanococcales*, 13.4±1.1% of *Methanobacteriales*, 11.9%±0.6% of *Methanomicrobiales*. In a previous study, 59±2.6% of members of the genus *Methanosaeta* and 40±1.3% *Methanobacteriales* were found in a full-scale UASB reactor sludge (Kolukirik, 2004).

The results indicated that *Methanosaeta* dominated the granules treating brewery wastewater (Table 5.6). Major hydrogenotrophic methanogens were *Methanococcales, Methanobacteriales, Methanomicrobiales* respectively. Acetoclastic methanogens, Methanosaeta sp. and Methanosarcina sp, can convert acetate to methane and carbon dioxide (Zinder, 1993). Approximately 70% of the methane formed during anaerobic processes is produced by members of the acetoclastic *Methanosarcina* and *Methanosaeta* species. Acetoclastic *Methanosaeta* spp. [only use acetate] (Huser et al., 1982) while *Methanosarcina* spp. which is the most versatile methanogen can use H_2/CO_2, methanol, methylated amines and pyruvate besides acetate (Jones, 1991). *Methanosaeta* sp. is generally dominant under typical loadings and reactor configurations especially in UASB and EGSB reactors (Sekiguchi et al., 1999) and these filamentous organisms are regarded as being important for the formation and maintenance of granular sludge (Merkel et al., 1999) and more stable reactor performance. It has been stated that the diversity of the methanogenic granule population depends mainly on the composition of the substrate, changes in temperature, pH stability and indicators as well as the solids retention time. The most dominant member of the archaeal cells in the brewery-degrading granule has been found to be the genus *Methanosaeta* (approximately 45% of total archaeal rRNA in EGSB reactor). This result stating dominance of *Methanosaeta* in anaerobic reactors verified previous findings (Sekiguchi et al., 1999) In a previous study, 59±2.6% of members of the genus *Methanosaeta* and 40±1.3% *Methanobacteriales* were found in a full-scale UASB reactor sludge (Kolukirik, 2004). It has been reported that the granule size and inner structure seem to play a more relevant role in EGSB reactors (Seghezzo, 1997) and microbial community of a biogranule is highly substrate dependent (Fang et al., 1995a, 1995b).

Table 5.6. Microbial population in EGSB reactor

	Seed2 (%)
Active cells *	86±1.6
*Archaea***	43.2±1.1
*Methanosaeta****	38.09±1.3
*Methanosarcina****	17.4±0.2
*Methanobacteriales****	13.4±1.1
*Methanococcales****	19.8±0.5
*Methanomicrobiales ****	11.9±0.6

* (The number of cells detected with UNIV1392)/(Total cell count using DAPI).

** (The number of cells detected with ARC915)/(The number of cells detected with UNIV1392).

*** Relative population in *Archaea.*

Among the hydrogenotrophic methanogens, *Methanococcales* was the predominant methanogen within the reactor (Table 5.6). It was previously reported that among the hydrogenotrophic methanogens, *Methanobacteriales* followed by *Methanomicrobiales* were dominant methanogens and *Methanococcales* were almost absent within both full-scale and lab-scale UASB reactors (Hofman-Bang et al., 2003). In some studies *Methanobacteriales* such as *Methanobacterium* and *Methanobrevibacter* have been reported to be the dominant hydrogen- and formate-consuming methanogens (Raskin et al., 1994), while other studies have shown that *Methanomicrobiales* can also be present in the sludge in high numbers and can even be more abundant than *Methanobacteriales* (Raskin et al., 1995). It has been previously reported that among the hydrogenotrophic methanogens, *Methanococcales* were almost absent within granular sludge (Hofman-Bang et al., 2003) or has been reported to play relatively small role (Raskin et al., 1995.) However, in this study, the relative abundance of *Methanococcales* was high in the anaerobic granular sludge among other hydrogenotrophic methanogens. Predominance of hydrogenotrophic methanogens is difficult to explain, since the ecological significance of

different hydrogen and formate utilizing methanogens and the competitiveness among these populations have not been studied in as much detail as for acetoclastic methanogens. (McMahon et al., 2001; Stams et al., 2003). In terms of kinetics, available Monod kinetic values are limited and do not provide a clear view of how the different H_2-utilizing methanogens compete for H_2 (Karadagli and Rittmann, 2005).

It is a contentious question of how diversity and dynamics of communities affect bioreactor performance. When overall results obtained from the anaerobic reactors inoculated with sludges having different quality and fed with same type of influent composition were compared, successful start-up of anaerobic reactors require a seed sludge with a well-balanced microbial community including diverse bacterial species and high methanogenic activity. Besides abundance of major methanogenic Achaeal groups in anaerobic sludges, a diverged bacterial groups having different metabolic capability may also play an important role in reactor performance. However, studies mostly concentrate on role of methanogenic *Archaea* and underestimate importance of bacterial diversity in anaerobic reactors. This may become more significant in case of treatment of complex wastewaters since more diverged bacterial community should be present in sludge.

5.2. Inoculum sludge characteristics used throughout reactor studies

As explained in previous section, it has been decided that an inoculum sludge taken from an EGSB reactor treating brewery wastewater should be used for further reactor studies including anaerobic solvent treatment. Therefore, a seed sludge was taken from the same EGSB reactor treating brewery wastewater in August 2007. The studies including methanogenic activity and microbial diversity for the selected seed sludge were repeated in order to set initial sludge characteristics.

5.2.1. Methanogenic activity of seed sludge

Maintenance of active methanogenic populations in anaerobic processes is critical for stable performance. Therefore, a seed sludge with high methanogenic activity and composition was used in the study. The seed sludge was taken from an EGSB reactor in August 2007. Two different substrates were used to determine specific methanogenic activity. Firstly, acetate was used as substrate in order to measure the potential acetoclastic methanogenic activity. In addition, a VFA mixture (2000 mg/L acetate, 500 mg/L propionate and, 500 mg/L butyrate) was used as substrate for determining overall methanogenic activity (Soto et al., 1993). Previously, acetate concentrations ranging from 1000 to 4000 mg/L have been tested in order to determine maximum acetoclastic activity and 2000 mg/L acetate concentration has been found to be maximum acetoclastic activity (Simsek, 2007). SMA tests were applied to the seed sludge that was taken from the EGSB reactor. The activity tests were repeated at least three times in order to determine precise and reproducible results and 5-10% changes in the SMA tests were assessed to be accepted. According to the results, maximum acetoclastic activity of the seed sludge was found to be 453 $mLCH_4$/gVSS/day at 2000 mg/L acetate (Figure 5.11). Compared to anaerobic sludge activities reported in the litrerature (>300 $mLCH_4$/gVSS/day), the seed sludge used in this study is in a good quality. Besides, a VFA mixture composed of 2000 mg/L acetate, 500 mg/L butyrate and 500 mg/L propionate was used as substrate in order to determine overall methanogenic activity. The overall methanogenic activity of the seed sludge was found to be 461 $mLCH_4$/gVSS/day (Figure 5.12).

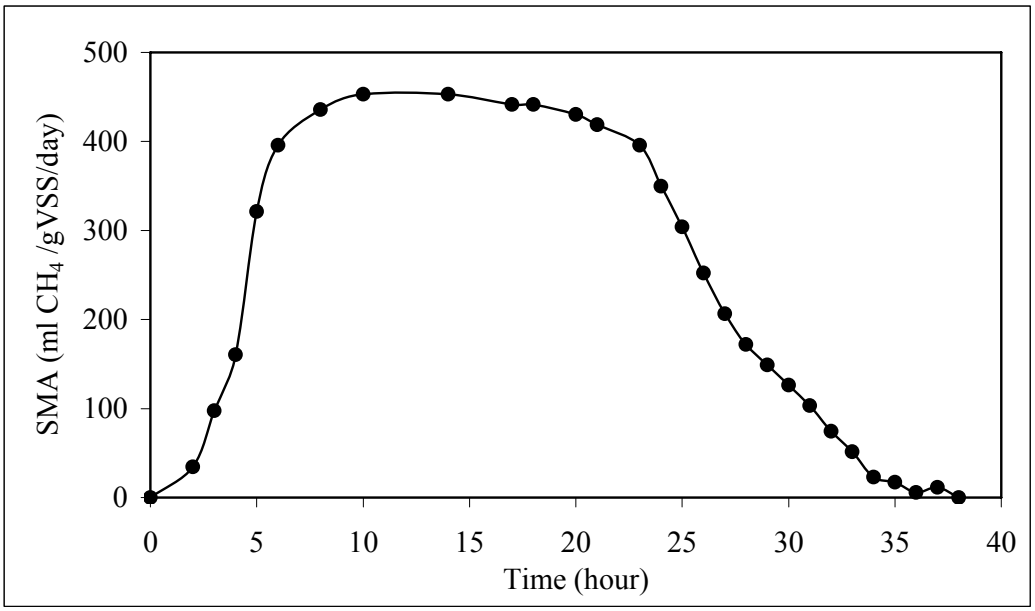


Figure 5.11. Acetoclastic methanogenic activity of the seed sludge (2000 mg/L acetate concentration).

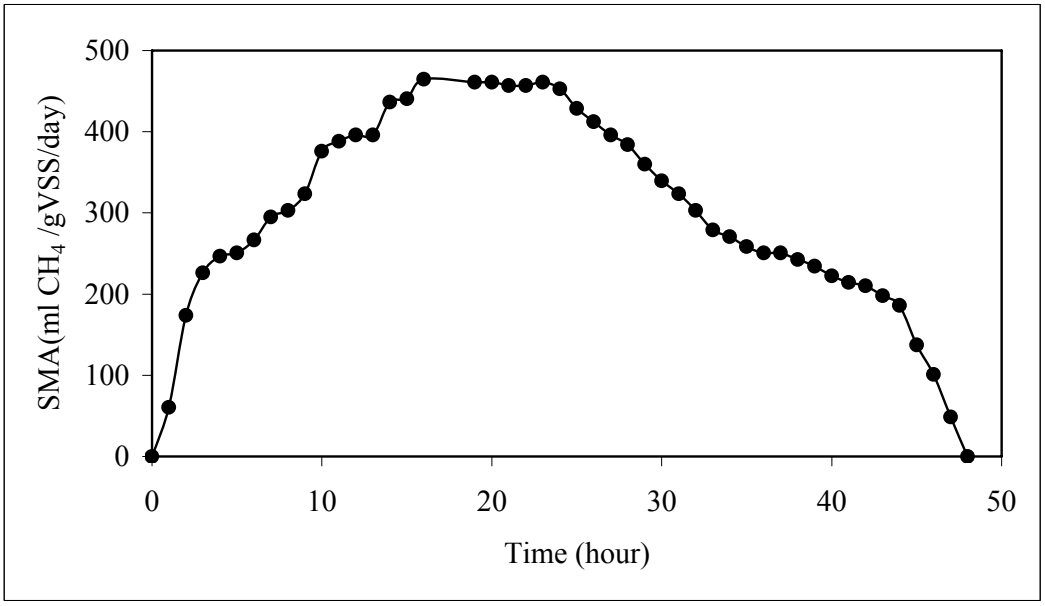


Figure 5.12. Overall methanogenic activity of the seed sludge (VFA mixture: 2000 mg/L acetate, 300 mg/L butyrate, 500 mg/L propionate).

Indirect methanogenic activity of the sludge has been also measured for propionate and butyrate. Maximum indirect methanogenic activity has been found to be 315 mL CH_4/gVSS/d (at butyrate concentration of 4000 mg/L) and 160 mLCH_4/gVSS/d (at 3000 mg/L propionate concentration), respectively.

Table 5.7. Maximum methanogenic activity of the seed sludge

	Acetate (2000 mg/L)	Butyrate (4000 mg/L)	Propionate (3000 mg/L)	VFA mixture*
SMA (mLCH_4/gVSS/day)	453	315	160	461

*(2000 mg/L acetate, 500 mg/L butyrate ve 500 mg/L propionate) (Soto vd., 1993)

5.2.2. FISH results of the seed sludge

Fluorescent oligonucleotide probes were used to identify relative abundance of archaea and phylogenetically defined groups of methanogens in the EGSB granules. Total active microorganisms of the seed sludge with Univ1392 probe was found to be 80.1±4.0% (mean ± standard deviation) (Table 5.2). 42.2±2.0% of the seed sludge consisted of Eubacteria (with Eubmix probe) whereas Archaeal population was detected as 62.3%±1.2% (with Arc915 probe). The Archaeal subpopulation composed of 32.4±0.8% of members of the genus *Methanosaeta* (With Mx825 probe), 8.2±1.5% *Methanosarcina* (with Ms821 probe), 17.1% ± 1.3% *Methanobacteriales* (with Mb310 probe), 12.5±1.1% *Methanococcales* (with Mc1109 probe), 14.2±1.6% *Methanomicrobiales* (with Mg1200 probe). Epifluorescence micrographs of the seed sludge are shown in Figure 5.13.

The Archaeal subpopulution in the seed sludge was found to be mostly dominated by *Methanosaeta* sp which is known to grow only on acetate (Table 5.8) indicating that the seed sludge used in this study is in good quality. The high percentage of the acetoclastic methanogens in the seed sludge also corresponded with the activity test results. Numerical dominance of the genus *Methanosaeta* compared to the other methanogens in anaerobic reactors has been reported previously (Ficker et al., 1999; Merkel et al., 1999; Sekiguchi et

al., 1999). Two acetate-utilizing methanogenic genera, *Methanosarcina* and *Methanosaeta*, have been identified as important methanogens in granular sludge from anaerobic reactors (de Zeeuw, 1984; Hulshoff, 1989; Schmidt, 1996). *Methanosaeta* sp. is generally dominant under typical loadings and reactor configurations especially in UASB and EGSB reactors (Sekiguchi et al., 1999) and these filamentous organisms are regarded as being important for the formation and maintenance of granular sludge (Merkel et al., 1999) and more stable reactor performance. *Methanosaeta spp*. was more numerous than *Methanosarcina spp*. in the seed sludge that is used in this study as shown in Table 5.8 (Schmidt et al., 1999). Besides acetate, *Methanosarcina* spp. is also capable of growing on substrates such as methanol, methylamines, and sometimes hydrogen and carbon dioxide. *Methanosaeta* spp. have a lower growth rate at high acetate concentrations than do *Methanosarcina spp*., but their affinity for acetate is higher (Zinder, 1990).

In the seed sludge, the percentage of *Methanobacteriales* relatives (8.4% of the active microbial community) was higher than *Methanomicrobiales* and *Methanococcales* relatives. In some studies *Methanobacteriales* such as *Methanobacterium* and *Methanobrevibacter* have been reported to be the dominant hydrogen- and formate-consuming methanogens (Raskin et al., 1994), while other studies have shown that *Methanomicrobiales* can also be present in the sludge in high numbers and can even be more abundant than *Methanobacteriales* (Raskin et al., 1995). It has been previously reported that among the hydrogenotrophic methanogens, *Methanococcales* were almost absent within granular sludge (Hofman-Bang et al., 2003) or has been reported to play relatively small role (Raskin et al., 1995.) The predominance of a subpopulation of hydrogenotrophic methanogens are difficult to explain, since the competition for common subsrates among different hydrogenotophic methanogens has been studied less extensively than the competition for acetate among acetoclastic methanogens (Raskin et al., 1996).

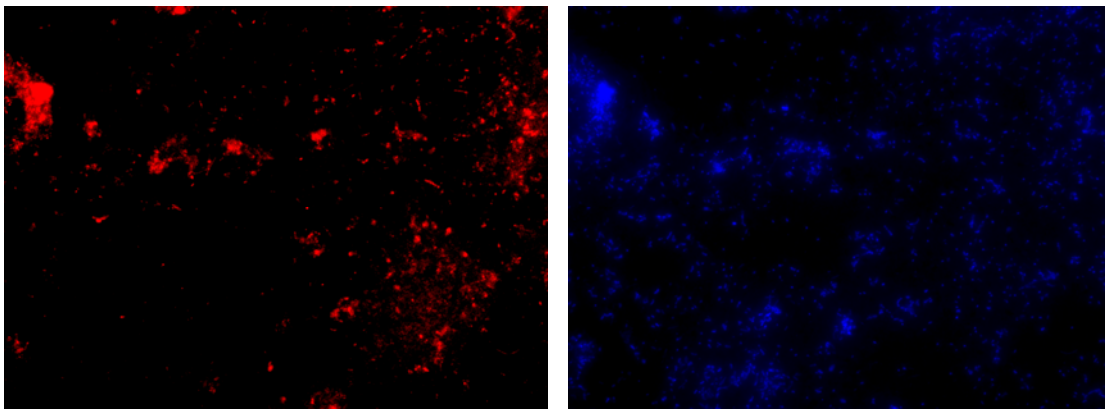
Table 5.8. FISH results of seed sludge

	%	Standardized
Univ*	80 ± 4,0	80
Bacteria**	42 ± 2,0	33.6
Archaea**	62 ± 1,0	49.6
Mx825***	32 ± 0.8	15.8
Ms821***	8 ± 1.5	4
MsMx***	61 ± 2	30.3
Ms1414***	31 ± 0.8	15.3
Mb310***	17 ± 1.3	8.4
Mc1109***	12 ± 1,1	5.9
Mg1200***	14,2 ± 1,6	7
Srb385	26,3 ± 1,8	8.8
Alfa proteobacteria	14,4 ± 1,6	4.8
Beta proteobacteria	19,0 ± 1,5	6.4
Gamma proteobacteria	21,2 ± 2,2	7.1
Non-eub	1,2 ± 0,9	0.9

* (The number of cells detected with UNIV1392)/(Total cell count using DAPI).

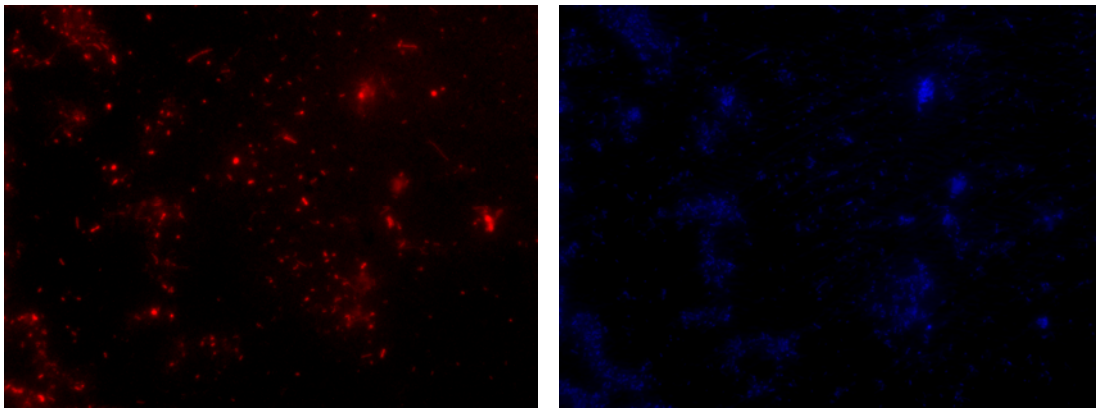
** (The number of cells detected with ARC915 or Eub 338) / (The number of cells detected with UNIV1392).

*** Relative population in *Archaea.*



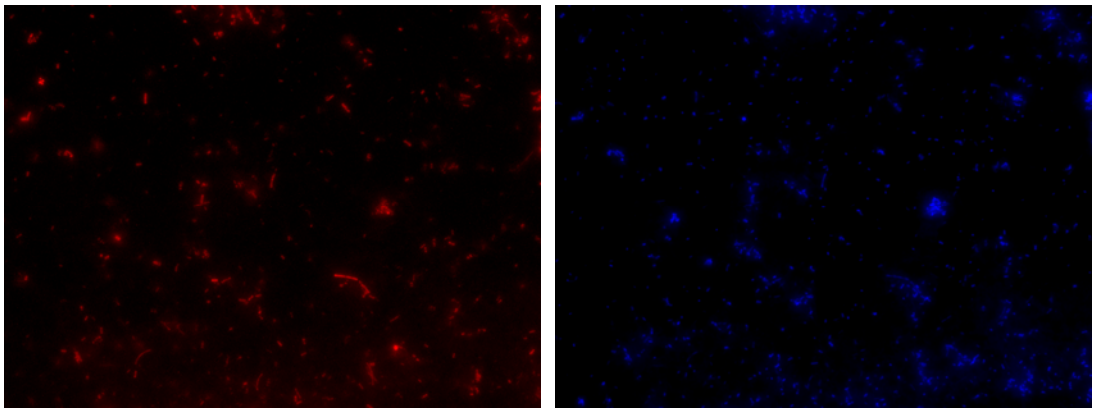
(a) (b)

Active cells hybridized with UNIV1392 probe



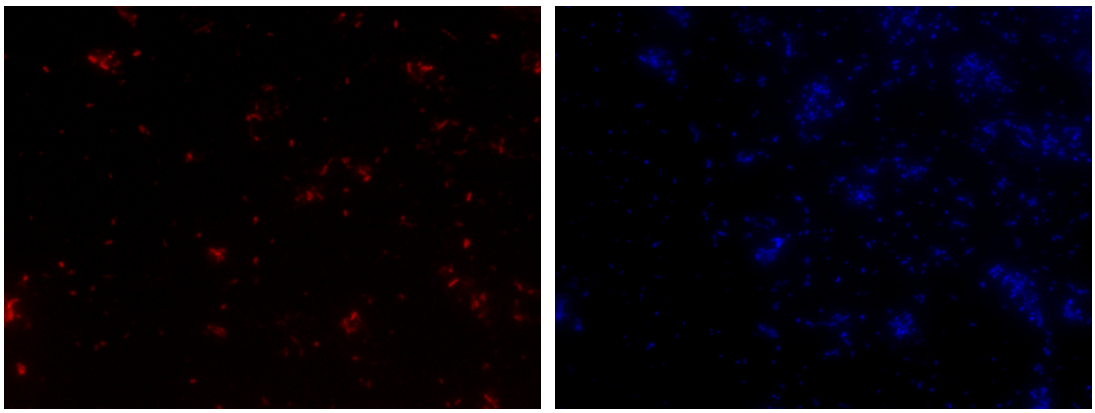
(a) (b)

Eubacteria hybridized with EUBMIX probe



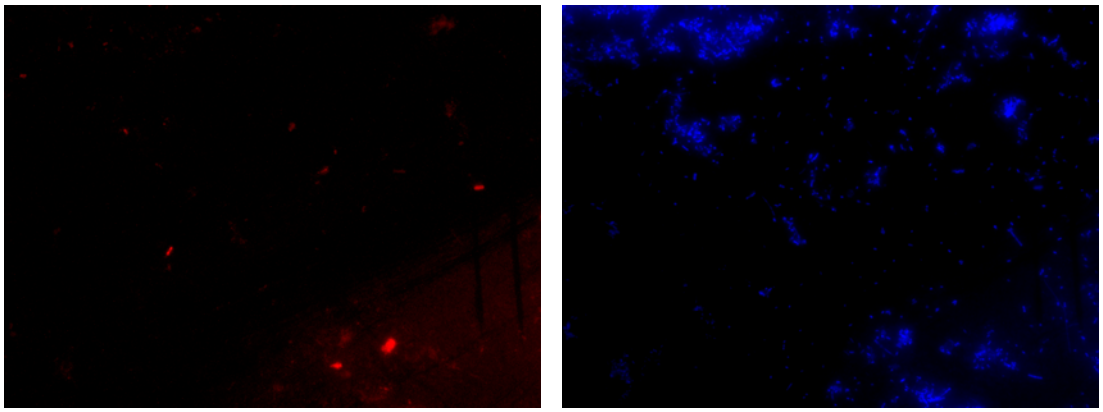
(a) *Archaea* hybridized with ARC195 probe (b) DAPI

Figure 5.13. Epifluorescence micrographs of the hybridized seed sludge samples. (a) Fluorescent and (b) DAPI images are in the same field (cont.).



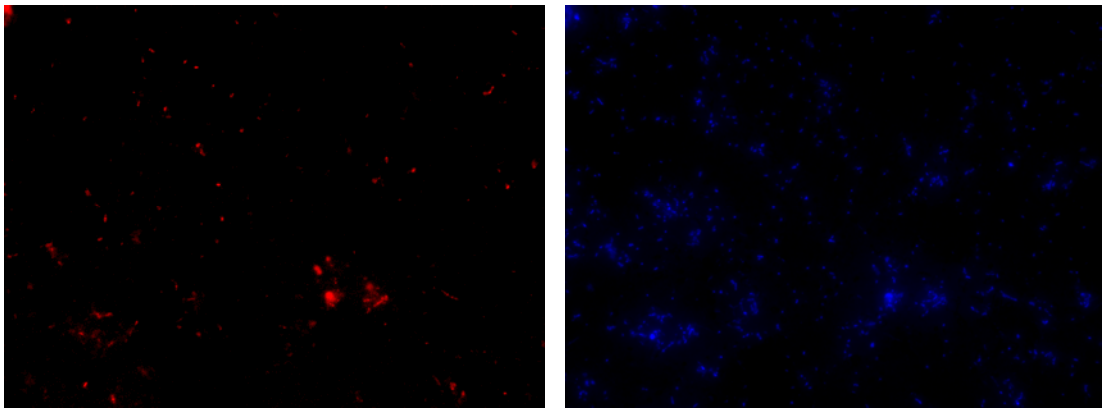
(a) (b)

Methanosaeta hybridized with MX825 probe



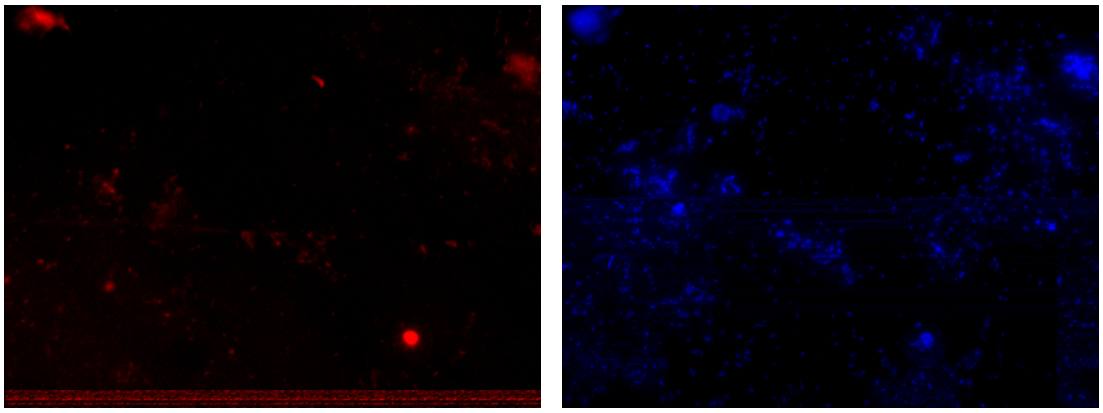
(a) (b)

Methanosarcina hybridized with MS821 probe



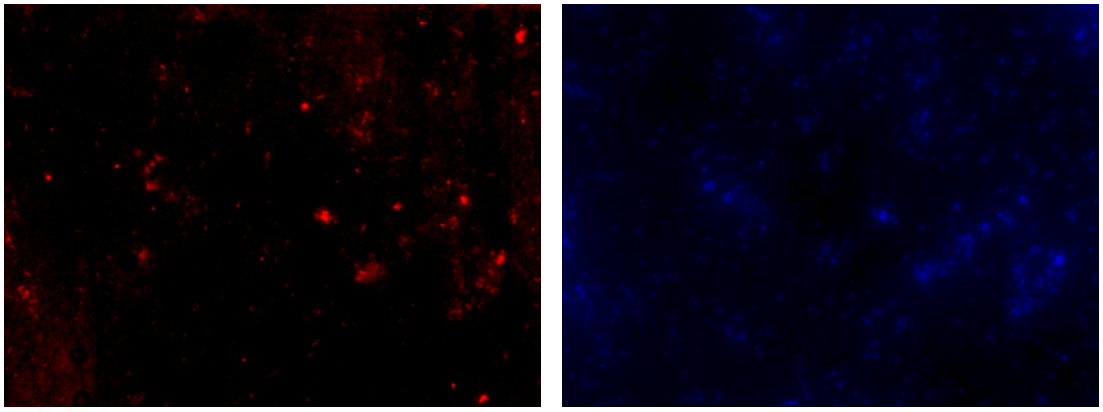
(a) *Methanobacteriales* hybridized with MB310 probe (b) DAPI

Figure 5.13. Epifluorescence micrographs of the hybridized seed sludge samples. (a) Fluorescent and (b) DAPI images are in the same field (cont.).



(a) (b)

Methanococcales hybridized with MC1109 probe



(a) (b)

Methanomicrobiales hybridized with MG1200 probe

Figure 5.13. Epifluorescence micrographs of the hybridized seed sludge samples. (a) Fluorescent and (b) DAPI images are in the same field.

5.3. Assessing IC_{50} for selected solvents on anaerobic sludge

Solvent-induced inhibition of acetoclastic methanogenic populations was determined using the SMA tests at different solvent concentrations as a preliminary study. SMA tests were carried out with the seed sludge from the EGSB reactor to determine IC_{50} concentrations of toluene, propanol and methanol concentrations (molarity) that resulted in 50% decrease in SMA. 2000 mg/L acetate was used as subsrate in the SMA test reactors and a control reactor without any solvent was used for each test unit. All activity assays contained 2 g VSS/L and were performed in triplicate.

Toluene concentrations ranging from 0.05 mM to 1.2 mM, propanol concentrations ranging from 0.1 M to 1 M and methanol concentrations ranging from 0.1 M to 1.5 M were used as test concentrations in SMA tests. IC_{50} concentrations of the sludge for toluene, propanol and methanol have been found to be 1.2 mM, 0.3 M and 0.4 M, respectively. The results are given in Figures 5.14-5.19. Similarly, IC_{50} value for methanol has been reported to be 400 mM for a nongranular sludge from a cattle rumen (Enright et al., 2005). The solvent concentrations that are found to be IC_{50} value, were used in the SMA tests to determine the effects of the solvents on methanogenic activity. Then, solvent-induced inhibition of acetoclastic and hydrogenotrophic methanogenic populations was determined for each sludge to evaluate the effect of solvents on these microbial populations by FISH studies.

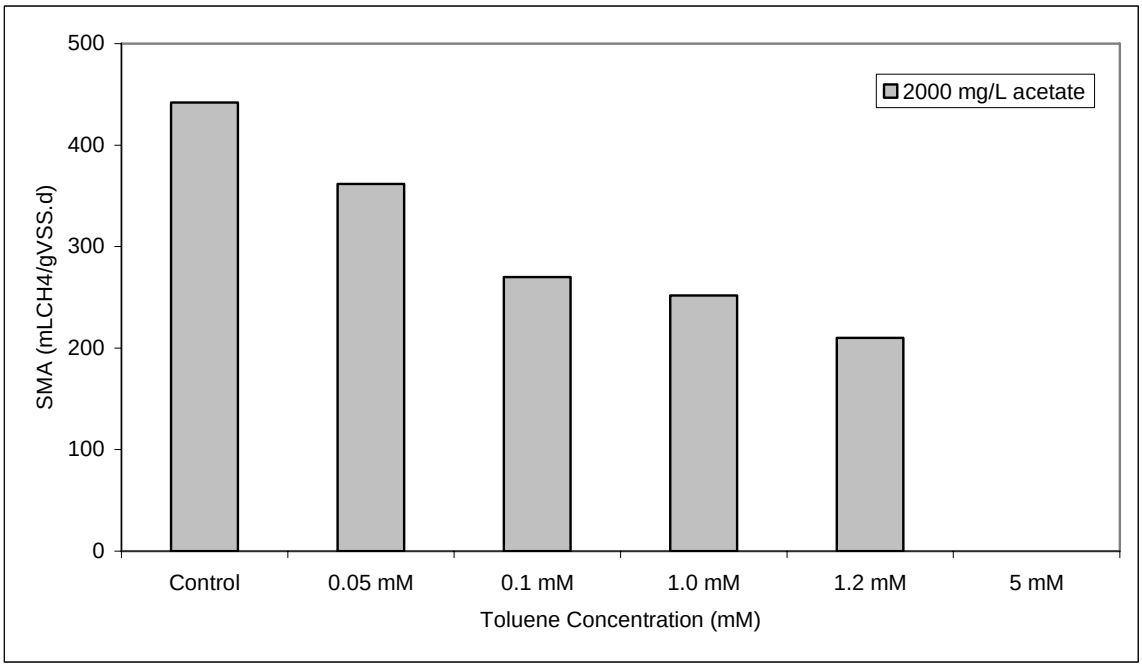


Figure 5.14. Specific methanogenic activity of seed sludge at various toluene concentrations

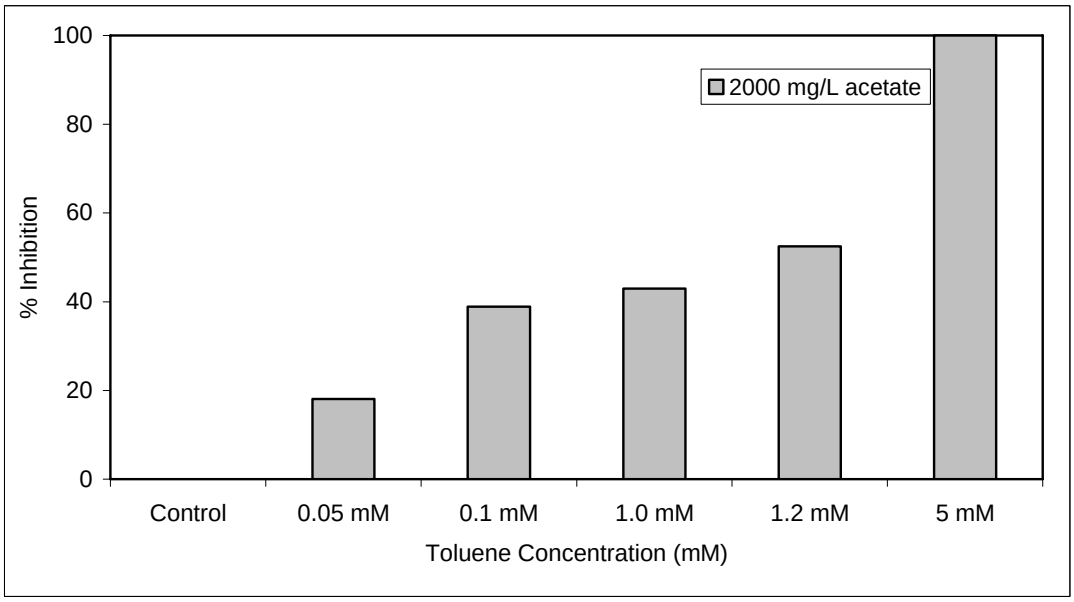


Figure 5.15. % Inhibition on SMA at various toluene concentrations

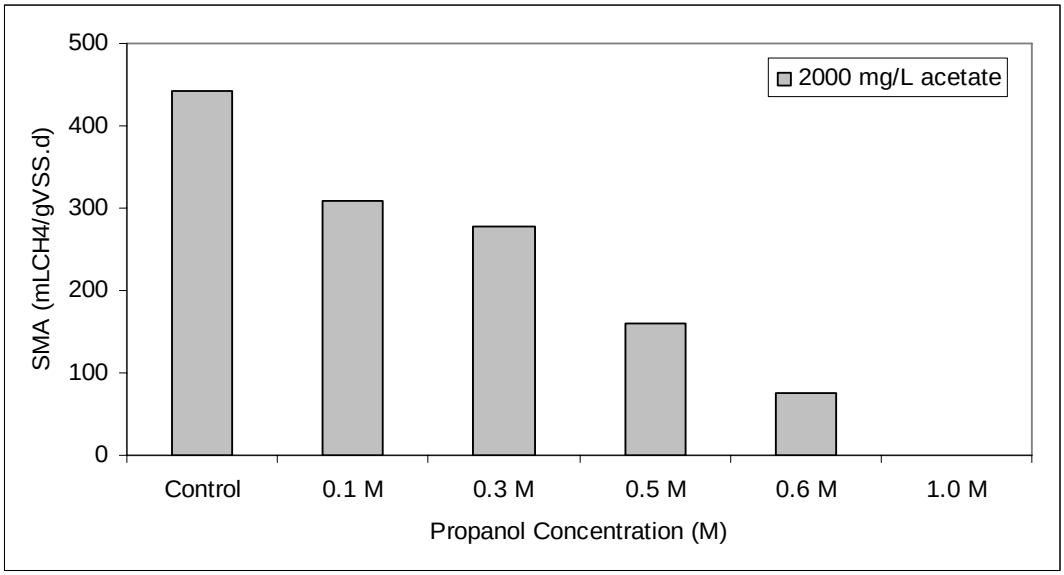


Figure 5.16. Specific methanogenic activity of seed sludge at various propanol concentrations

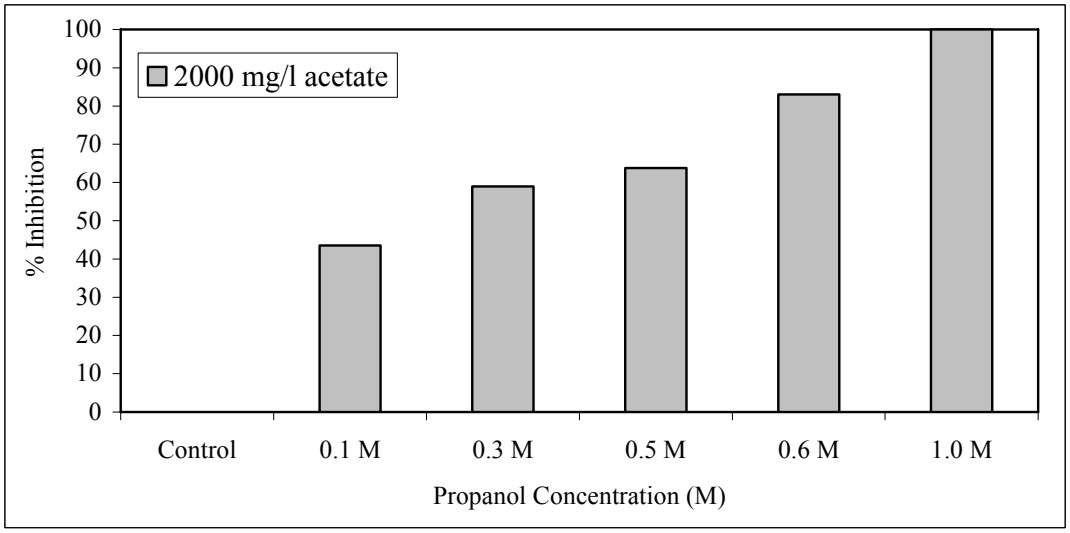


Figure 5.17. % Inhibition on SMA at various propanol concentrations

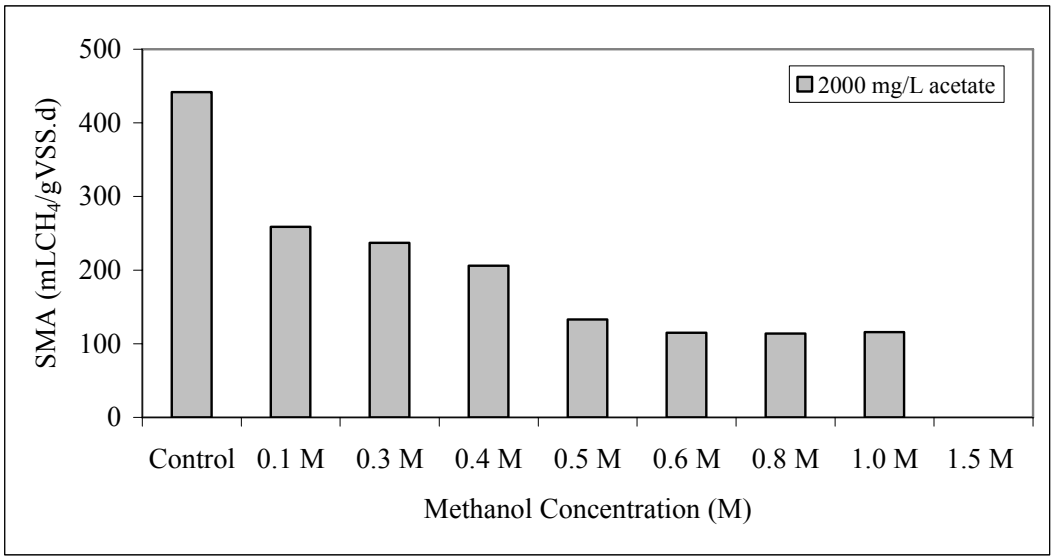


Figure 5.18. Specific methanogenic activity of seed sludge at various methanol concentrations

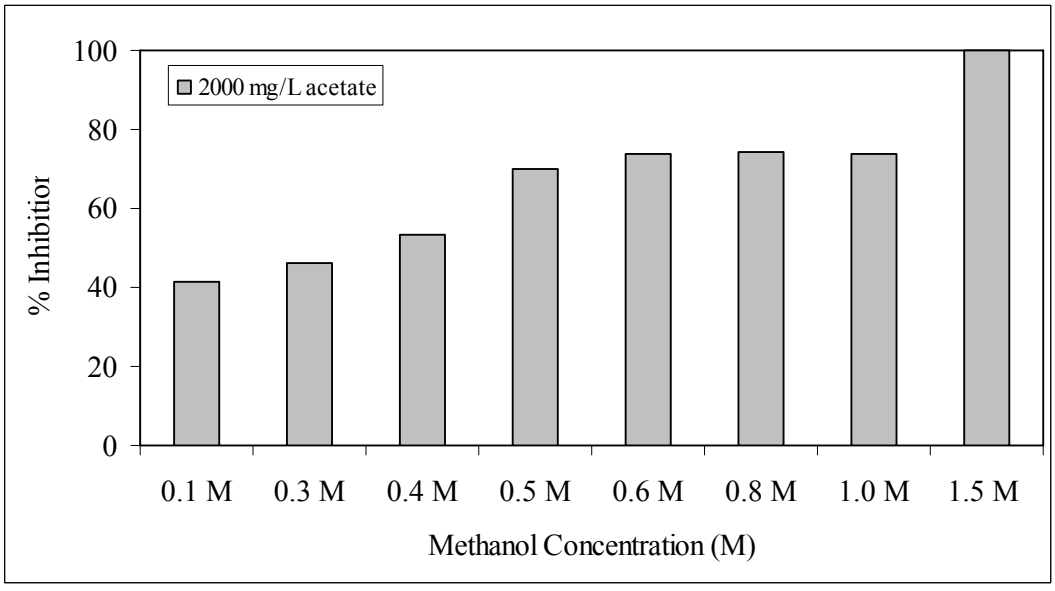


Figure 5.19. % Inhibition on SMA at various methanol concentrations

5.4. Single Phase and Two-Phase Reactor Operations

The anaerobic seed sludge which has a high acetoclastic methanogenic capacity of 453 mLCH_4/gVSS/day was inoculated into anaerobic sequencing batch reactors including single phase and two phase reactor. Single phase reactor and acidogenic reactor being the first stage of two-phase reactor were fed with a glucose-based synthetic wastewater. The methane reactor was fed with the effluent of acidogenic reactor. Sludge samples taken from the reactors at the end of the operation period were subjected to solvents at preliminary determined IC_{50} concentrations in order to determine effects of selected solvents on activity and microbial diversity of different phases of anaerobic digestion.

5.4.1. Performance of the single phase lab- scale anaerobic batch reactor

A lab-scale anaerobic batch reactor which was inoculated with the seed sludge taken from a full-scale EGSB reactor was operated with a synthetic wastewater as a preliminary-study in order to use the anaerobic sludge at the end of the operation period for SMA tests and FISH studies. The sludge taken at the end of the operational period of 47 days was used in the SMA tests and FISH studies to evaluate the effects of selected solvents on activity and microbial community of the sludges. The lab-scale anaerobic batch reactor which has a active volume of 1.6 L was operated for 47 days at 35±2°C with glucose as substrate at a hydraulic retention time of 36 hours and a sludge retention time of 30 days. During the operation time, the pH was kept in a range between 6.8 and 7.4 with $NaHCO_3$ addition. Influent COD of the anaerobic reactor was increased gradually from 2500 mg/L to 9000 mg/L during the operation period. Efficient and stable COD removal in a range of 88-98% was maintained during the operation period. The methane content of the biogas remained relatively stable at 70%. Figure 5.20 shows the performance of the lab-scale anaerobic batch reactor.

The SMA tests were applied to the sludge samples taken at the end of the operational period of the anaerobic batch reactor. Maximum acetoclastic and maximum overall methanogenic activity of the lab-scale anaerobic batch reactor sludge, which will be used

for further inhibition studies were found to be 437 $mLCH_4$/gVSS/day at 2000 mg/L acetate and 490 $mLCH_4$/gVSS/day respectively as illustrated in Figures 5.21 and 5.22.

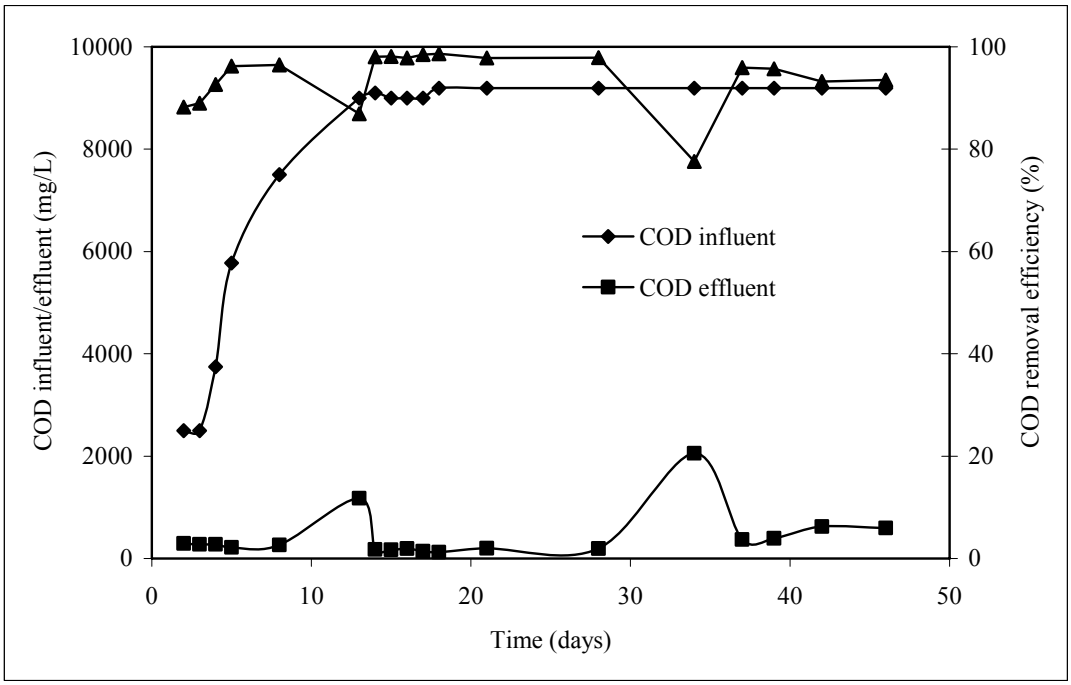


Figure 5.20. Performance of single phase lab-scale anaerobic batch reactor

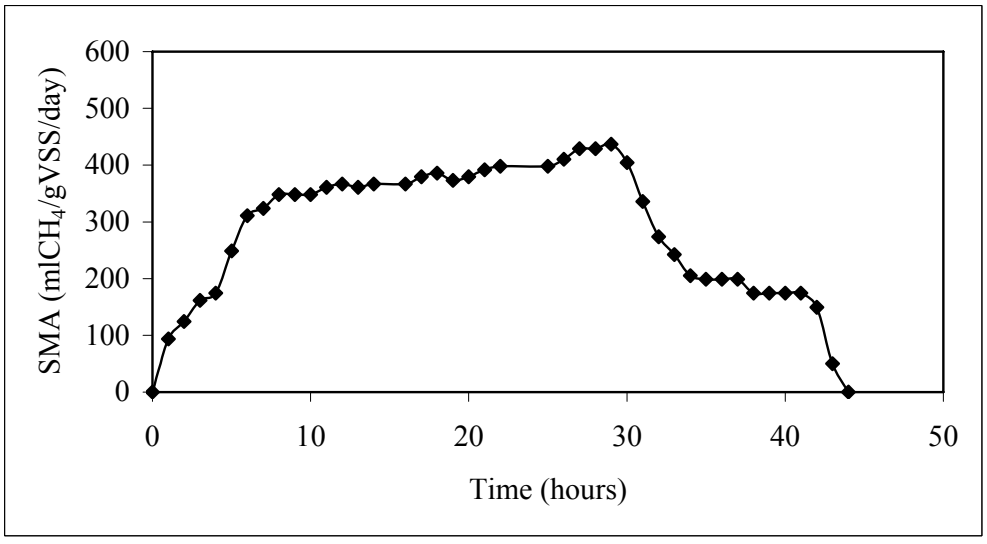


Figure 5.21. Acetoclastic methanogenic activity of the lab-scale anaerobic batch reactor

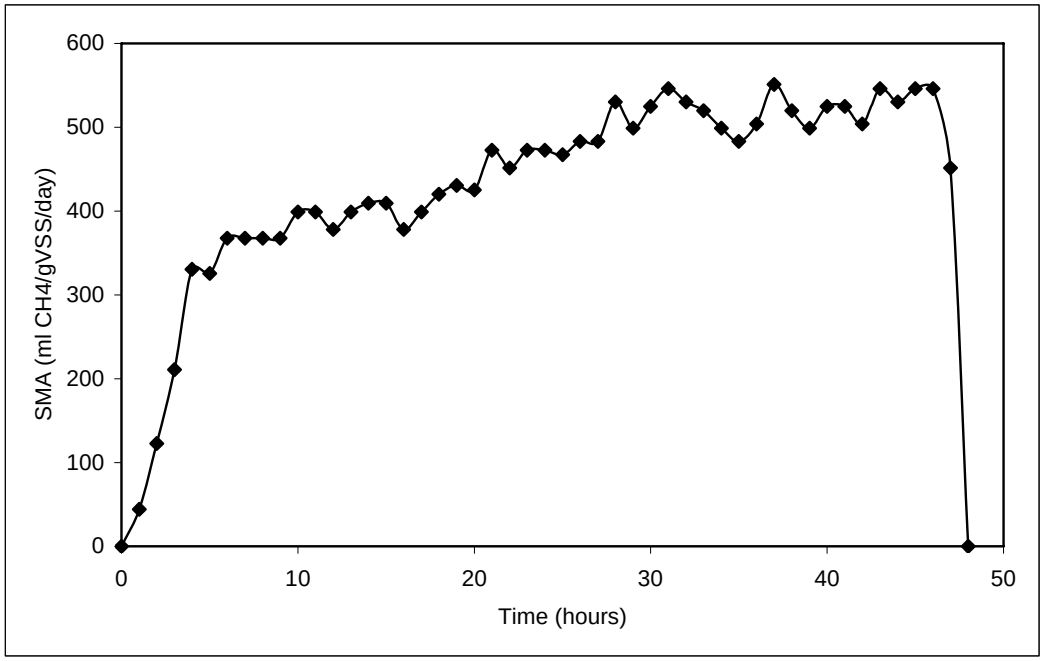


Figure 5.22. Overall methanogenic activity of the lab-scale anaerobic batch reactor

5.4.2. Performance of the Two-Phase Anaerobic Reactor

5.4.2.1. Acid Reactor. The acidogenic reactor was gradually loaded with increasing concentration of glucose in the synthetic feed from 2500 to 9000 mg/L. The acidogenic reactor was operated on a 2 cycle/day for a total of 51 days. The pH of acidogenic reactor was controlled to an average of 5.0-5.5. Figure 5.23 shows the performance of acidogenic reactor during the study. Due to stability problems in the reactor, influent COD was decreased from 9000 mg/L to approximately 6000 mg/L and influent concentration was maintained at this value at the remaining part of the study. After day 25, COD removal efficiency ranged between 15-20%. It was expected that the acidogenic reactor would not remove significant amounts of COD since the acidified wastewater and the raw wastewater COD concentrations would be approximately equal. The reactor was operated at a S/X ratio of 0.62, pH in a range of 5.5±0.1. The degree of acidification achieved was approximately 30% at these conditions. It has been recommended that the degree of acidification should be in a range of 20–40% to maintain stability of process (Lettinga and

Hulshof, 1991). As VFA concentration is the most widely used parameter to assess acidification, the combined COD equivalent of each individual VFA was used to express the total COD of VFA in the effluent (COD of VFAeff) and this was compared to the soluble COD of the influent (Soluble CODinf) and the degree of acidification calculated by Eq. (5.1). The COD equivalents and percentage of organic carbon for individual VFA are given in Figure 5.24. The main VFAs were found to be acetic, butyric and caproic acids in the reactor.

$$\text{Degree of acidification (\%)} = \frac{\text{COD of VFA (mg/L) eff}}{\text{soluble COD (mg/L) inf}} \times 100 \qquad (5.1.)$$

The objective of the acidogenic reactor was to acidify the synthetic wastewater in an effort to improve the performance of the methanogenic reactor by increasing the COD removal efficiency and to promote the growth of acid-forming microorganismsin the acid reactor and methanogenic *Archaea* in methane reactor. In addition, it was hypothesized that the acidogenic reactor may increase the stability of the two-phase system by reducing the effect of shock loadings to the methanogenic reactor. Operating conditions for the acidogenic reactor were set based on several studies reported in the literature evaluating the performance of acid-phase reactors treating various wastewaters (Ince, 1998; Anderson et al., 1994). In the previous studies, effects of pH, HRT and temperature on substrate degradation, degree of acidogenic conversion from COD to VFAs, changes in the distribution of major VFAs produced, overall COD removal efficiencies were evaluated.

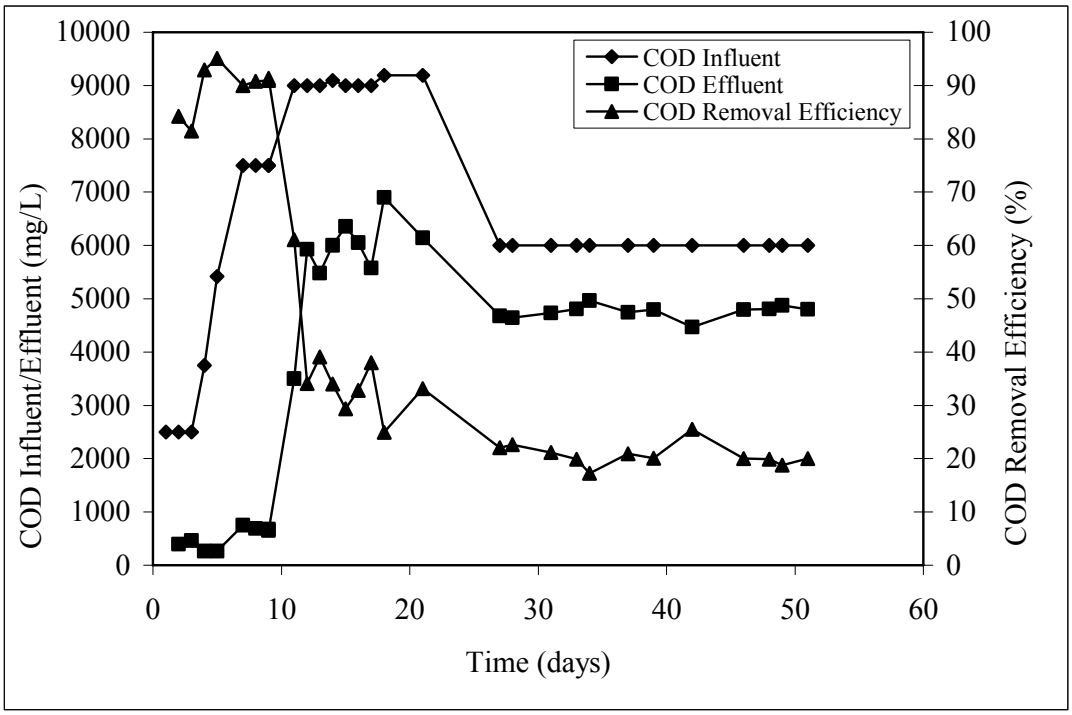


Figure 5.23. Performance of lab-scale anaerobic batch acid reactor

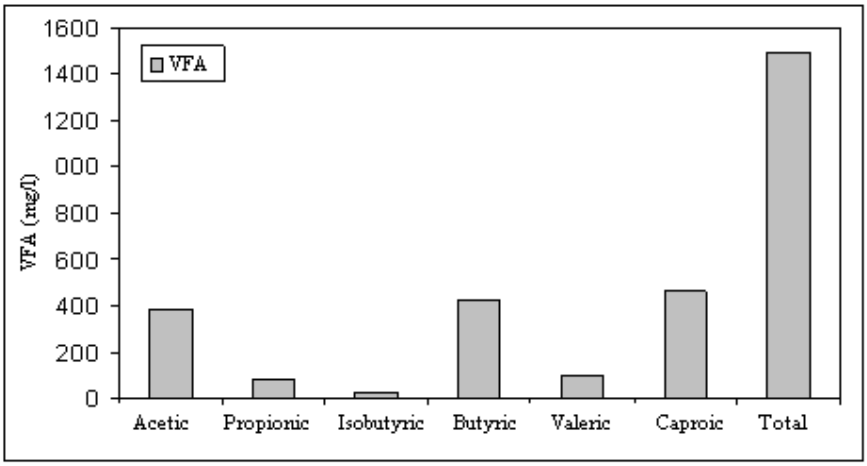


Figure 5.24. VFA in acid reactor at the end of the acid reactor.

5.4.2.2. Methane Reactor. The methanogenic reactor was operated to optimize the growth of methanogenic *Archaea*. The methanogenic reactor was fed with the acidified effluent wastewater from the acidogenic reactor and was operated on a 1-day cycle for a total of 38 days at a S/X ratio of 0.58. The COD concentration of the acidified wastewater fluctuated throughout the study. The pH of the aciodgenic reactor effluent was arranged to 7.0 and fed to the methanogenic reactor. The pH in the methane reactor was maintained between 7.0 to 7.5 in the methane reactor by adding alkalinity source. A COD removal efficiency over 95% was obtained. Figure 5.25 shows the performance of lab-scale anaerobic batch methane reactor.

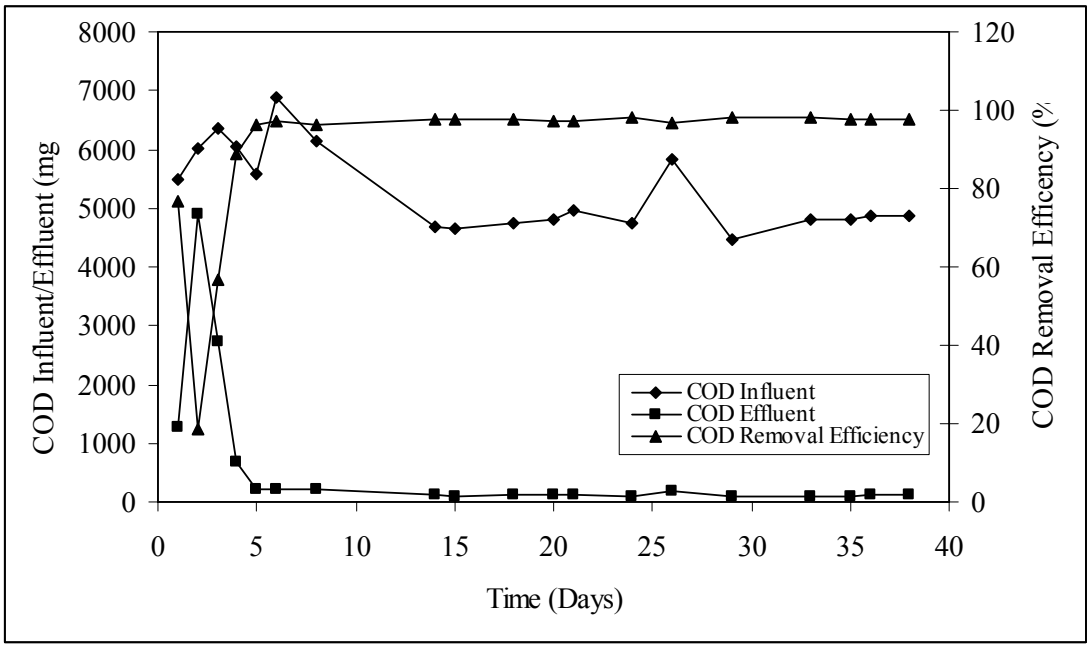


Figure 5.25. Performance of lab-scale anaerobic batch methane reactor

5.4.3. FISH results of sludge from the single phase and two-phase anaerobic batch reactors

At the end of the operation period of single phase and two phase anaerobic reactors, microbial composition of the sludge samples was characterized by FISH. Table 5.9 summarizes FISH results of the sludge samples from lab scale anaerobic batch reactors and Figure 29 shows the distribution of *Archaeal* subpopulation (%) in anaerobic batch reactor sludges. 53.2±1.5% of the cells in the lab-scale anaerobic batch reactor sludge from acid phase gave positive signal with UNIV1392 probe, that is, 53.2% of the microorganisms were metobolically active. This ratio was 77.9±1.6% for methane phase and 73.2±1.2% for single phase operation. Archaeal population was detected as 44.2±2.6% for acid phase, 88.7±1.4% for methane phase and 57.1±0.8% for single phase operation, respectively. Some epifluorescence micrographs of the anaerobic sludge from the acid reactor are shown in Appendix A. Compared to the seed sludge there was a slight increase in the percentage of Eubacteria and a slight decrease in Archaeal population in the single phase anaerobic reactor sludge. However, a decrease in archaeal population in the acid reactor was observed whereas there was an increase in the methane reactor. FISH results showed that the phase separation has been succesfully maintained in the reactors (Acid reactor- 44.2±2.6% Archaea, 56.5±2.0% eubacteria; Methane reactor- 88.7±1.4% Archaea, 28.1±4.2% bacteria; Single phase reactor- 57.1±0.8% Archaea, 49.6±1.1% eubacteria).

The Archaeal subpopulation from the single phase anaerobic reactor composed of 54.2±0.8% of members of the genus *Methanosaeta* (With Mx825 probe), 30.1±1.4% *Methanosarcina* (with Ms821 probe), 14.2 ± 2.2 *Methanobacteriales* (with Mb310 probe), 8.6±0.3% *Methanococcales* (with Mc1109 probe), 8.2±1.7% *Methanomicrobiales* (with Mg1200 probe). *Methanosaeta* spp. show a 42% increase in the anaerobic batch reactor sludge compared to the seed sludge. Besides *Methanosarcina spp*. increased from 4.0% to 12.6%. An increase in the relative abundance of acetoclastic methanogens was accompanied by a corresponding decrease in the relative abundunce of hydrogenotrophic methanogens (Figure 5.16). In the methane reactor, archaeal subpopulation composed of 76.9±1.1% of members of the genus *Methanosaeta,* 26.7±3.2% *Methanosarcina,*

15.5±1.2% *Methanobacteriales,* 10.6±1.3% *Methanococcales and* 18.2±1.0% *Methanomicrobiales.* The elevated amount of *Methanosaeta* sp. in total community at the end of the operation of the anaerobic reactor shows that anaerobic reactor performed well (over 95% COD removal efficiency) and stabilizion occured, the results were correlated with the literature stating that *Methanosaeta* sp. improves granulation and result in more stable reactor performance. Since only 53.2% of the total microbial community was active in the acid reactor, the FISH results were standardized against active population in order to compare sub-population levels in the anaerobic reactors operated at different phases (Table 5.9).

Table 5.9. FISH results of single and two-phase anaerobic batch reactors.

	Seed Sludge	Two-Phase		Single Phase Reactor
		Acid Reactor	Methane Reactor	
Univ*	80 ± 4.0	53.2 ± 1.5	77.9 ± 1.6	73.2 ± 1.2
Bacteria**	42 ± 2.0	56.5 ± 2.0	28.1 ± 4.2	49.6 ± 1.1
Archaea**	62 ± 1.0	44.2 ± 2.6	88.7 ± 1.4	57.1 ± 0.8
Mx825***	32 ± 0.8	52.8 ± 1.8	76.9 ± 1.1	54.2 ± 0.8
Ms821***	8 ± 1.5	6.1 ± 1.2	26.7 ± 3.2	30.1 ± 1.4
MsMx***	61 ± 2	53.5 ± 2.3	78.4 ± 1.3	57.2 ± 1.2
Ms1414***	31 ± 0.8	15.2 ± 2.2	12.3 ± 2.2	12.1 ± 1.5
Mb310***	17 ± 1.3	18.2 ± 1.3	15.5 ± 1.2	14.2 ± 2.2
Mc1109***	12 ± 1.1	12.1 ± 1.5	10.6 ± 1.3	8.6 ± 0.3
Mg1200***	14.2 ± 1.6	10.2 ± 1.4	18.2 ± 1.0	8.2 ± 1.7
Srb385	26.3 ± 1.8	42.7 ± 1.3	18.2 ± 2.9	12.2 ± 2.1
Alfa proteobacteria	14.4 ± 1.6	24.2 ± 3.3	8.1 ± 1.3	12.4 ± 2.1
Beta proteobacteria	19.0 ± 1.5	36.1 ± 1.6	15.5 ± 1.9	17.5 ± 1.5
Gama proteobacteria	21.2 ± 2.2	19.2 ± 1.4	14.4 ± 1.6	19.4 ± 1.7
Non-eub	1.2 ± 0.9	2.4 ± 0.4	2.4 ± 0.3	2.1 ± 1.0

* (The number of cells detected with UNIV1392)/(Total cell count using DAPI).

** (The number of cells detected with ARC915 or Eub 338) / (The number of cells detected with UNIV1392).

*** Relative population in *Archaea.*

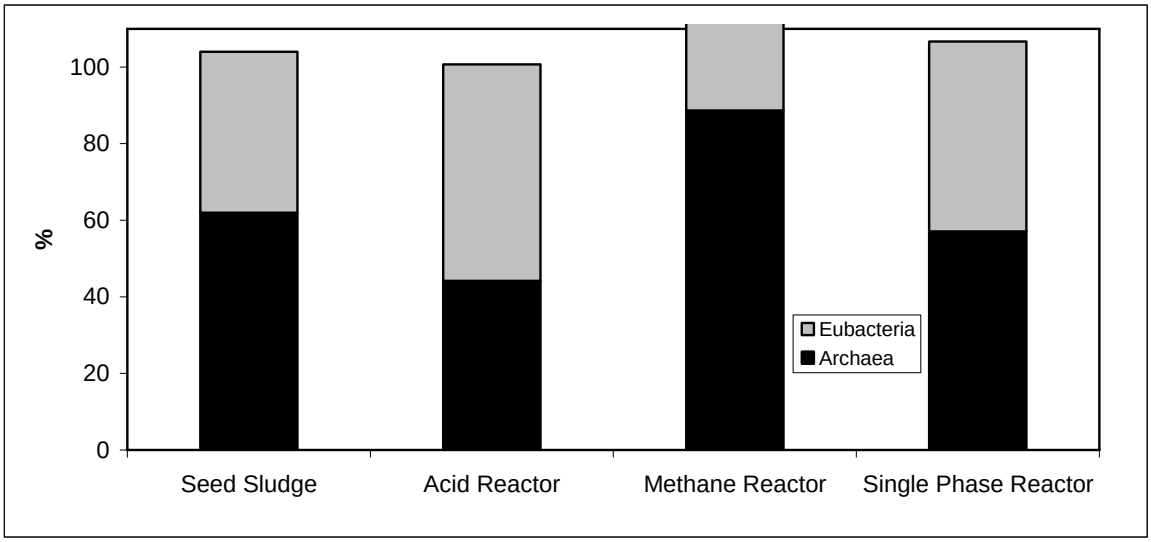


Figure 5.26. Archaeal and eubacterial composition of the sludge taken from single phase and two-phase reactor.

Table 5.10. Standardized FISH results of single and two-phase anaerobic batch reactors' sludges.

Probe	Seed Sludge	Acid Reactor	Methane Reactor	Single Phase Reactor
Mx825	15.8	12.4	53.1	22.6
Ms821	4	1.4	18.5	12.6
MsMx	30.3	12.6	54.2	23.9
Ms1414	15.3	3.6	8.5	1.7
Mb310	8.4	4.3	10.5	5.9
Mc1109	5.9	2.8	7.3	3.6
Mg1200	7	2.4	12.6	3.4
Srb385	8.8	12.8	4	4.4
Alfa proteobacteria	4.8	7.3	1.8	4.5
Beta proteobacteria	6.4	10.9	3.4	6.3
Gama proteobacteria	7.1	19.2	3.1	7

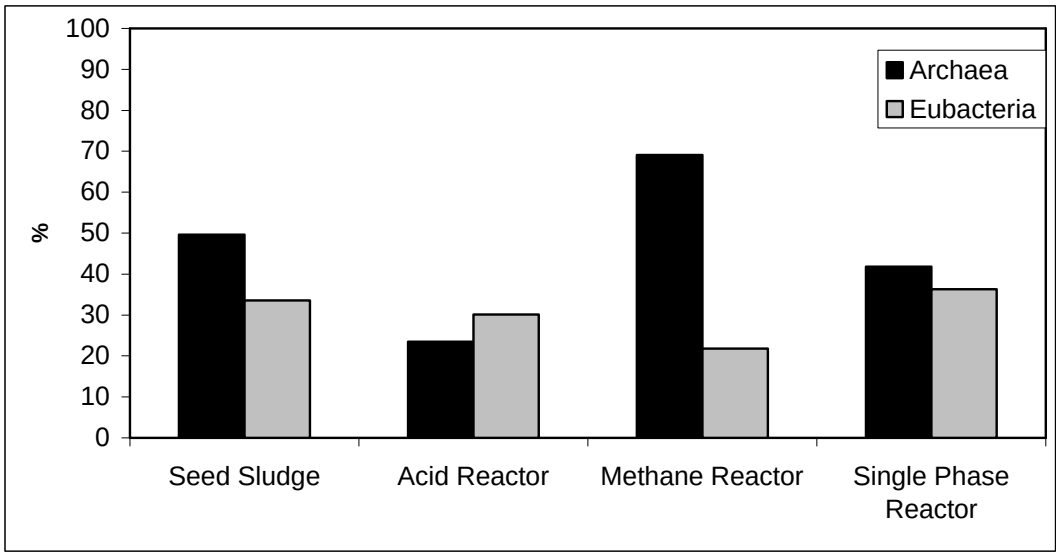


Figure 5.27. Distribution of *Archaeal* and bacterial composition (%) in single and two-phase anaerobic batch reactors' sludges (standardized).

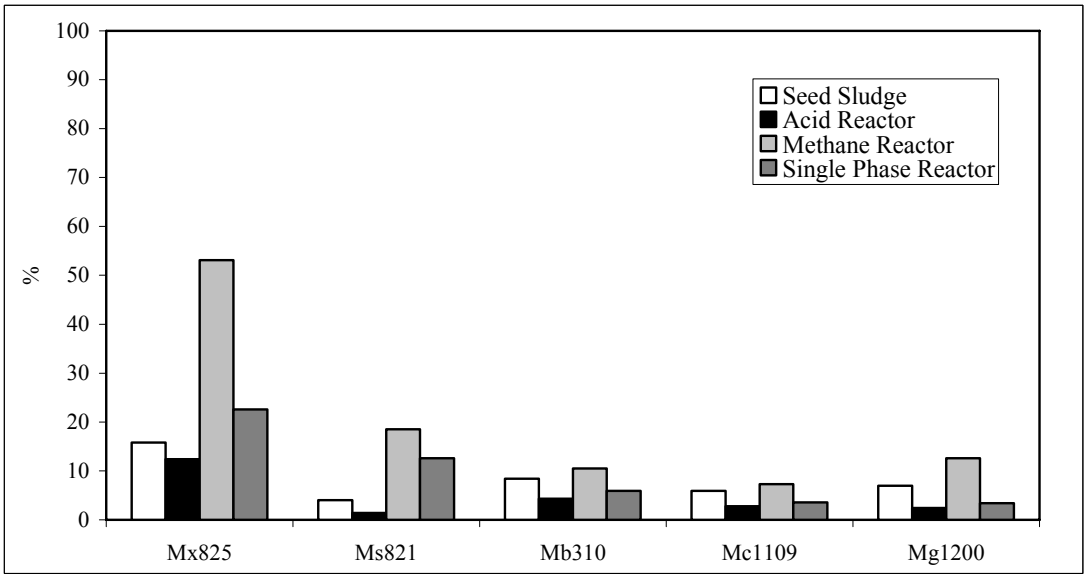


Figure 5.28. Distribution of *Archaeal* subpopulation (%) in single and two-phase anaerobic batch reactors' sludges (standardized).

In the scope of this thesis, quantification of alfa proteobacteria, beta proteobacteria and gamma proteobacteria was done for the first time in single phase and two phase anaerobic digestion. As can be seen from the Figure 5.29, gamma proteobacteria were the predominant compared to alfa and beta proteobacteria. The relative ratio of the groups decreased significantly in the methane reactor.

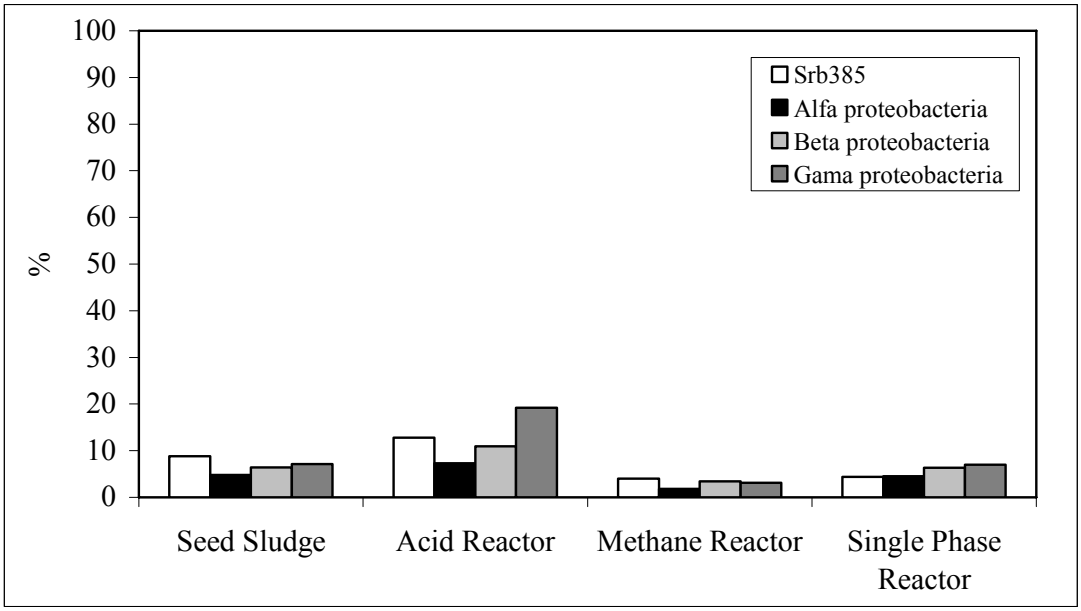


Figure 5.29. Distribution of bacterial subpopulation (%) in single and two-phase anaerobic batch reactors' sludges (standardized)

5.5. Effect of Solvents and Solvent Mixtures on Methanogenic Activity and Microbial Composition of Anaerobic Sludge Taken From Single Phase and Two-phase

5.5.1. Single phase reactor

Methanogenic activity tests had been carried out to determine the effect of solvents-methanol, toluene and isopropanol on anaerobic sludge which was taken from the lab-scale anaerobic batch reactor operated in single phase. IC_{50} concentrations of methanol, propanol and toluene were applied to SMA test reactors. Acetate (2000 mg/L) and VFA mixture were used as substrates in order to determine effect of solvents on acetoclastic and total methanogenic activity, respectively. Table 5.11 summarizes the effect of IC_{50} concentrations of solvents on acetoclastic methanogenic activity. According to SMA test result, acetoclastic methanogenic activity of the sludge decreased from 412 $mLCH_4$/gVSS/day to 252 $mLCH_4$/gVSS/day for methanol, 229 $mLCH_4$/gVSS/day for propanol, 211 $mLCH_4$/gVSS/day for toluene, 128 $mLCH_4$/gVSS/day for methanol-propanol mixture, respectively (Figure 5.30).

Table 5.11. Effect of IC_{50} concentrations of solvents on acetoclastic methanogenic activity test of the sludge taken from single phase anaerobic reactor

Substrate (acetate)	**Maximum activity** $mLCH_4$/gVSS/day	**std. dev.**
Control	412	10,7
0.4 M Methanol	252	12,8
0.4 M Propanol	229	14,2
1.2 mM Toluene	211	10,4
0.2 M Methanol-0.2 M Propanol Mixture	128	18

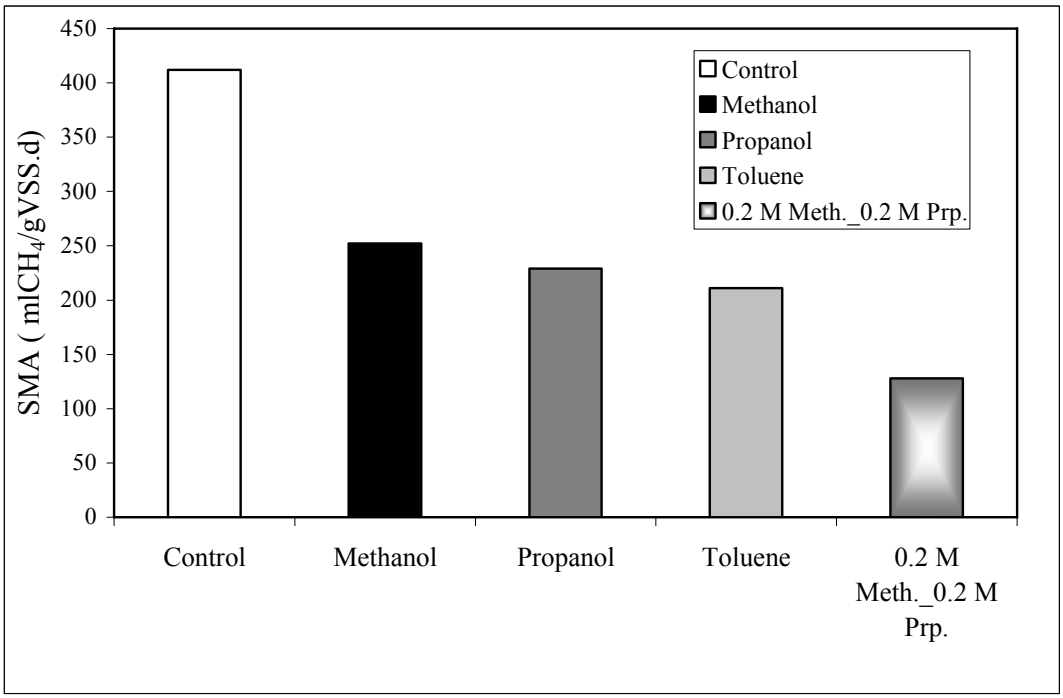


Figure 5.30. Effect of IC_{50} concentrations of solvents on acetoclastic methanogenic activity test of the sludge taken from single phase anaerobic reactor

IC_{50} concentrations of methanol, propanol and toluene were applied to SMA test reactors in order to determine the effect of solvents on total methanogenic activity (Table 5.12). Total methanogenic activity of the sludge decreased from 548 $mLCH_4$/gVSS/day to 221 $mLCH_4$/gVSS/day for methanol, 240 $mLCH_4$/gVSS/day for propanol, 272 $mLCH_4$/gVSS/day for toluene, 129 $mLCH_4$/gVSS/day for methanol-propanol mixture, respectively (Figure 5.31).

Table 5.12. Effect of IC_{50} concentrations of solvents on total methanogenic activity test of the sludge taken from single phase anaerobic reactor

Substrate (VFA mixture)	Maximum activity $mLCH_4/gVSS/day$	std. dev.
Control	548	12,4
0.4 M Methanol	221	10,1
0.4 M Propanol	240	12,6
1.2 mM Toluene	272	14,2
0.2 M Methanol-0.2 M Propanol Mixture	129	12,8

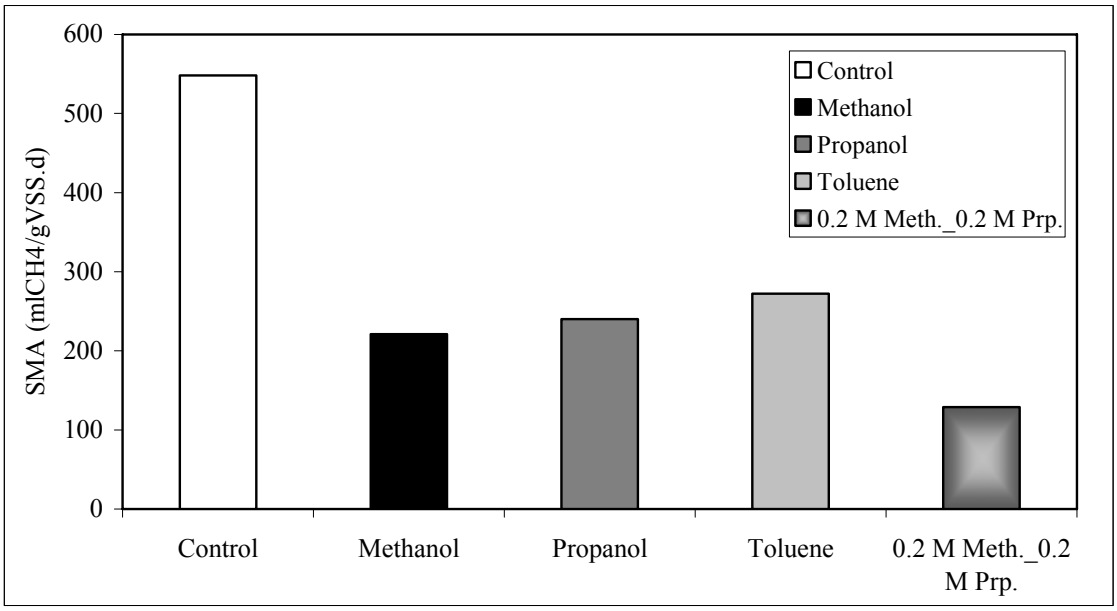


Figure 5.31. Effect of IC_{50} concentrations of solvents on total methanogenic activity test of the sludge taken from single phase anaerobic reactor

FISH was applied to the sludge samples taken from the solvent-added SMA test reactors in order to determine the changes in microbial community in the sludge samples. The microbial community structure of the SMA samples was characterized using FISH

(Table 5.13). FISH results were standardized against active populaton in order to make comparison between the samples taken from the different reactors operated in single phase and two phase (Table 5.14). UNIV1392 probe showed that 73.2% of the microorganisms in the control reactor were metabolically active. This ratio in the SMA reactors fed with IC_{50} concentrations of the solvents was 64.8 ± 2.8 for methanol, 71.4 ± 2.4 for toluene, 57.4 ± 2.1 for isopropanol. As can be seen from the table, the most pronounced effect on active population was caused by IC_{50} concentration of isopropanol followed by methanol. The active population in the toluene-added SMA reactor did not show a significant change compared to control reactor.

Table 5.13. FISH results of the sludge taken from single phase anaerobic reactor and the solvent-added SMA test reactors

	Control	0.4 M Methanol	1.2 mM Toluene	0.4 M isopropanol
Univ	73.2 ± 1.2	64.8 ± 2.8	71.4 ± 2.4	57.4 ± 2.1
Archaea*	57.1 ± 0.8	48.0 ± 1.9	50.3 ± 2.4	49.8 ± 2.5
Bacteria*	49.6 ± 1.1	51.1 ± 2.5	51.5 ± 3.4	48.5 ± 3.2
*M.saeta***	54.2 ± 0.8	36.5 ± 1.4	51.3 ± 1.2	32.2 ± 1.4
*M.sarcina***	30.1 ± 1.4	19.1 ± 1.4	16.5 ± 1.4	17.6 ± 2.3
*M.bacteriales***	14.2 ± 2.2	27.4 ± 2.3	31.6 ± 2.1	31.5 ± 2.2
*M.coccales***	8.6 ± 0.3	9.4 ± 1.6	3.1 ± 0.7	10.5 ± 1.4
*M.microbiales***	8.2 ± 1.7	11.3 ± 1.1	5.6 ± 1.2	11.5 ± 2.1

According to standardized FISH results, the most pronounced effect of the solvents tested on methanogenic archaea was caused by IC_{50} concentration of the methanol and isopropanol. *Methanosaeta* and *Methanosarcina* were the most effected species from methanol. The relative population of *Methanosaeta* showed a decrease from 22.6% to 6.1% whereas *Methanosarcina* decreased from 12.6% to 3.4%. These results corresponded with the activity test results indicating adverse effect of the solvents on anaerobic sludge. Among hydrogenotrophic methanogens, the relative ratio of *Methanomicrobiales* was not affected by the solvents. Acetoclastic *Methanosaeta* was not significantly affected by toluene compared to control reactor. However, *Methanosarcina* genus showed

approximately 50% decrease in the toluene added SMA reactor compared to control reactor. *Methanobacteriales* showed a better resistance to stress conditions like inhibitory effect of the solvents.

Table 5.14. Standardized FISH results of the sludge taken from single phase anaerobic reactor and from the solvent-added SMA test reactors

	Control	0.4M Methanol	1.2 mM Toluen	0.4 M isopropanol
M.saeta	22.6	6.1	18.5	9.2
M.sarcina	12.6	3.4	6	5
M.bacteriales	5.9	1.6	11.4	9
M.coccales	3.6	2.5	1.1	3
M.microbiales	3.4	3.1	2	3.3

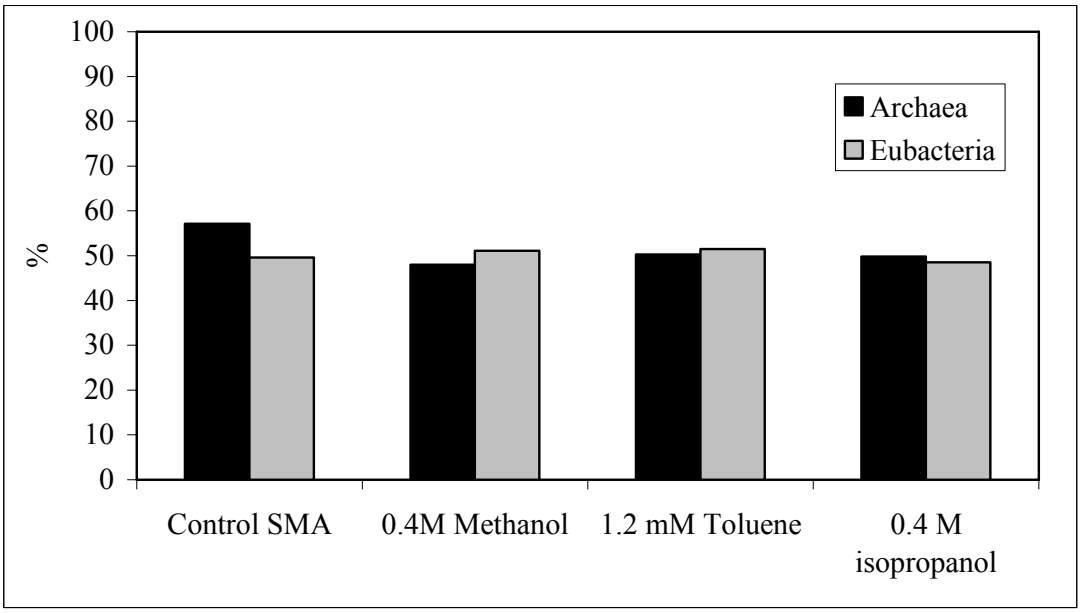


Figure 5.32. Distribution of microbial composition (%) in control and solvent-added SMA test reactors

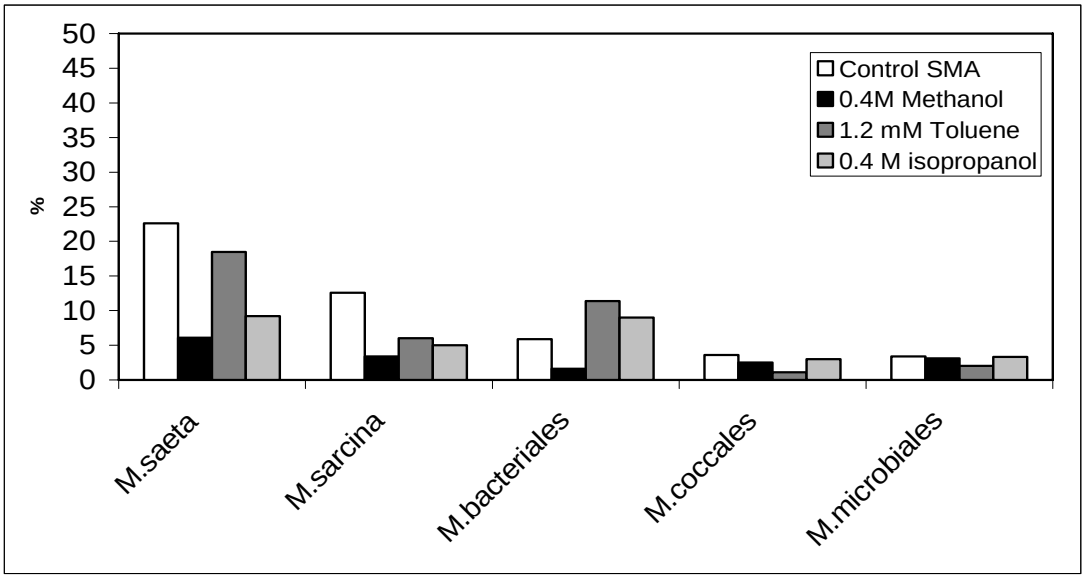


Figure 5.33. Standardized FISH results of the sludge from the contol and solvent-added SMA test reactors

5.5.2. Two-phase reactor

5.5.2.1. Acid Reactor. A non-methanogenic activity test with glucose was carried out for the sludge taken from acid reactor for selected organic solvents at IC_{50} concentrations. Initial COD values were very high. However, a part of the solvents is volatile and lost from the reactor to the biogas. Figure 5.34 shows the non-methanogenic activity test for the sludge taken from acid reactor. VFA changes during the tests was also measured (Figure 5.35-5.38). Major VFAs were found to be as acetic acid and butyric acid at the test carried out with IC_{50} concentrations of methanol and toluene. On the other hand, major VFAs were butyric acid, caproic acids and heptanoic acids during the non-methanogenc activity test carried out isopropanol. As can be seen from both non-methanogenic activity tests and VFA changes during the tests, the activity of the sludge from acid reactor was mostly affected by IC_{50} concentrations of isopropanol and methanol, respectively.

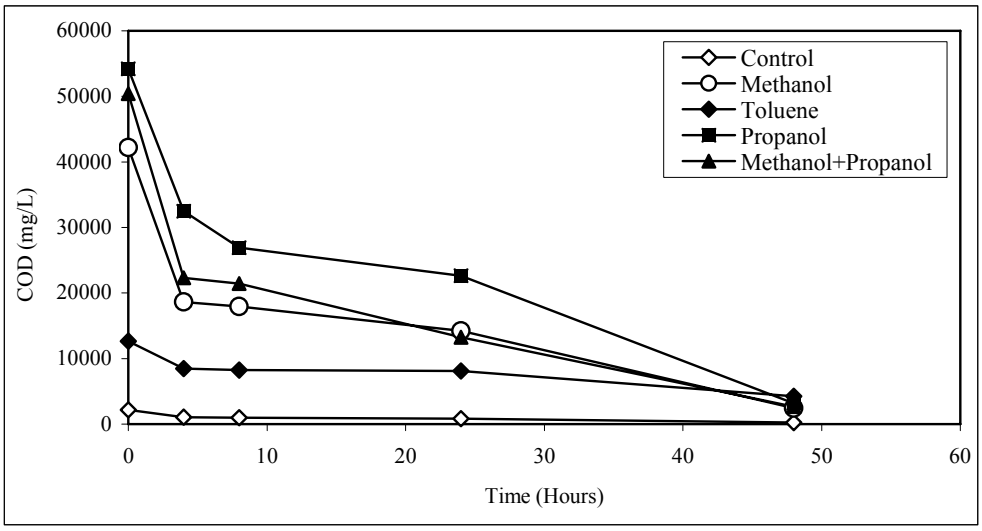


Figure 5.34. Non-methanogenic activity test for the sludge taken from acid reactor at IC_{50} concentrations of selected solvents.

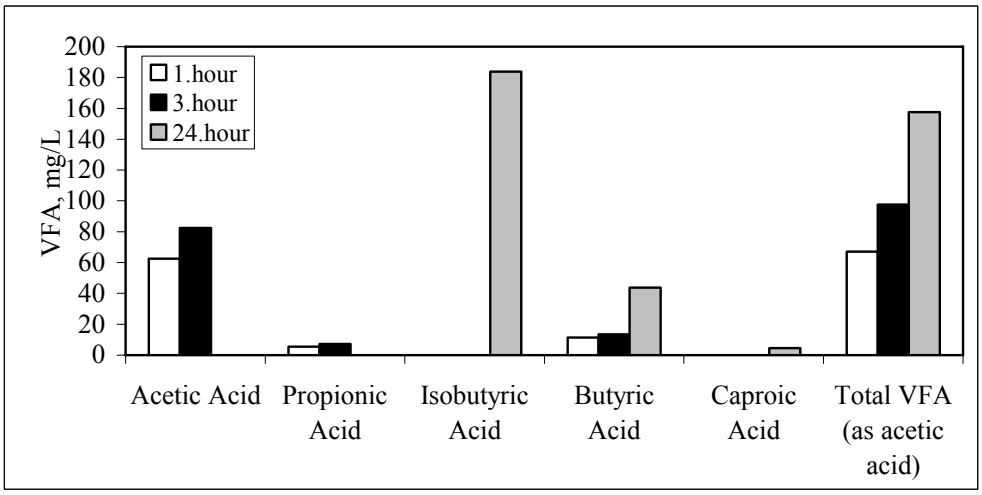


Figure 5.35. VFA changes during non-methanogenic activity tests (Control)

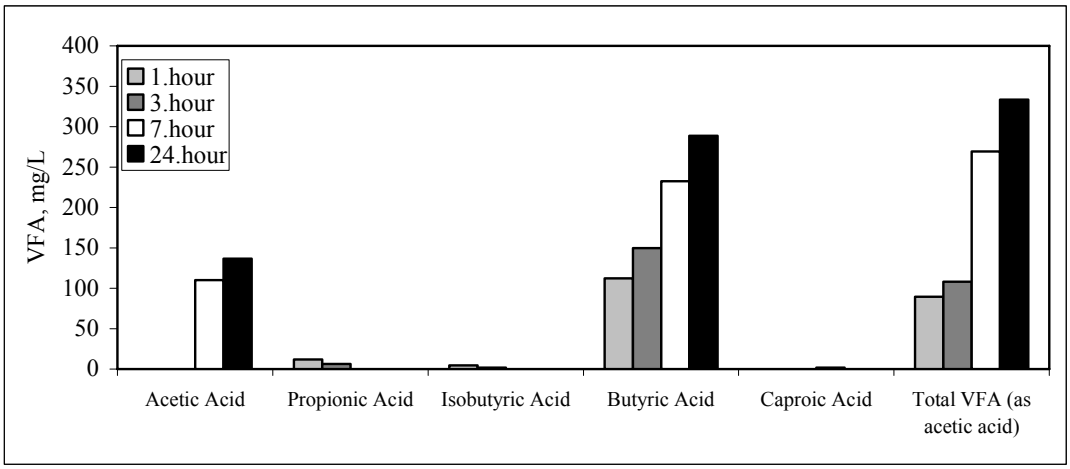


Figure 5.36. VFA changes during non-methanogenic activity tests (Methanol)

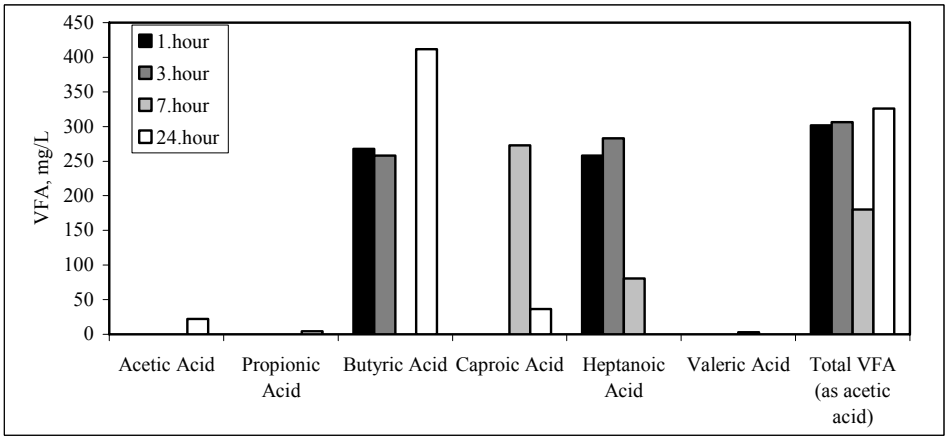


Figure 5.37. VFA changes during non-methanogenic activity tests (Propanol)

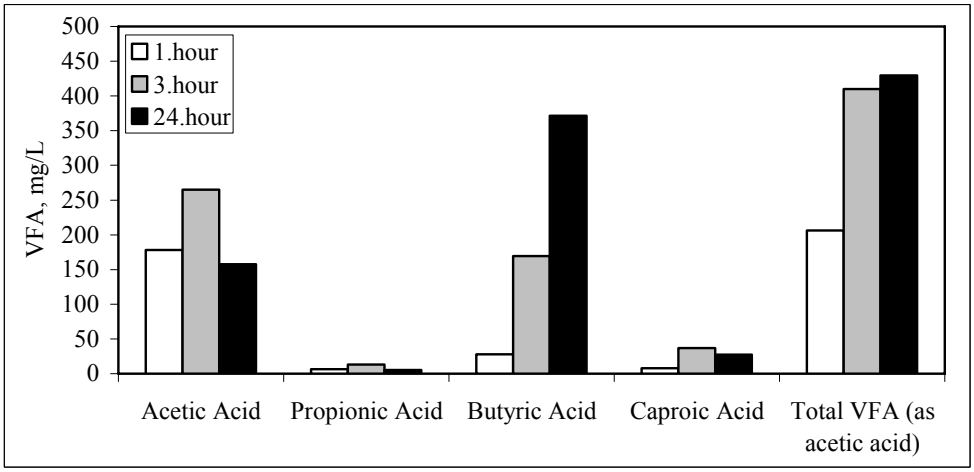


Figure 5.38. VFA changes during non-methanogenic activity tests (Toluene)

5.5.2.2. Methane Reactor. Methanogenic activity tests had been carried out to determine the effect of solvents-methanol, toluene and isopropanol on anaerobic sludge which was taken from the lab-scale anaerobic batch reactor operated in methane phase. IC_{50} concentrations of methanol, propanol and toluene were applied to SMA test reactors fed with acetate and VFA mixture in order to determine effect of the solvents on acetoclastic and total methanogenic activity, respectively. According to SMA test result, acetoclastic methanogenic activity of the sludge decreased from 341 mL CH_4/gVSS/day to 194 mLCH_4/gVSS/day for methanol, 207 mL CH_4/gVSS/day for propanol and 248 mLCH_4/gVSS/day for toluene, respectively (Figure 5.39).

Table 5.15. Effect of IC_{50} concentrations of solvents on acetoclastic methanogenic activity test of the sludge taken from methane phase anaerobic reactor

Substrate (acetate)	**Maximum activity** mLCH_4/gVSS/day	**std. dev.**
Control	341	10,7
0.4 M Methanol	194	13,1
0.4 M Propanol	207	11,9
1.2 mM Toluene	248	12,5

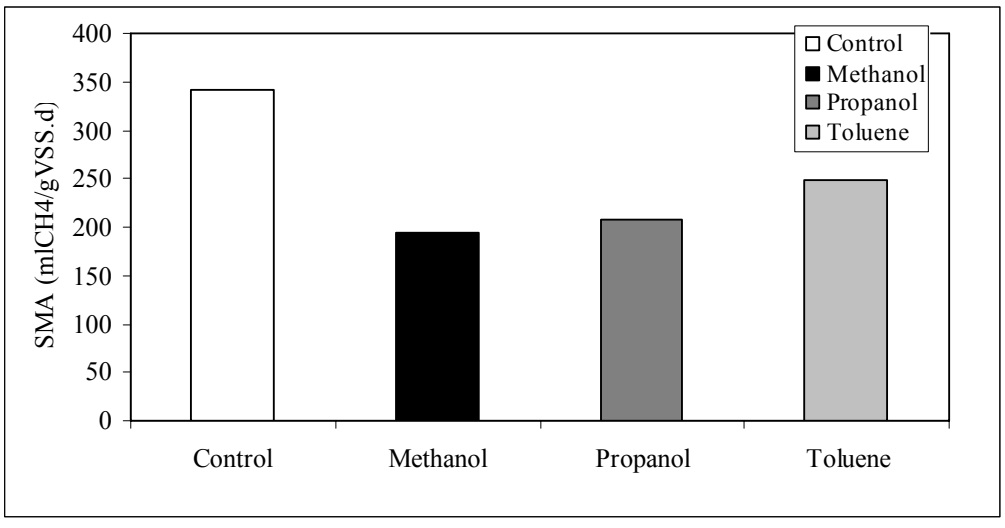


Figure 5.39. Effect of IC_{50} concentrations of solvents on acetoclastic methanogenic activity test of the sludge taken from methane reactor

Table 5.16. Effect of IC_{50} concentrations of solvents on total methanogenic activity of the sludge taken from methane phase anaerobic reactor

Substrate (VFA mixture)	**Maximum activity** $mLCH_4/gVSS/day$	**std. dev.**
Control	398	11.7
0.4 M Methanol	192	13.6
0.4 M Propanol	195	14.2
1.2 mM Toluene	367	9.2

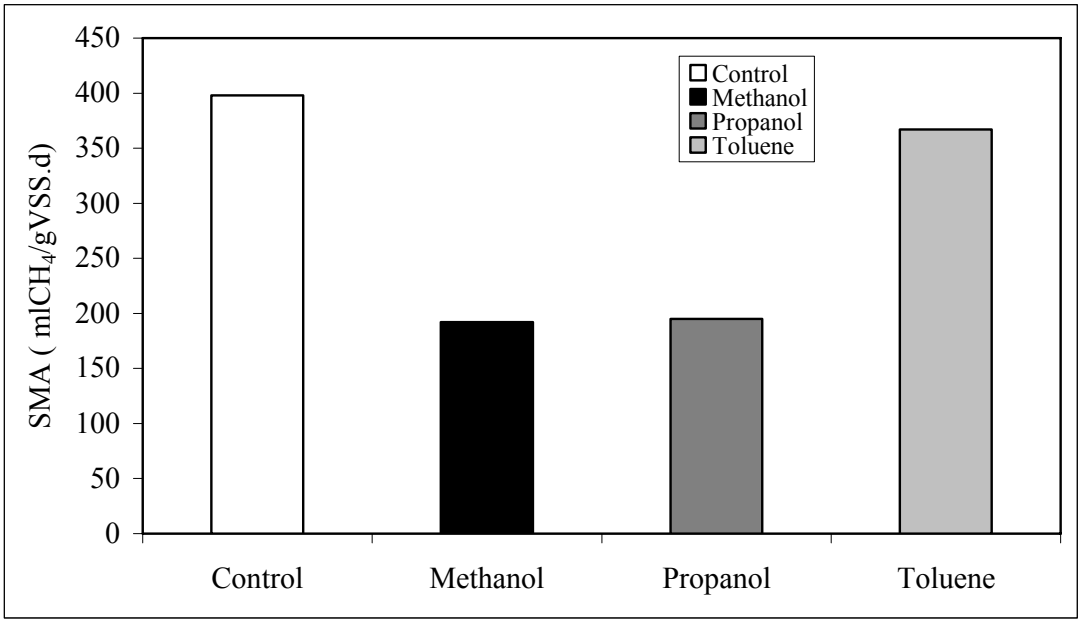


Figure 5.40. Effect of IC_{50} concentrations of solvents on total methanogenic activity of the sludge taken from methane reactor

FISH studies (Table 5.17) were also carried out for the sludge in order to determine effect of the selected solvents on microbial dynamics in methane reactor.

Table 5.17. FISH results of the sludge taken from methane phase anaerobic reactor and the solvent-added SMA test reactors

	Control (%)	0.4M Methanol (%)	1.2 mM Toluene (%)	0.4 M isopropanol (%)
Univ	77.9 ± 1.6	60.6 ± 3.4	74.4 ± 1.4	56.3 ± 2.3
Bacteria	28.1 ± 4.2	46.2 ± 2.8	52.5 ± 2.6	46.2 ± 2.1
Archaea	88.7 ± 1.4	57.8 ± 2.4	52.4 ± 3.1	54.7 ± 3.3
M.saeta	76.9 ± 1,1	34.0 ± 1.9	36.2 ± 2.2	35.6 ± 2.5
M.sarcina	26.7 ± 3,2	14.4 ± 1.6	13.2 ± 2.5	15.4 ± 2.1
M.bacteriales	15.5 ± 1,2	25.7 ± 3.6	27.1 ± 3.1	29.3 ± 1.7
M.coccales	10.6 ± 1.3	14.5 ± 2.7	8.2 ± 2.2	15.3 ± 1.4
M.microbiales	18.2 ± 1.0	17.3 ± 1.9	9.4 ±1.1	17.9 ± 2.2

According to standardized FISH results, the most pronounced effect of the solvents tested on methanogenic archaea was caused by IC_{50} concentration of the isopropanol among the others. *Methanosaeta* and *Methanosarcina* were the most effected species from isopropanol. The relative population of *Methanosaeta* showed a decrease from 53.16% to 11% whereas *Methanosarcina* decreased from 18.5% to 4.7%. The relative ratio of acetoclastic *Methanosaeta* significantly decreased in the toluene-added SMA reactor compared to control reactor. Among hydrogenotrophic methanogens, *Methanobacteriales* was not affected by the solvents.

Table 5.18. Standardized FISH results of the sludge taken from methane phase anaerobic reactor and the solvent-added SMA test reactors.

	Control (%)	0.4M Methanol (%)	1.2 mM Toluene (%)	0.4 M isopropanol (%)
M.saeta	53.1	16.0	14.5	11
M.sarcina	18.5	6.8	5.3	4.7
M.bacteriales	10.5	12.1	10.8	9.0
M.coccales	7.3	6.8	3.3	4.7
M.microbiales	12.6	8.2	3.8	5.5

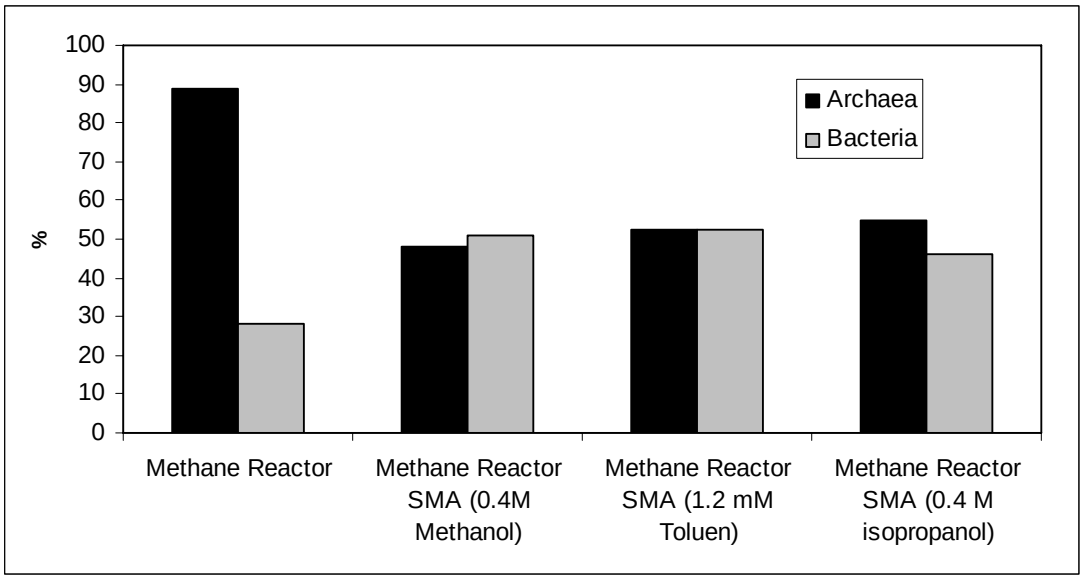


Figure 5.41. Distribution of microbial composition (%) in control and SMA test reactors

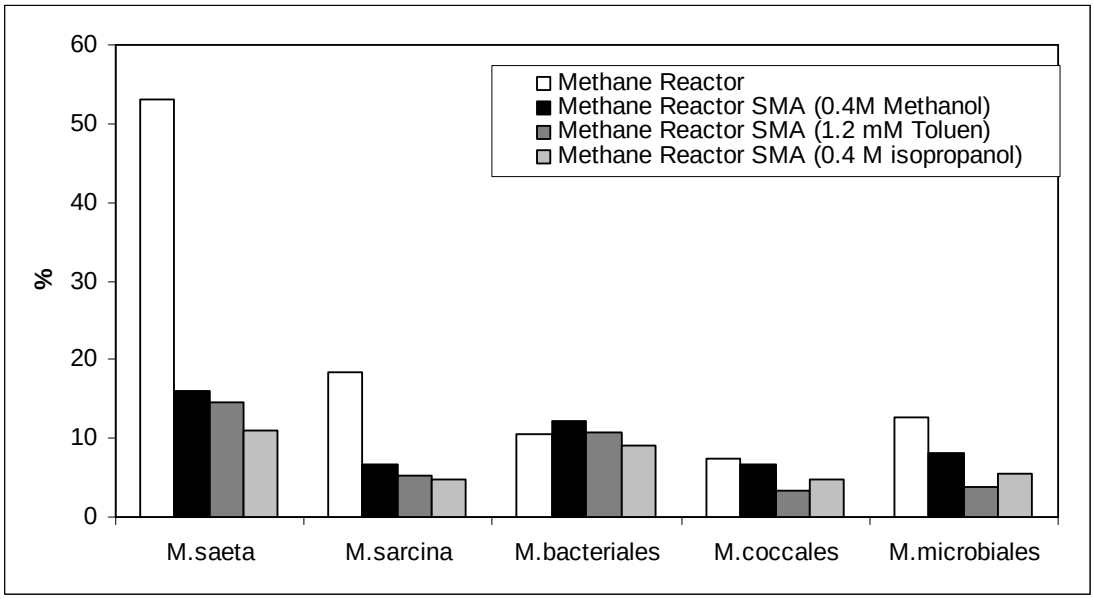


Figure 5.42. Standardized FISH results of control and SMA test reactor sludges

Activity test results of lab-scale anaerobic reactor showed that the reactor performed well since methanogenic activity has not shown a significant decrease compared to seed sludge with a acetoclastic methanogenic activity of 341 $mLCH_4$/gVSS/day, and an overall methanogenic activity of 398 $mLCH_4$/gVSS/day. Besides, FISH results sustain this statement with an increase in relative abundance of *Methanosaeta* spp. which is known to improve granulation and maintain more stable reactor performances according to the literature.

5.5.3. Comparison of Effects of Solvents on Microbial Ecology and Activity of the Sludge from Single Phase and Two-Phase Anaerobic Bioreactors

Organic solvents such as methanol, toluene and isopropanol which are mostly used for dissolving several compounds in a variety of industries cause several environmental problems and should be removed from the waste streams using treatment technologies. In recent years, anaerobic treatment has been applied for this kind of wastewater. However, there are still some concerns in the application of anaerobic treatment processes for solvent containing wastewaters due to possible detrimental effect of the compounds on the activity

and methanogenic population in the conventional anaerobic reactors. It has been stated that the two-phase systems have several advantages over conventional single-phase processes due to protection against inhibitory compounds. In conventional single-phase systems, all anaerobic degradation steps of the organic matter take place in the same reactor. On the other hand, the hydrolytic and acidogenic bacteria are separated from methanogenic *Archaea* in separate reactors in the two-phase systems. It was also reported in another study that the system configuration has a direct effect on the performance when substrate complexity is in question. While substrate complexity becomes an important factor affecting single-stage CSTR process efficiency, it is less important in the other process configurations such as two-stage and UASB (Azbar et al., 2000). Therefore, the systems can be used in the treatment of wastewaters containing inhibitory compounds. Several studies comparing the single-phase and two-phase anaerobic digestion processes of various synthetic wastewaters stated that phase separation can significantly improve the performance of the methanogenic reactor as a result of the optimized conditions for acidogenesis and methanogenesis in each reactor. Since the nutrient and growth requirements of the acidogenic and methanogenic organisms are different, the two-phase systems can be operated to provide optimal conditions for the microorganisms in each phase for stable and high performance. Two-phase AD were applied to brewery wastewater (Ahn et al., 2001), dairy wastewater (Ince, 1998), chemical synthesis based pharmaceutical wastewater containing organic solvents (Oktem et al., 2006; Chen et al., 2008), biogasification of: wastewater treatment sludge (Ghosh et al., 1995), organic fractions of municipal solid wastes (Argelier et al., 1998), industrial wastes and sludge (Ghosh et al., 1985). In a current study, a combination of two-phase anaerobic digestion (TPAD) which comprised a CSTR and a UASB-AF reactor, working as the acidogenic and methanogenic phases, respectively and an aerobic membrane reactor was demonstrated as an applicable option for the treatment of chemical synthesis-based pharmaceutical wastewater (Chen et al., 2008). Adverse effects of toxic organic substances including acrylic acid and pentachlorophenol on acid phase have been investigated by Qu and Bhattacharya, (1996) and Pringer and Bhattacharya, (1999). Although the treatment of organic solvents in anaerobic acid reactor has been studied (Oktem et al., 2006), relevant literature concerning effect of organic solvents on the two-phase anaerobic systems including acidogenic and methanogenic phase is still scarce. If acidogenesis can be shown to resitant to organic toxicants, it could be used to reduce toxicity of a wastewater and thus

make it more amenable to the second, methanogenic treatment phase (Fox and Pohland, 1994). The acid-phase digestion products, such as volatile fatty acids (VFA), CO_2 and H_2 can be greatly influenced by operational and design parameters such as hydraulic retention time (HRT), solid retention time (SRT), environmental factors such as pH, temperature, oxidation–reduction potential (ORP), as well as reactor configuration, wastewater characteristics and availability of trace minerals (Yu and Fang, 2001).

When the results of effects of selected solvents on methanogenic activity test and FISH studies for the sludges taken from single-phase and methane reactor were compared, it can be seen that acetoclastic and methanogenic activity did not show a significant change for the sludges taken from different phases whereas FISH results of methanogens showed significant variations for the selected solvents. Figure 5.43 and Figure 5.44 show effects of IC_{50} solvent concentrations on the activity and methanogenic composition of the sludge taken from single phase and methane reactor, respectively. Vitality of the methanogens under IC_{50} concentrations of methanol and isopropanol was higher in the sludge taken from methane reactor compared to single phase reactor (Figure 5.45). The situation was more evident for methanol. On the other hand, methanogens showed a better response to toluene in the sludge taken from single phase reactor. The results could be due to different microbiological characteristics of the sludge taken from different phases. Based on the discussion above, it can be concluded that from the study, two-stage anaerobic reactor configurations can be used for solvent containing wastewaters especially for methanol.

Table 5.19. Effects of IC_{50} solvent concentrations on the activity of the sludge taken from single phase and methane reactor

	Acetoclastic methanogenic activity SMA, mLCH_4/gVSS/day			**Total methanogenic activity SMA, mLCH_4/gVSS/day**	
Solvent	Sludge	Single Phase Reactor (R1)	Methane Reactor (R2M)	Single Phase Reactor (R1)	Methane Reactor (R2M)
Toluene (1.2 mM)	224	211	248	272	367
Propanol (0.4 M)	286	229	207	240	195
Metanol (0.4 M)	239	252	194	221	192

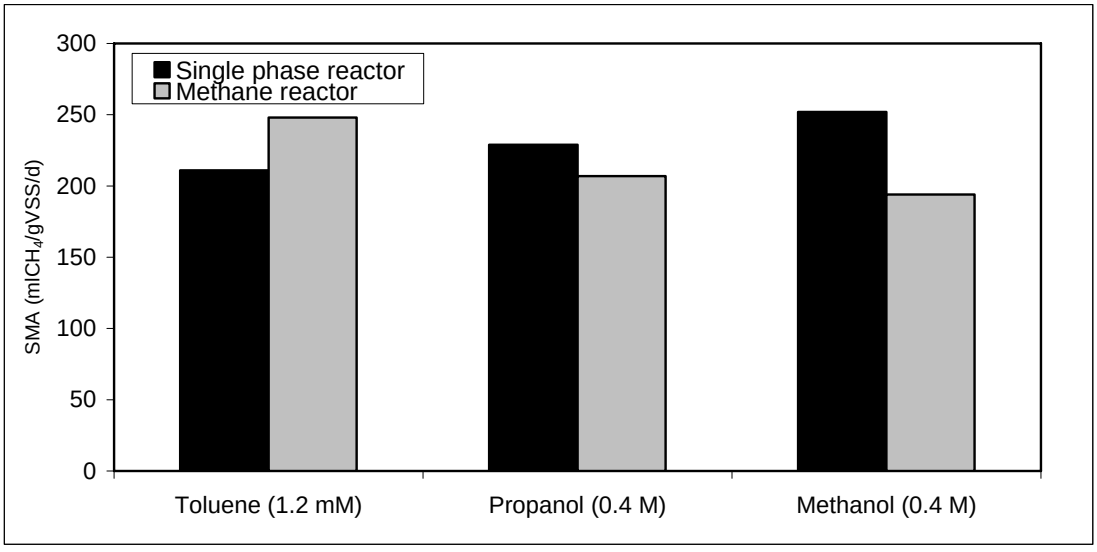


Figure 5.43. Effects of IC_{50} solvent concentrations on the activity of the sludge taken from single phase and methane reactor

Table 5.20. Effects of IC_{50} solvent concentrations on the methanogenic population in the sludges taken from single phase and methane reactor

	Methane	**Single - phase**	**Methane**	**Single - phase**	**Methane**	**Single - phase**
	Methanol		**Toluene**		**Isopropanol**	
M.saeta	6.1	16.0	14.5	18.5	11.0	9.2
M.sarcina	3.4	6.8	5.3	6.0	4.7	5.0
M.bacteriales	1.6	12.1	10.8	11.4	9.0	9.0
M.coccales	2.5	6.8	3.3	1.1	4.7	3.0
M.microbiales	3.1	8.2	3.8	2.0	5.5	3.3

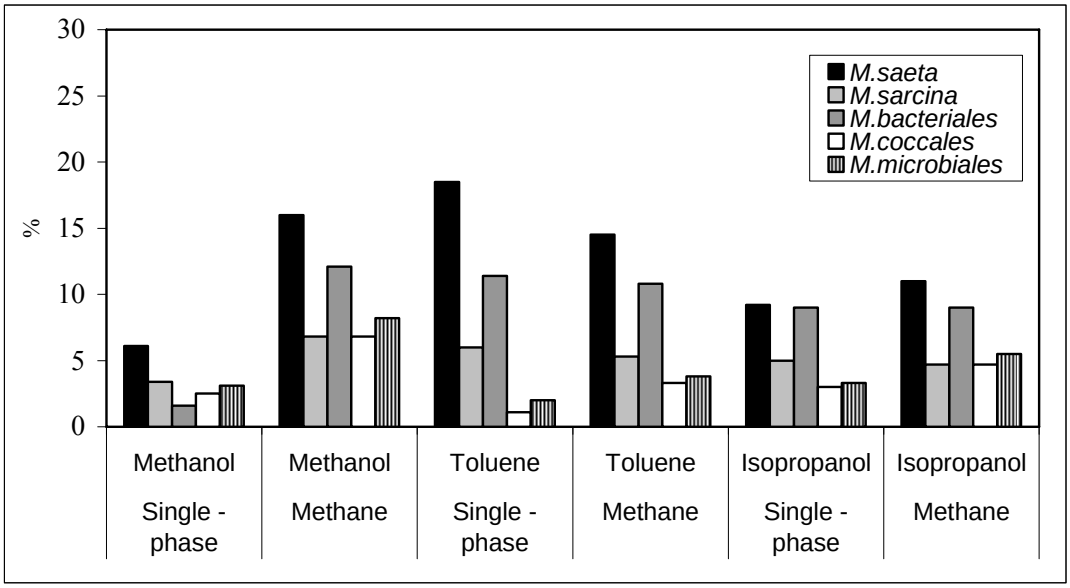


Figure 5.44. Effects of IC_{50} solvent concentrations on the methanogenic population in the sludges taken from single phase and methane reactor

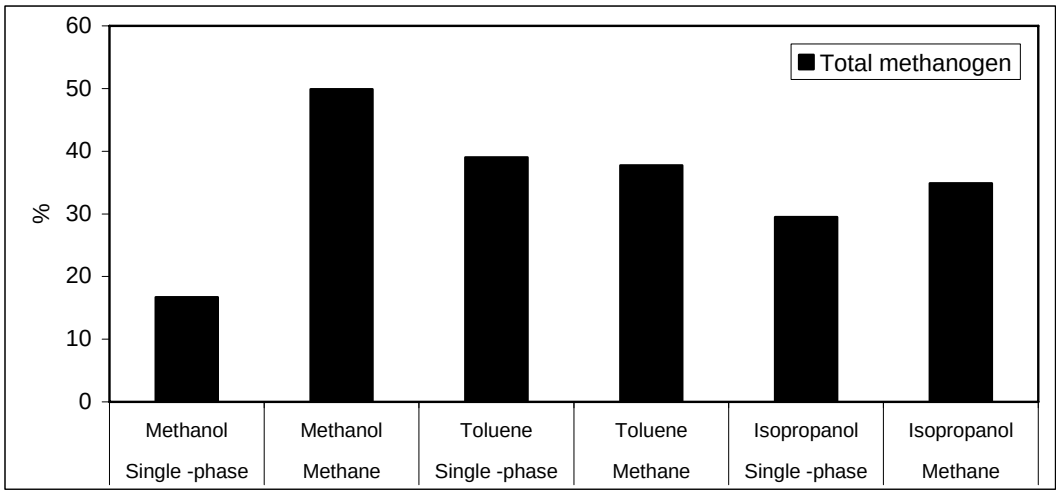


Figure 5.45. Effects of IC_{50} solvent concentrations on total methanogens in the sludges taken from single phase and methane reactor

5.6. Microbial Population Changes during Anaerobic Treatment of Toluene-containing Synthetic Wastewater in an ASBR

ASBR reactors fed with solvent containing wastewater were initially inoculated from an anaerobic stock reactor fed with glucose (data not shown). The ASBR reactor was firstly fed with a glucose-based synthetic wastewater in order to acclimatize the sludge to new reactor conditions. A rapid and successful start-up was observed for the reactor with COD removal efficiency over 95%. Since glucose is a readily degradable, soluble carbohydrate that does not, itself, limit the rate of anaerobic biodegradation (Noike et al., 1985), the compound is commonly used as a carbonaceous substrate for acclimization periods to inhibitory wastewaters (Oz et al., 2003). After the operation period with glucose, toluene was added to glucose-based synthetic wastewater at a concentration of 0.3 mM at each cycle during the operation period with the solvent. (Figure 5.46). Toluene concentration applied to the reactor was less than IC_{50} concentration determined in batch studies by taking into consideration of possible detrimental effect of toluene on the reactor.

The ASBR was operated with one cycle/day including the following four discrete steps: a feed step (0.5 h), a react step (22 h), a settling step (1 h), and a decant step (0.5 h). The ASBRs were generally operated with a long cycle time compared with aerobic SBRs. Under these operating conditions, the So/Xo ratio was 0.59 gCODt/gVSS. The reactor was operated with a SRT of 70 days and a HRT of 34 hours. VSS concentration in the reactor ranged between 6000-6530 mg/L during the operation period. The importance of the role of SRT in guarding the systems against failure has been previously shown (Bhattacharya and Parkin, 1988). Maintenance of proper SRT is extremely important in allowing for acclimation to toxicants. It has been shown that systems with high SRTs tolerated higher concentration of formaldehyde and methylene chloride (Bhattacharya and Parkin, 1988). Systems with low SRTs showed higher VFA concentrations. Speece and Parkin (1983) studied chloroform at 12.5, 25 and 50 days and showed that the longest SRT of 50 days provided the greatest process stability in the presence of toxicants shock to the biomass. These studies indicate the importance of SRT in minimizing the toxic effects of the compounds. It has been also reported that microbial populations in the reactor systems providing longer solid retention time (SRT) such as attached systems are more protected

against irregularities in operational conditions such as shock loadings etc or inhibitory compounds in wastewater (Henze and Harremoes, 1983).

At the beginning of the operation period with toluene, COD removal efficiency of the reactor was not affected by toluene addition to the synthetic wastewater. However, after day 34 a gradual deterioration in the reactor performance was observed with a COD removal efficiency falling to 70% (Figure 5.46). A sudden decline in the performance of the reactor was observed after day 52 with a COD removal efficiency of 40% (Figure 5.46). At the same period, methane yield was approximately 0.17 $m^3CH_4/kgCOD_{removed}$. An example of the evolution of the COD during a cycle (day 66) is presented in Figure 5.47. The rate of COD decrease was greatest at the start of the cycle, just after the feed period, and then decreased with time.

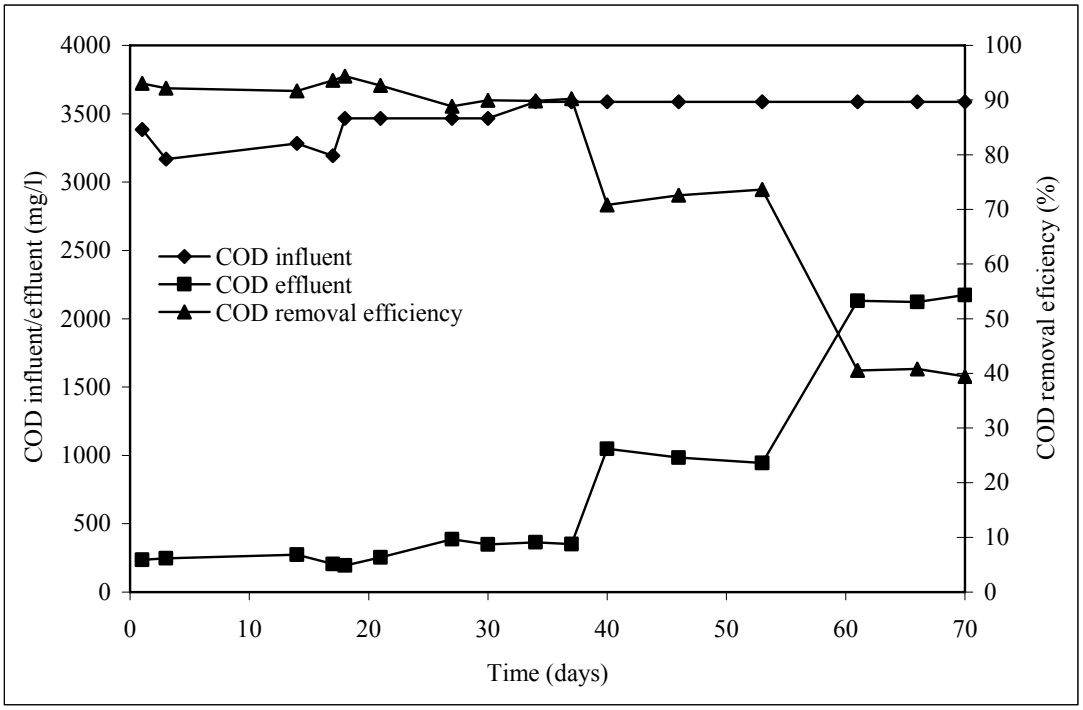


Figure 5.46. Performance of the ASBR fed with toluene-containing synthetic wastewater with time.

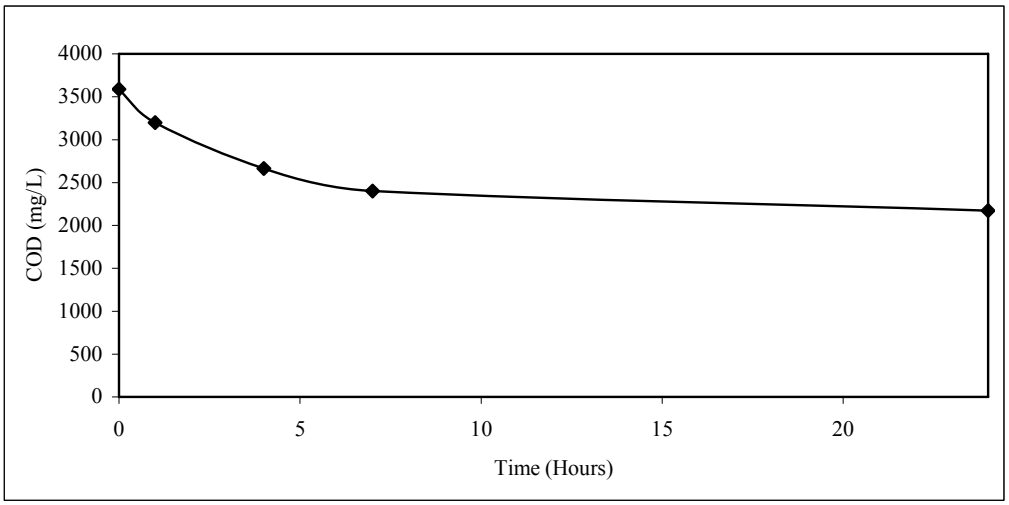


Figure 5.47. Changes in COD concentration during a cycle time in the ASBR fed with toluene-containing synthetic wastewater (day 66).

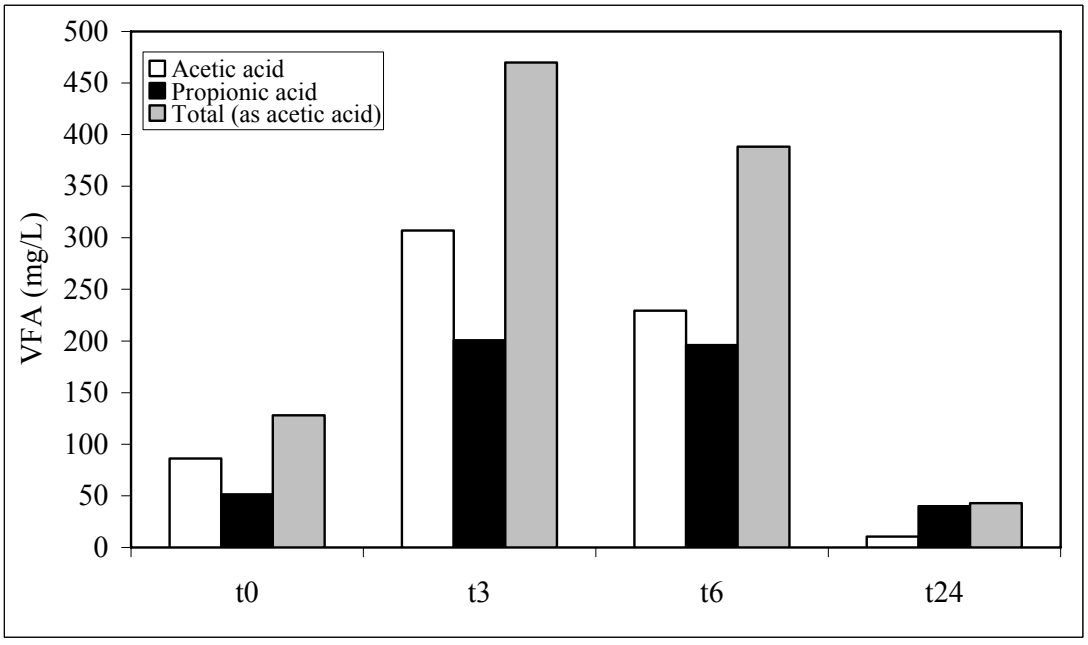


Figure 5.48. Changes in VFA during a cycle time in the ASBR fed with toluene-containing synthetic wastewater.

5.6.1. FISH results of the ASBR reactor operated with toluene-containing synthetic wastewater

After feeding the anaerobic reactor with toluene containing synthetic wastewater, percentage of active population in the reactor decreased over the period of operation of the reactor. Although the reactor performed well on day 31, active population decreased to approximately 48% and the decrease in the active population continued during the operation. The decrease in active population was not reflected in the reactor performance. However, after day 52, the deterioration of both reactor performance and the relative population of active methanogenic populations become more severe. Since the active population in the anaerobic reactor decreased over time with the effect of toluene, the FISH results were standardized against active total population in order to make a comparison between the sludge samples taken from the reactor over time. It has been reported that variations in process type, operating conditions and reactor performance resulted in changes in the composition rather than the size of the archaeal communities present in anaerobic reactors (Hofman-Bang et al., 2003; Leclerc et al., 2004). Apparently, this is not the case in the reactors treating inhibitory wastewaters when the number of total active population is taken into the consideration for the calculation of percentage of archaeal community.

Figure 5.49 shows the changes in major methanogenic groups during the operation of ASBR fed with toluene. The most pronounced effect among methanogenic *Archaea* has been observed in *Methanomicrobiales* species. Although this group was approximately 14% of the archaeal population in the seed sludge, after feeding the ASBR with toluene, this group has not been observed in the sludge samples taken from the reactor using FISH. The same adverse effect was seen in *Methanococcales* species. However, *Methanobacteriales* species were dominant H_2 and formate utilizing methanogen in the anaerobic reactor during the study. In some other studies, the dominancy of *Methanobacteriales* species over *Methanomicrobiales* has been reported (Kolukirik et al., 2007). Predominance of *Methanobacteriales* among the hydrogenotrophic methanogens is difficult to explain, since the ecological significance of different hydrogen and formate

utilizing methanogens and the competitiveness among the populations has not been studied in as much detail as for acetoclastic methanogens. Up to now, how the different $_{H2}$-utilizing methanogens compete for H_2 and formate in anaerobic sludges has not been revealed yet. It has been reported that this competition may result from the availability of the substrates in the reactor and other variables. However, it is not clear that why *Methanomicrobiales* spp. lost completely their activity in the reactor fed with toluene. Exposure of bioreactor sludge to toluene resulted in the selection of some resistant organisms to this toxic substance. At the end of the operation with toluene, dominant species in the reactor sludge (standardized against total active population) were *Methanosaeta* (12.3%), *Methanobacteriales* (5.6%), *Methanosarcina* (3.6%) and *Methanococcales* (1.4%) respectively. Although, with the introduction of toluene to the reactor, a decrease in the relative abundance of members of the acetoclastic genus *Methanosaeta* from 15.5% to 7.5% of the total active population then an increase to 12.3 over time in the reactor indicating acclimization, the relative abundance of the species could not reach the size in the seed sludge. Numerical dominance of the genus *Methanosaeta* compared to other methanogens in anaerobic reactor exposed to toluene has been observed. A methanogenic culture, which has been maintained for 10 years with toluene as the sole carbon source and electron donor, is dominated by two archaeal species (members of the *Methanosaeta* and *Methanospirillum* genera), a eubacterial species belonging to the genus *Desulfotomaculum*, and a eubacterial organism whose 16S rRNA sequence does not correspond well to known species (Ficker et al., 1999).

There are many studies assessing the effect of different amendments of toluene on anaerobic reactor sludges under sulfate reducing, nitrate reducing and methanogenic conditions (Cattony et al., 2005; Martinez et al., 2006; Mrowiec et al., 2005; Grbic-Galic and Vogel, 1987). However, the effect of toluene on potential methanogenic activity was not assessed in those studies. Our study is the first to report that toluene addition up to 0.3 mM causes serious losses in potential acetoclastic methanogenic activity of the anaerobic sludge. From the operational stand point of anaerobic reactors, such activity losses may not be reflected in the reactor performance. In case of longer exposure of the anaerobic sludge to toluene, the reactor could not achieve desired organic matter removal due to significant losses in the potential activity of acetoclastic methanogens.

Table 5.21. FISH results of the sludge taken from the ASBR fed with toluene-containing synthetic wastewater.

	Seed sludge		Glucose-fed		Day 31		Day 53		Day 66	
	%	±	%	±	%	±	%	±	%	±
Active cells	80	4	84	3.2	47.7	3.2	48.1	3.1	42.6	2.4
Eubacteria*	42	2	48	2.1	46.9	2.4	45.1	2.1	40.5	2.8
Archaea*	62	1	54	2.2	49.6	2.5	53.3	2.5	59.1	1.4
*M.saeta***	32	0.8	34	3.4	31.7	1.8	36.4	1.9	48.8	1.4
*M.sarcina***	8	1.5	17	1.3	12.8	1.2	15.2	1.4	14.2	1.6
*M.microbiales***	14.2	1.6	22	1.2	0	-	0	-	0	-
*M.coccales***	12	1.1	12	1.4	10.5	1.1	7.8	1.2	5.4	1.1
*M.bacteriales***	17	1.3	21	1.4	22.4	1.6	23.3	1.9	22.1	1.9

Table 5.22. Standardized FISH results of the sludge taken from the ASBR fed with toluene-containing synthetic wastewater.

	Seed Sludge	**Glucose-fed**	**Day 31**	**Day 53**	**Day 66**
*Methanosaeta**	15,9	15.5	7.5	9.3	12.3
*Methanosarcina**	4.0	7.8	3.0	3.9	3.6
*Methanomicrobiales**	7.0	10	ND**	ND**	ND**
*Methanococcales**	5.9	5.5	2.5	2.0	1.4
*Methanobacteriales**	8.4	9.6	5.3	6.0	5.6

**ND: Not detected

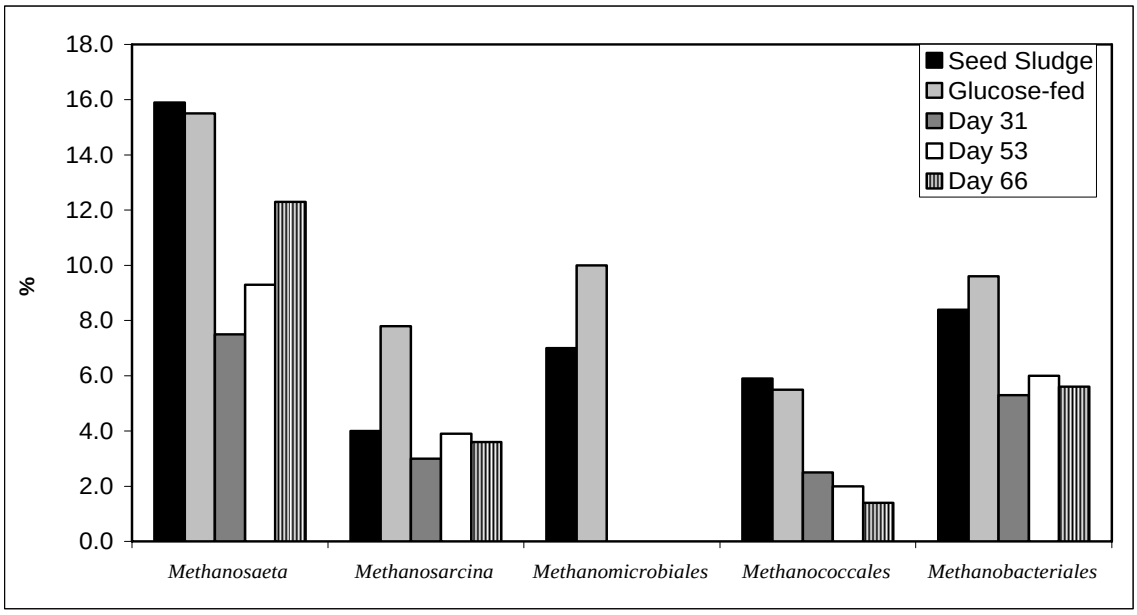


Figure 5.49. Changes in major methanogenic groups during the operation of ASBR fed with toluene.

5.6.2. DGGE results of the ASBR reactor operated with toluene-containing synthetic wastewater

Archaeal and bacterial community structure during the operating period with toluene were screened by DGGE analysis of PCR amplified 16S rRNA gene fragments and sequence analysis of excised bands. When the reactor was fed with toluene containing wastewater, a decrease in the diversity of the sludge was observed. Bacterial diversity (Figure 5.50) decreased from 15 to 9 operational taxonomic unit (OTUs) as archaeal diversity (Figure 5.51) decreased from 8 to 7. However, bacterial DGGE fingerprinting data showed that number of OTUs in the reactor sludges fed with toluene-containing wastewater increased in the course of time (From 8 to 14) and decreased then to 12 at the end of the operation period. Archaeal diversity of the reactor sludge showed the same tendency like bacterial diversity; diversity oscillates from 6 to 9 then to 5 OTUs.

The data obtained from Bionumerics software analysis of DGGE gel photos were used to construct a relation analysis tree of samples. A focused analysis using the

Bionumerics software package showed a number of low-intensity bands in all samples. These low-intensity bands can be detected by software programs and may be responsible for the differences between the samples especially in bacterial community profile. Figure 5.52 and Figure 5.53 show the phylogenetic tree constructed (based on Dice coefficient and Pearson coefficient) from the bacterial and Archaeal DGGE profiles.

Bacterial communities of reactor sludge fed with toluene-containing wastewater form distinctive clusters at 64% in relation analysis with Dice coefficient. In Pearson coefficient analysis, the reactor samples including T2, T3, T4, T5 and glucose-fed control reactor formed a cluster at 75%. Bacterial community profiles clustered at 44% in Dice correlation coefficient, as profile cluster at 49% in Pearson correlation coefficient. Close similarity values and similar clustering behaviors obtained using Dice and Pearson correlation coefficient implied that microbial communities in the reactors show similar tendencies in terms of OTU type's present and relative abundance of these OTUs.

The effect of OTU types present was more noticeable in relation analysis of archaeal communities. In both correlation analysis communities of the reactor form distinctive clusters (90% for the Pearson coefficient and 65% for the Dice coefficient). Opposite to bacterial community profiles, Archaeal community profiles clustered at higher point in Pearson coefficient analyses than Dice (57% for Dice, 86% for Pearson coefficients). Relation analysis obtained using Pearson correlation coefficient show relative OTU abundance is a more distinctive criterion than OTU types in the archaeal community in the toluene-fed reactor.

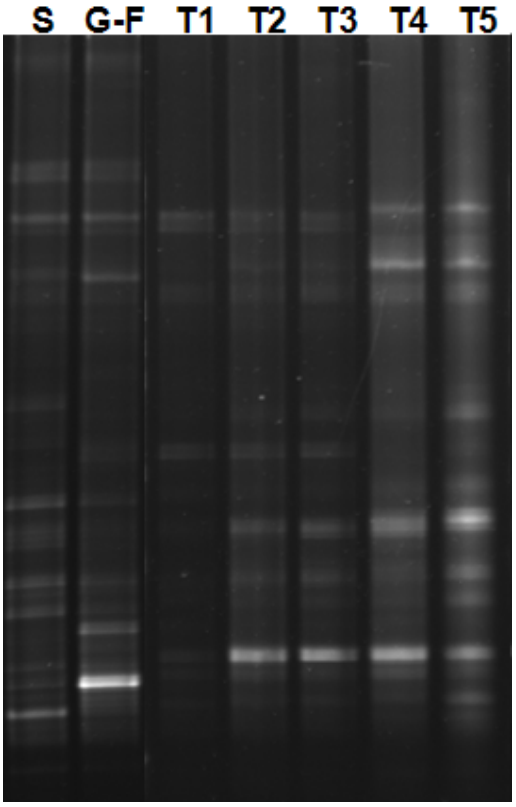


Figure 5.50. Analysis of bacterial community in the Toluene reactor samples by DGGE

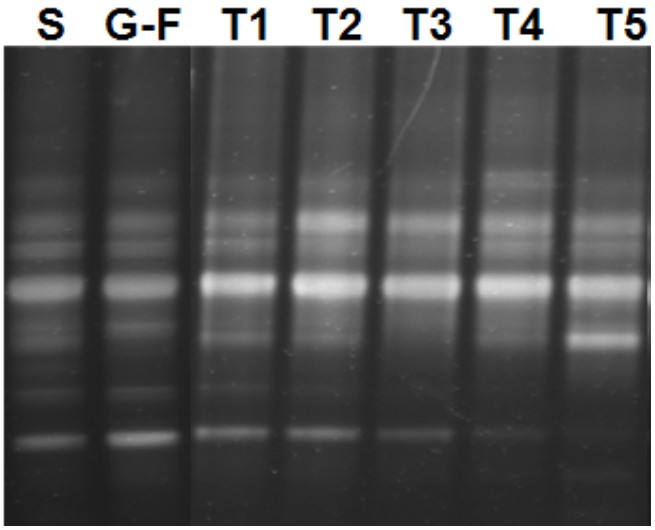


Figure 5.51. Analysis of Archaeal community in the Toluene reactor samples by DGGE.

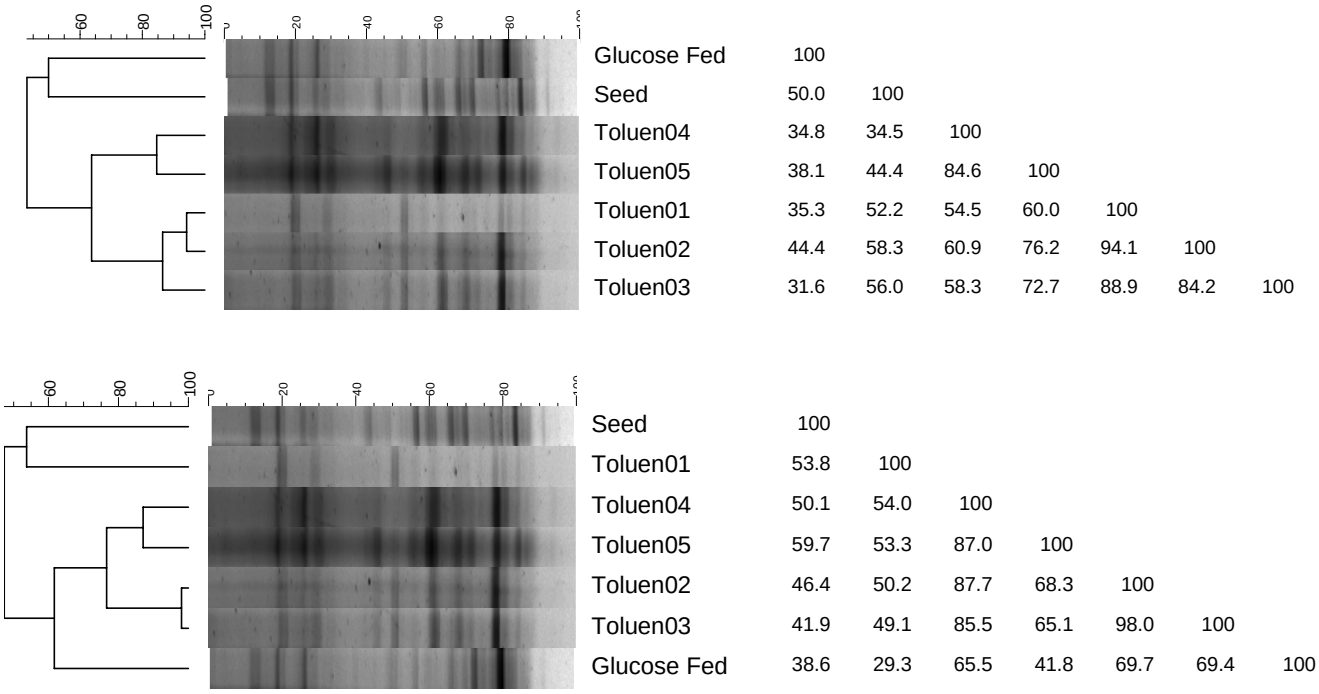


Figure 5.52. Phylogenetic tree constructed (based on Dice coefficient and Pearson coefficient) from the bacterial DGGE profiles in the ASBR reactor fed with toluene.

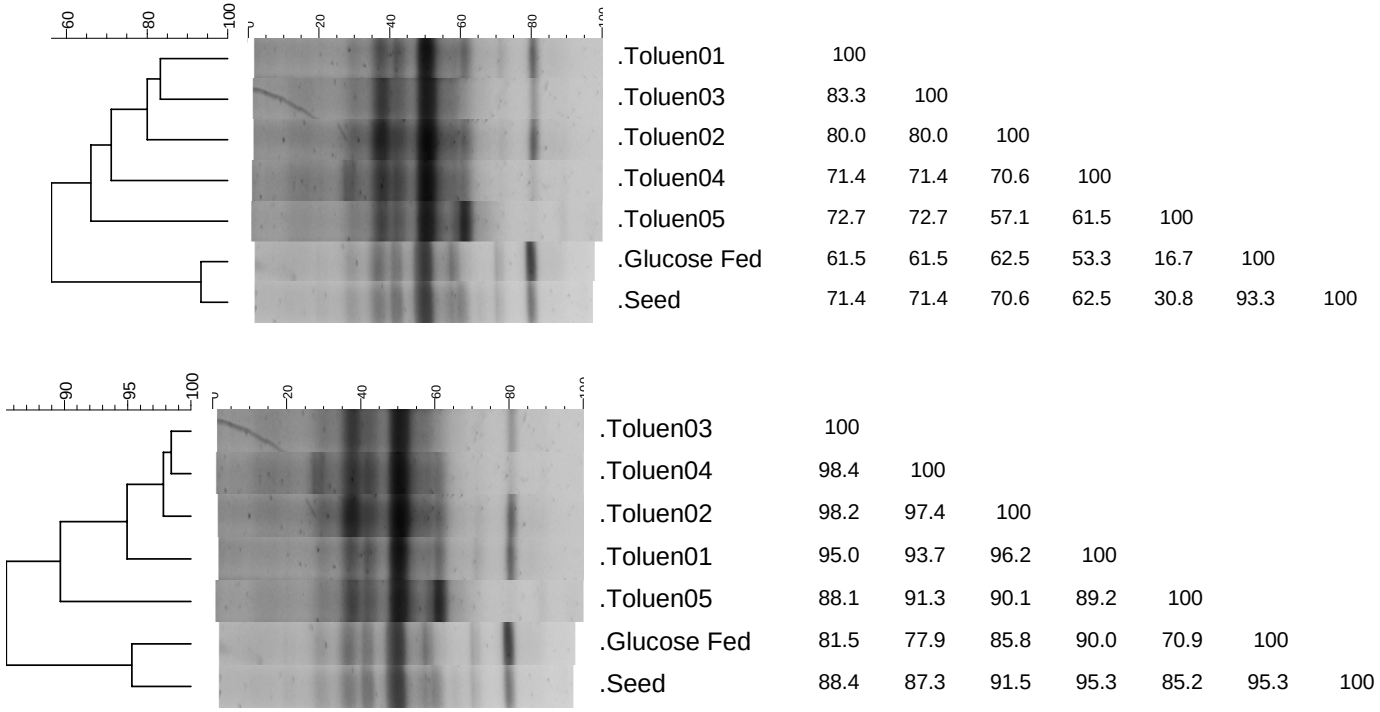


Figure 5.53. Phylogenetic tree constructed (based on Dice coefficient and Pearson coefficient) from the archaeal DGGE profiles in the ASBR reactor fed with toluene.

Total 9 bands were analyzed from bacterial DGGE gel of the sludge sample taken from the reactor fed with toluene-containing wastewater as 6 bands were analyzed from Archaeal DGGE gel. Bands were amplified and sequenced to assess the identity of community members. Table 5.23 and Table 5.25 show the bacterial and Archaeal phylotypes identified in the reactor, respectively. Table 5.24 and Table 5.26 show the closest matches of phylotypes from bacterial and Archaeal community in the samples. According to the data obtained from bacterial sequence analysis, *DeltaProteobacteria* related species made up 3/9 sequences as both *Chloroflexi* and *Firmicutes* related species made up 2/9 sequences. Sequences related to *Actinobacteria* and *Aquificae* were also found. Syntrophic proteobacteria were responsible in sulfate reduction coupled with fatty acid oxidation, especially propionate, in presence of H_2 using microorganisms mainly hydrogenotrophic methanogens. Those bacteria play significant roles in start up of bioreactors and presence of these bacteria indicates an efficient reactor performance. In all reactors, bands with sequences related to syntrophic bacteria were found. Although *Firmicutes* mainly hosted fermentative bacteria, different metabolic pathways are present within this group. *Dehalococcoides sp*. which is described as VC-transforming bacteria (He et al.,2003) and PCE-dechlorinating bacteria (Löffler et al., 2000) has been found to be present in seed, glucose-fed reactor. The group disappeared at the beginning of the reactor operation with toluene and then reappeared in the reactor samples at the end of the operation period (T4-T5). Fermentative *Clostridium* species were present in toluene-fed reactor as a propionate and butyrate producing Firmicutes, *Acidaminococcus intestinalis*, was found in the stock glucose-fed reactor and control reactor as well as the reactor fed with toluene at the beginning of the operation (T1-T3). However, they are not found through the end of the operation period with toluene. Both bacteria belonging to *Chloroflexi* and *Aquificae* present in both reactor communities, glucose-fed and the reactor samples (T1-T5), as the former mainly utilizes protein and limited sugars the latter is responsible for sulfur compounds reduction with H_2 oxidation.

The syntrophic benzoate degrader *Syntrophus aciditrophicus* metabolizes benzoate to acetate, hydrogen, and CO_2 in cocultures with a hydrogen-using methanogen or a sulfate reducer (Jackson et al., 1999). The ability of *Syntrophus aciditrophicus* to ferment benzoate may have ecological significance since benzoate (or benzoyl coenzyme A) is a central intermediate in anaerobic degradation of many natural and xenobiotic aromatic

compounds. It has been previously reported that anaerobic metabolism of benzoate to acetate, CO_2, and hydrogen or formate in the absence of light or terminal electron acceptors is thermodynamically unfavorable and degradation proceeds only if the concentration of hydrogen produced during benzoate oxidation is continuously maintained at a low level by a hydrogen-using microorganism (Jackson et al., 1999, Schink, 1997). This kind of mutual cooperation between two species to degrade a single substrate via interspecies hydrogen transfer is called syntrophism. In syntrophic cocultures, hydrogen-utilizing bacteria are needed to maintain low hydrogen levels and are not directly involved in the metabolism of the original substrate. Also, many syntrophic microorganisms can grow in the absence of hydrogen-utilizing partners on unsaturated substrate analogues by using dismutation reactions in which part of the original substrate is used as an electron acceptor. It has been recently reported that *S. aciditrophicus* is able to grow and metabolize benzoate in the absence of a hydrogen-utilizing partner and can be grown in a monoculture by oxidizing about one half the benzoate to acetate and CO_2 and reducing the other half to cyclohexane carboxylate (Elshahed and McInerney, 2001).

Archaeal communities in the reactor samples (T1-T5) were found to be as acetate-utilizing *Methanosaeta concilii* (*Methanosarcinales*), H_2/CO_2-formate utilizing *Methanolineatarda and Methanoregula boonei* (*Methanomicrobiales*) and *Methanomethylovarans*-using methylated compounds- (*Methanosarcinales*). At the end of the operation period with toluene, the Archaeal community of the reactor was dominated by *Methanosaeta concilii* and *Methanomethylovorans* belonging to *Methanosarcinales* group and *Methanolineatarda* belonging to *Methanomicrobiales* whereas Methanogenic archaeon F1/B-2 (*Methanosarcinales*), *Methanoregula boonei* (*Methanomicrobiales*), Methanolobus sp. HigM (*Methanosarcinales*) were eliminated from the reactor. It has been reported that a toluene-degrading consortium was composed of dominant species resembling *Methanospirillum* sp. and *Methanosaeta* sp. and a spore-forming sulfate reducer, *Desulfotomaculum* and a syntrophic benzoate degrader (Ficker et al., 1999). Thereore, it can be deduced that *Methanosaeta* sp. is resistant to toluene and play a significant role in acetate degradation in the toluene-fed cultures.

Table 5.23. Identification of phylotypes from bacterial community of the ASBR reactor fed with toluene.

Accession number	Closest relative	% sim.	Phylogeny	Metabolism	S	G-F	T1	T2	T3	T4	T5
CP000612	*Dehalococcoides sp. BHI80-15*	88	Chloroflexi	H_2 Oxidation	X	X	0	0	0	X	X
CP000478	*Syntrophobacter fumaroxidans MPOB*	85	Deltaproteo.	VFA oxidation	X	0	X	X	X	X	X
AY651787	*Syntrophobacter sulfatireducens*	88	Deltaproteo.	VFA oxidation	X	X	X	X	X	X	X
CP000252	*Syntrophus aciditrophicus SB*	83	Deltaproteo.	VFA oxidation	X	X	0	X	0	X	X
CP001080	*Sulfurihydrogenibium sp. YO3AOP1*	88	Aquificae	H_2 oxidation sulfur compounds reduction	X	X	X	X	X	X	X
CP001078	*Clostridium botulinum E3 str. Alaska E43*	95	Firmicutes	Fermentation	0	0	X	X	X	X	X
BX248359	*Corynebacterium diphtheriae*	84	Actinobacteria	Fermentation	X	0	X	X	X	X	X
AB243673	*Longilinea arvoryzae*	91	Chloroflexi	Protein and sugar degradation	0	0	X	X	X	X	X
AF473835	*Acidaminococcus intestinalis*	87	Firmicutes	Amino acid degradation to VFA	0	X	X	X	X	0	0
Number of OTU's on DGGE gel					**15**	**9**	**8**	**9**	**10**	**14**	**12**

* S : seed sludge
* GF : glucose-fed reactor sludge

Table 5.24. Closest matches of phylotypes from bacterial community of the ASBR reactor fed with toluene (Environmental Database)

Accession number	Closest relative	% sim.	Isolation sources	S	G-F	T1	T2	T3	T4	T5
AF423186	Bacterium EU6	89	toluene-degrading methanogenic consortium	X	X	0	0	0	X	X
AY426441	clone B16	93	full-scale anaerobic UASB bioreactor treating paper mill wastewater	X	0	X	X	X	X	X
AB266988	clone: QpjB112fl	97	mesophilic UASB sludge granules	X	X	X	X	X	X	X
AJ306771	clone SHA-42	88	1,2-Dichlorpropan dechlorierend en Mischkultur	X	X	0	X	0	X	X
AB266990	clone: HgtB7fl.	98	mesophilic UASB sludge granules	X	X	X	X	X	X	X
EF034988	Clostridia bacterium	99	Spitsbergen permafrost soil	0	0	X	X	X	X	X
AB267014	clone: YkB4fl.	87	mesophilic UASB sludge granules	X	0	X	X	X	X	X
EF053078	clone PEU-21	95	diethyl phthalate and phenol degrading UASB reactor	0	0	X	X	X	X	X
EU888006	Clostridia bacterium clone L33	99	reactor	0	X	X	X	X	0	0
Number of OTU's on DGGE gel				15	9	8	9	10	14	12

Table 5.25. Identification of phylotypes from archaeal community of the ASBR reactor fed with toluene.

Accession number	Closest relative	% sim.	Phylogeny	Metabolism	S	R	T1	T2	T3	T4	T5
AY422331	*Methanogenic archaeon F1/B-2*	88	*M.sarcinales*	Acetate	X	X	X	0	0	0	0
CP000780	*Candidatus Methanoregula boonei 6A8*	86	*M.microbiales*	H_2+CO_2-Formate	X	X	X	X	X	X	0
AB370247	*Methanolobus sp. HigM*	83	*M.sarcinales*	Methylated c.	X	X	0	X	X	0	0
X51423	*Methanosaeta concilii*	96	*M.sarcinales*	Acetate	X	X	X	X	X	X	X
AB162774	*Methanolinea tarda*	88	*M.microbiales*	H_2+CO_2-Formate	X	X	X	X	X	X	X
	Methanomethylovorans hollandica	92	*M.sarcinales*	Methylated c.	X	X	X	X	X	X	X
Number of OTU's on DGGE gel					**8**	**7**	**6**	**9**	**6**	**8**	**5**

* S : seed sludge
* GF : glucose-fed reactor sludge

Table 5.26. Closest matches of phylotypes from archaeal community of the ASBR reactor fed with toluene (Environmental Database)

Accession number	Closest relative	% sim.	Isolation source	S	R	T1	T2	T3	T4	T5
EU591674	clone MCSArc_H3	92	methanogenic reactor	X	X	X	0	0	0	0
AF229776	uncultured archaeon TA03	84	anaerobic granular sludge	X	X	X	X	X	X	0
AY454755	U. Methanolobus clone E_C06	85	estuary sediment	X	X	0	X	X	0	0
AB077216	clone:KuA6.	82	oil-contaminated groundwater	X	X	X	X	X	X	X
AB329663	clone: UASB26.	96	UASB granule	X	X	X	X	X	X	X
AB195851	uncultured archaeon	93	VFA degrading AB	X	X	X	X	X	X	X
Number of OTU's on DGGE gel				**8**	**7**	**6**	**9**	**6**	**8**	**5**

* S : seed sludge
*GF : glucose-fed reactor sludge

5.7. Microbial Population Changes during Anaerobic Treatment of Methanol - containing Synthetic Wastewater in an ASBR

The reactor was firstly fed with a glucose-based synthetic wastewater in order to acclimatize the sludge and COD removal efficiency over %95 was obtained. After the operation period with glucose, methanol was added to glucose-based synthetic wastewater at a concentration of 0.1 M at each cycle during the operation period with the solvent (Figure 5.54). The methanol concentration applied to the reactor was less than IC_{50} concentration determined in batch studies by taking into consideration of possible detrimental effect of methanol on the reactor.

The ASBR was operated with one cycle/day including the following four discrete steps: a feed step (0.5 h), a react step (22 h), a settling step (1 h), and a decant step (0.5 h). The reactor was operated with a SRT of 70 days and a HRT of 34 hours. At the beginning of the operation period with methanol containinig synthetic wastewater, COD removal efficiency of the reactor was decreased and then showed a significant improvement. VSS concentration in the reactor ranged between approximately 5000-6800 mg/L. However, a sudden decline in the performance of the reactor was observed after day 52 with a COD removal efficiency of 50% (Figure 5.54) and then 35% after day 65. An example of the evolution of the COD during a cycle at the end of the operation period is presented in Figure 5.55. At the same period, methane yield was approximately 0.10 m^3CH_4/kg $COD_{removed}$.

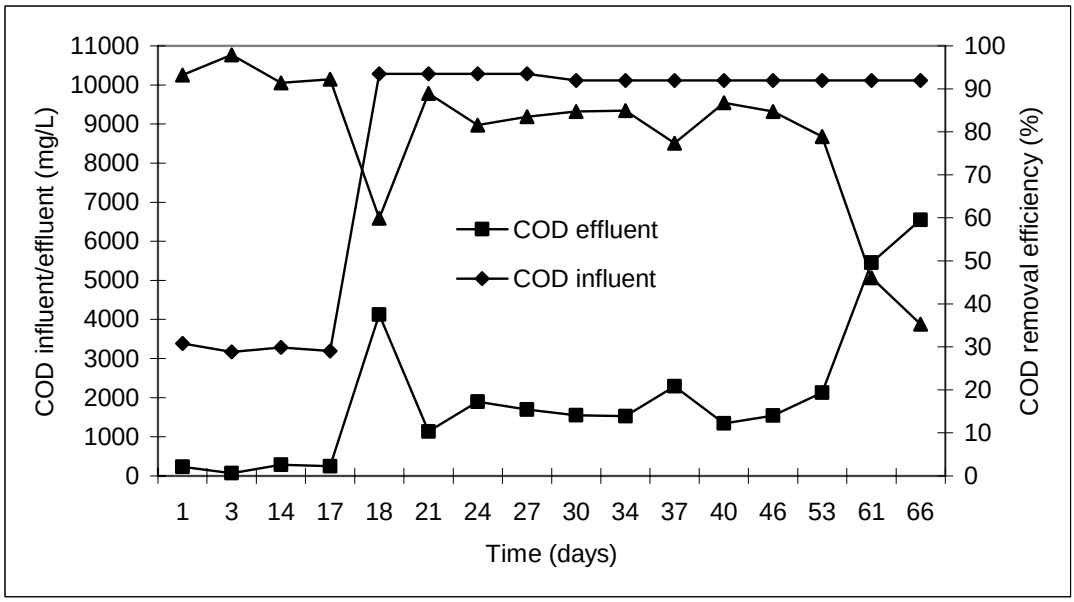


Figure 5.54. Performance of the ASBR fed with methanol-containing synthetic wastewater with time.

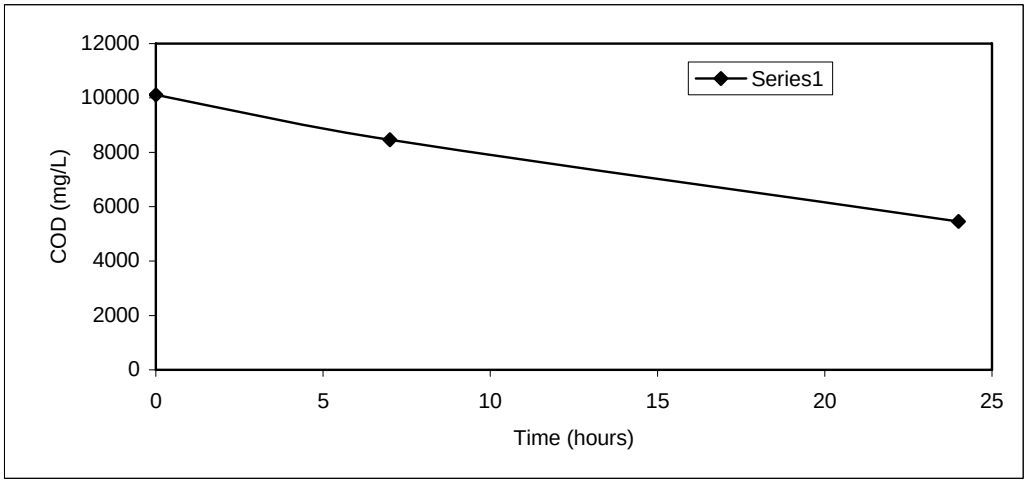


Figure 5.55. Changes in COD concentration during the cycle time at the end of the operation period of the ASBR fed with methanol-containing synthetic wastewater.

5.7.1. FISH results of the ASBR reactor operated with methanol-containing synthetic wastewater

After feeding the anaerobic reactor with methanol containing synthetic wastewater, the percentage of active population in the reactor decreased from 84±3.2% to 46.1±4.1% and then to 40.7±3.9% over the period of operation of the reactor. Table 5.27 shows the FISH results of the sludge taken from the ASBR fed with methanol-containing synthetic wastewater as a function of time. Although the reactor performed well on day 31 with a COD removal efficiency of 85% active population decreased to approximately %46 and the decrease in the active population continued during the operation. The decrease in the active population did not reflected in the reactor performance in advance. However, after day 61, the deterioration of both reactor performance and the relative population of active methanogenic populations become more severe. Since active population in the anaerobic reactor decreased over time with the effect of methanol, the FISH results were standardized against active total population (Table 5.28) in order to make a comparison between the sludge samples taken from the reactor over time.

Table 5.27. FISH results of the sludge taken from the ASBR fed with methanol-containing synthetic wastewater.

	Seed Sludge		**Glucose-fed**		**Day 31**		**Day 53**		**Day 66**	
	%	±	%	±	%	±	%	±	%	±
Active Cells	80.0	4.0	84.0	3.2	46.1	4.1	42.4	4.2	40.7	3.9
Eubacteria*	42.0	2.0	48.0	2.1	22.1	3.8	20.3	2.9	20.1	2.7
Archaea*	62.0	1.0	54.0	2.2	74.1	2.9	74.8	3.6	76.4	2.8
*M.saeta***	32.0	0.8	34.0	3.4	52.7	3.2	50.5	3.5	56.7	2.4
*M.sarcina***	8.0	1.5	17.0	1.3	18.8	2.4	21.2	2.5	19.4	2.4
*M.microbiales***	14.2	1.6	22.0	1.2	3.2	1.2	2.1	1.9	2.2	1.1
*M.coccales***	12.0	1.1	12.0	1.4	4.4	1.1	5.2	1.5	6.4	1.4
*M.bacteriales***	17.0	1.3	21.0	1.4	25.4	2.6	29.1	3.1	29.6	2.8

Table 5.28. Standardized FISH results of the sludge taken from the ASBR fed with methanol-containing synthetic wastewater.

	Seed Sludge	**Glucose-fed**	**Day 31**	**Day 53**	**Day 66**
Methanosaeta	15.9	15.5	18.0	16.0	17.6
Methanosarcina	4.0	7.8	6.4	6.7	6.0
Methanomicrobiales	7.0	10.0	1.1	0.7	0.7
Methanococcales	5.9	5.5	1.5	1.6	2.0
Methanobacteriales	8.4	9.6	8.7	9.2	9.2

Figure 5.56 shows standardized FISH results of the sludge taken from the ASBR fed with methanol-containing synthetic wastewater. At the end of the operation with methanol, dominant species in the reactor sludge (standardized against total active population) were *Methanosaeta* (17.6%), *Methanobacteriales* (9.2%), *Methanosarcina* (6.0%), *Methanomicrobiales* (0.7%) and *Methanococcales* (2%) respectively. *Methanosarcina* was also not affected from methanol. Besides acetate, *Methanosarcina* sp. is also capable of growing on substrates such as methanol, methylamines, and sometimes hydrogen and carbon dioxide. The second major methanogen was hydrogenotrophic *Methanobacteriales* and relative ratio of the methanogen remained almost same throughout the operating period. *Methanomicrobiales* was the most affected hydrogenotrophic methanogen from methanol.

Methanosaeta was not affected from methanol and remained as major methanogen in the reactor. Under anaerobic conditions, methanol potentially supports a complex food chain composed of a variety of trophic groups. Methylotrophic methanogens can directly convert methanol into methane (Jarrel and Kalmokoff, 1988). Methylotrophic acetogens produce acetate and butyrate but this conversion is limited by the availability of inorganic carbon (Ljungdahl, 1986). While the conversion of methanol to H_2/CO_2 is usually thermodynamically unfavourable, acetogens are also able to generate H_2/CO_2 from methanol in a syntrophic partnership with hydrogen consumers, *e.g.* sulfate-reducing bacteria (Cord-Ruwisch and Ollivier, 1986; DiStefano et al., 1992; Heijthuijsen and Hansen, 1986). Thus, methanol can indirectly support hydrogenotrophic and acetoclastic

methanogens via acetate or H_2/CO_2 generation by acetogenic bacteria. Since methanol is both methanogenic and an acetogenic substrate (Rocheleau et al., 1999), dominancy of the genus *Methanosaeta* in anaerobic granules fed with methanol is an expected result. This has been also reported previously (Rocheleau et al., 1999). Acetate produced from the conversion of methanol by the homoacetogens diffused along a decreasing gradient in the anaerobic granules, explaining why the obligate acetoclastic *Methanosaeta concilii* was also present in significant numbers on the inside of the *Methanosarcina barkeri* layer and randomly distributed in the core of the granule.

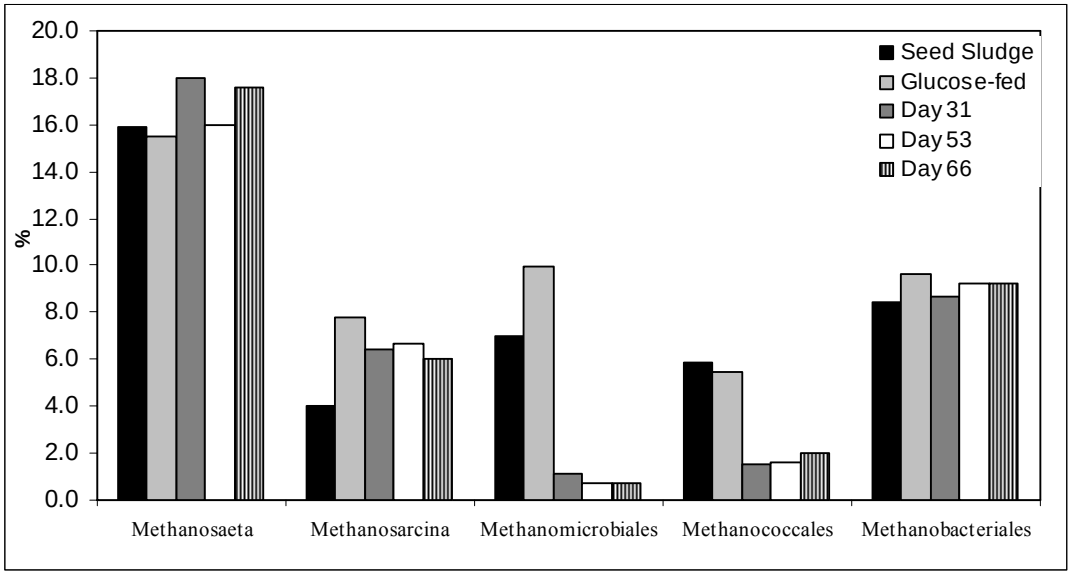


Figure 5.56. FISH results of the sludge taken from the ASBR fed with methanol-containing synthetic wastewater (Standardized).

5.7.2. DGGE results of the ASBR reactor operated with methanol-containing synthetic wastewater

Archaeal and bacterial community structure during the operating period with toluene were screened by DGGE analysis of PCR amplified 16S rRNA gene fragments and sequence analysis of excised bands When the reactor was fed with methanol containing wastewater, a change in the diversity of the reactor sludge was observed. Bacterial diversity (Figure 5.57) decreased from 15 to 9 (operational taxonomic unit) OTUs as archaeal diversity (Figure 5.58) decreased from 8 to 7. However, bacterial DGGE fingerprinting data showed that number of OTUs in the reactor sludges fed with methanol-containing wastewater increased from 9 to 15 in the course of time. Archaeal diversity of the reactor sludge increased to 7 OTUs. The data obtained from Bionumerics software analysis of DGGE gel photos were used to construct a relation analysis tree of samples (Figure 5.59 and 5.60). Relation Analysis trees constructed from Bacterial and Archaeal DGGE profiles were shown along with similarity matrices obtained using both band position based Dice and curve based (band intensity profile) Pearson correlation coefficients.

Bacterial communities of Methanol reactors form distinctive clusters at 64% in relation analysis with Dice coefficient except Methanol2. In Pearson coefficient analysis a cluster of Methanol reactors except Methanol5 was observed at 60%. Bacterial community profile clustered at 45% in Dice correlation coefficient, as profile cluster at 40% in Pearson correlation efficient. Substantially higher similarities and clustering obtained using Dice correlation coefficient implied that microbial communities in the reactors were more similar in terms of OTU type's present rather than relative abundance of similar OTUs. The effect of OTU types present was more noticeable in relation analysis of archaeal communities. In both correlation analysis communities of methanol-fed reactor form distinctive clusters (90% for the Pearson coefficient and 80% for the Dice coefficient). Opposite to bacterial community profiles, archaeal community profiles clustered at higher point in Pearson coefficient analyses than Dice (57% for Dice, 61% for Pearson coefficients). Relation analysis obtained using Pearson correlation coefficient show relative OTU abundance is a more distinctive criterion than OTU types in archaeal community.

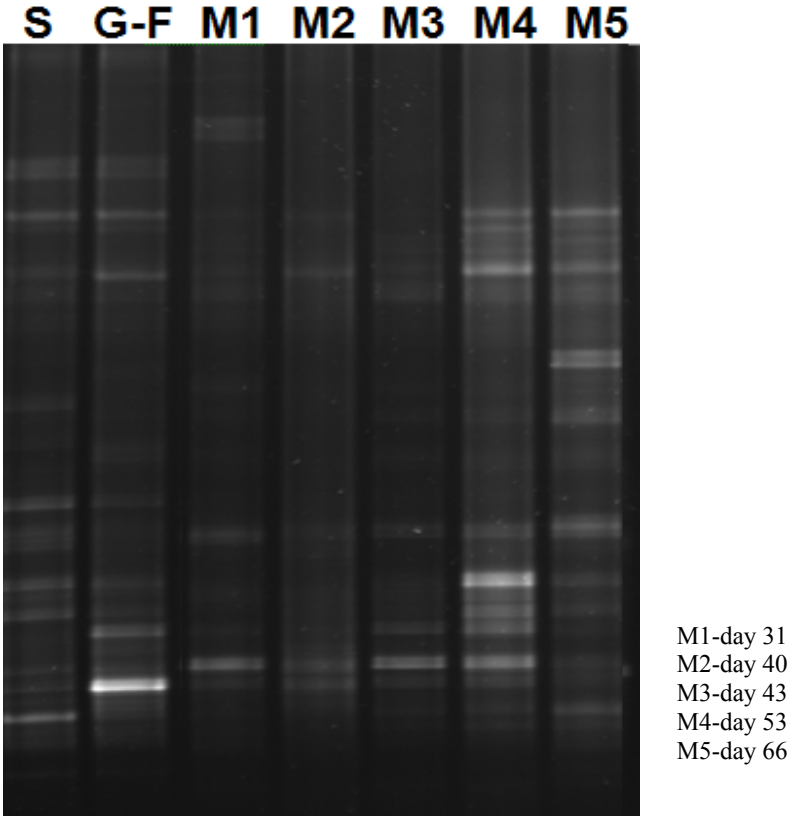


Figure 5.57. Analysis of bacterial community in the ASBR fed with methanol-containing synthetic wastewater by DGGE

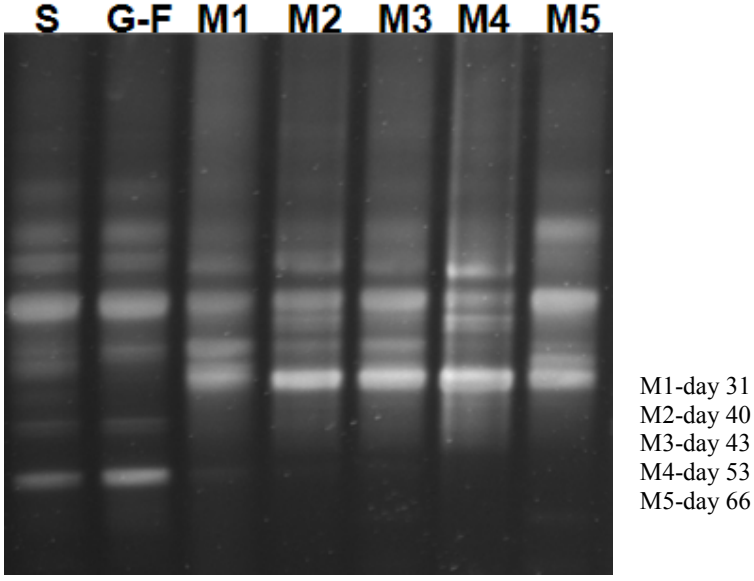


Figure 5.58. Analysis of Archaeal community in the ASBR fed with methanol-containing synthetic wastewater by DGGE.

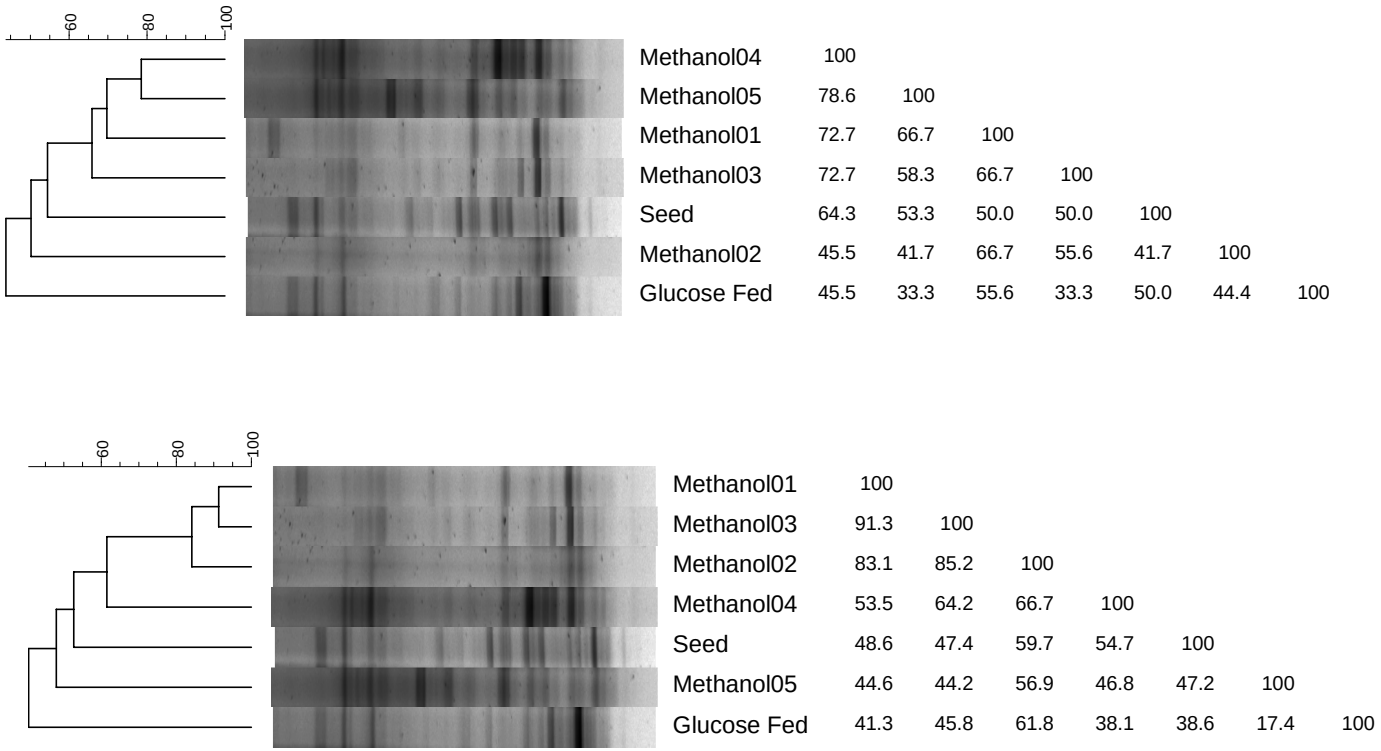


Figure 5.59. Phylogenetic tree constructed (based on Dice coefficient and Pearson coefficient) from the bacterial DGGE profiles in the ASBR fed with methanol-containing synthetic wastewater (M1-day 31, M2-day 40, M3-day 43, M4-day 53, M5-day 66)

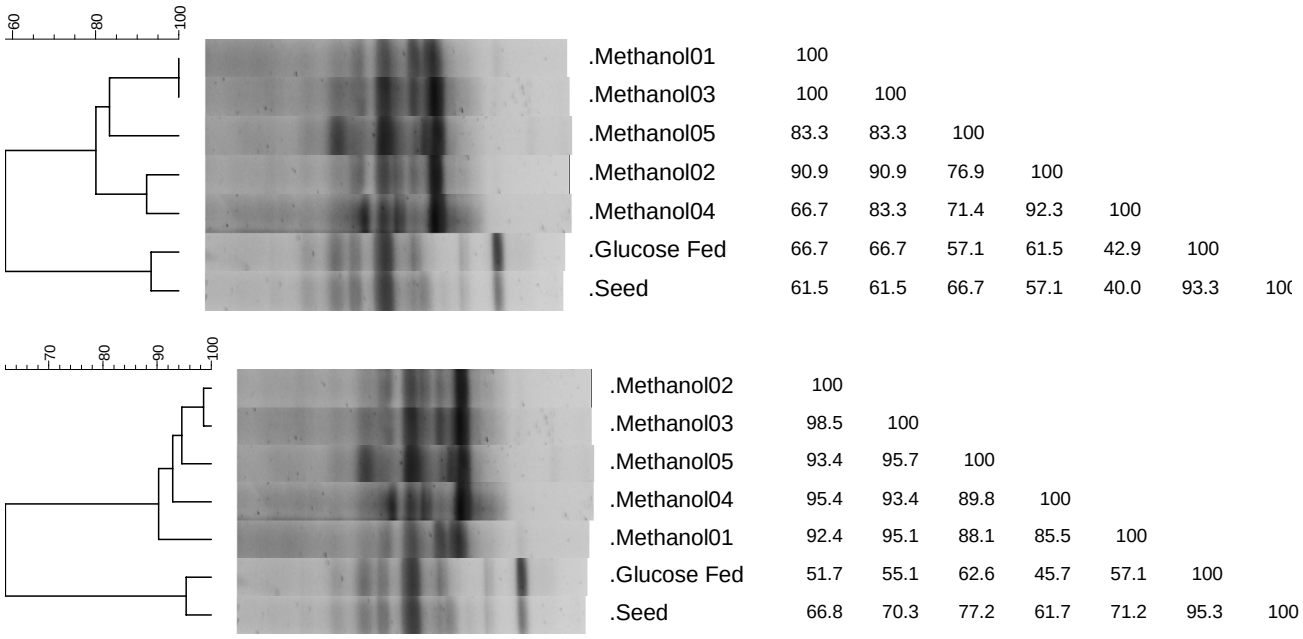


Figure 5.60. Phylogenetic tree constructed (based on Dice coefficient and Pearson coefficient) from the Archaeal DGGE profiles in the ASBR fed with methanol-containing synthetic wastewater (M1-day 31, M2-day 40, M3-day 43, M4-day 53, M5-day 66).

Total 10 bands were analyzed from bacterial DGGE gel of the reactor samples fed with methanol as 6 bands were analyzed from archaeal DGGE gel. Bands were amplified and sequenced to assess the identity of community members. According sequence analysis data, *Deltaproteobacteria* dominated reactor communities (4/10), and followed by *Firmicutes* and *Chloroflexi* by 2/10 of sequences. In a study, *Firmicutes*, Proteobacteria and a green sulphur bacteria were found to be abundant in a reactor treating solvent-containing wastewater (methanol) in psycrophilic conditions (Enright et al., 2007). These findings are in good agreement with previously documented anaerobic methanol enrichments (Roest et al., 2005), in which Firmicutes-like bacterial sequences were described. Furthermore, it has been also reported that the homoacetogenic species oxidized methanol completely to CO_2 when grown in the presence of hydrogenotrophic methanogens (Roest et al., 2005). Therefore, homoacetogenic bacteria in the solvent-degrading bioreactors could be in syntrophic association with the hydrogenotrophic methanogens, which are mainly reliant on interspecies H_2 transfer (Enright et al., 2007). However, this approach to assigning bacterial functionality has limited application, and need further investigations. The failure in the reactor treating methanol could be due to the decrease in the activity of H_2 utilizing methanogens especially *Methanomicrobiales*. There is limited information on the functionality and role of bacterial species and interactions between syntrophic associations in solvent treating reactors. This subject needs further investigations using combined application of radiotracer incubations, fluorescence microscopy and substrate uptake analysis to elucidate the exact ecophysiology of these organisms in solvent-treating bioreactors.

Sequences related to *Actinobacteria* and *Aquificae* were also found. Syntrophic proteobacteria were responsible in sulfate reduction coupled with volatile fatty acid oxidation, especially propionate, in presence of H_2 using microorganism mainly hydrogenotrophic methanogens. Those bacteria play significant roles in start up of bioreactors and presence of these bacteria indicates an efficient reactor performance. In all reactors, bands with sequences related to syntrophic bacteria were found. Hydrogentrophic Sulfate reduceing proteobacteria, *Desulfonauticus submarinus*, was found in the all samples taken from the reactor. It has been reported that some cultures of acetate oxidizing iron reducers such as the genus Geobacter could oxidize acetate in co-culture with hydrogen utilizing sulfate or nitrate reducers in the absence of oxidized form of iron (Cord-

Ruwisch et al., 1998). In anaerobic conditions, acetate is converted to methane by the acetate-cleaving methanogens, *Methanosarcina* and *Methanosaeta*. It is still believed that they are the primary acetate consumers in reactors. However, several studies showed that anaerobic reactors harbor diverse organisms even when they metabolize a simple substrate such as acetate (Schnüner et al., 1999). In order to understand the dynamics in the anaerobic reactors, population dynamics of the organisms and their competition with the acetate-cleaving methanogens in the sludge communities should be studied extensively.

Fermentative *Clostridium* species were not present in seed sludge and glucose-fed control reactor. However, the group was found to be in the reactors fed with methanol. Bacteria belonging to *Chloroflexi* and *Aquificae* present in both reactor communities, as the former mainly utilizes protein and limited sugars the latter is responsible for sulfur compounds reduction with H_2 oxidation. The *Chloroflexi* have long been recognized as an evolutionary and environmentally significant group of bacteria (Hugenholtz et al., 1998; Sekiguchi et al., 2001, 2003). Cultivation-independent 16S rRNA studies suggest that many species of the *Chloroflexi* thrive in anaerobic dechlorinating enrichments (Chandler et al., 1998; Hugenholtz et al., 1998) and anaerobic digesters (Chouari et al., 2005). In another study Sekiguchi and colleagues (1998) found that *Chloroflexi* clone sequences were predominant in clone libraries constructed using genomic DNA extracted from both mesophilic and thermophilic methanogenic sludge granules.

Syntrophobacter sulfatireducens, Syntrophobacter fumaroxidans MPOB and *Syntrophus aciditrophicus* SB have been found to be in seed and methanol-fed reactors. Syntrophic propionate degradation by *Syntrophobacter fumaroxidans* occurs only if the partner used both hydrogen and formate. Some gram-negative bacteria affiliated with the *Deltaproteobacteria* are capable of syntrophic metabolism (Jackson *et al.,* 1999). The first syntrophic propionate oxidizer described was *Syntrophobacter wolinii (*Boone and Bryant, 1980) which was isolated from primary anaerobic digestor sludge. Three other *Syntrophobacter* species have been described, *Syntrophobacter pfennigii* from an anaerobic sludge of a sewage plant, (Wallrabenstein et al., 1995). *Syntrophobacter fumaroxidans* isolated from an anaerobic sludge blanket reactor treating wastewater from a sugar refinery (Harmsen et al.,1998) and two strains of *Syntrophobacter sulfatireducens*

TB8106 and WZH410 isolated from the anaerobic sludge of a reactor treating brewery wastewater and a reactor treating bean-curd wastewater, respectively (Chen et al., 2005).

Archaeal communities in Methanol reactors were dominated by *Methanosarcinales* group. Two members of *Methanosarcinaceae* present in Methanol reactors not in glucose-fed control reactor. *Methanosaeta* sp. was present in all samples. Presence of a methyl compounds degrading *Methanosarcinales* and H_2+CO_2-Formate using *Methanomicrobiales* was also confirmed in all reactors. *Methanolobus* which is a coccoid methanogen growing only on methanol and methylamines and belonging to *Methansarcinales* was present in all samples taken from the anaerobic reactor fed with methanol-containing synthetic wastewater. Since DGGE is a semi-quantative method, based on the archaeal DGGE gel it can be said that the group was dominant (M3-M4) in the reactor. Another *Methanomicrobiales* species was present in seed sludge, glucose-fed control reactor but not in the reactor fed with methanol.

Table 5.29. Identification of phylotypes from bacterial community in the ASBR fed with methanol-containing synthetic wastewater.

Accession number	Closest relative	% sim.	Phylogeny	Metabolism	S	R	M1	M2	M3	M4	M5
CP000478	*Syntrophobacter fumaroxidans MPOB*	85	Deltaproteo.	VFA oxidation	X	0	X	0	X	0	X
AY651787	*Syntrophobacter sulfatireducens*	88	Deltaproteo.	VFA oxidation	X	X	X	X	0	X	X
CP000252	*Syntrophus aciditrophicus SB*	83	Deltaproteo.	VFA oxidation	X	X	X	X	X	X	X
EF527427	*Desulfonauticus submarinus*	85	Deltaproteo.	hydrogenotrophic, sulfate-reduction	0	X	X	X	X	X	X
CP001078	*Clostridium botulinum E3 str. Alaska E43*	95	Firmicutes	Fermentation	0	0	X	X	X	X	X
BX248359	*Corynebacterium diphtheriae*	84	Actinobacteria	Fermentation	X	0	X	X	X	X	X
AB243673	*Longilinea arvoryzae*	91	Chloroflexi	Protein and sugar degradation	0	0	X	X	X	X	X
AF473835	*Acidaminococcus intestinalis*	87	Firmicutes	Amino acid degradation to VFA	0	X	0	0	0	X	X
CP001080	*Sulfurihydrogenibium sp. YO3AOP1*	88	Aquificae	H_2 oxidation sulfur compounds reduction	X	X	X	X	X	X	X
Number of OTU's on DGGE gel					**15**	**9**	**9**	**9**	**9**	**13**	**15**

Table 5.30. Closest matches of phylotypes from bacterial community in the ASBR fed with methanol-containing synthetic wastewater (Environmental Database)

Accession number	Closest match	% sim.	Isolation source	S	R	M1	M2	M3	M4	M5
AY426441	clone B16	93	UASB bioreactor treating paper mill wastewater	X	0	X	0	X	0	X
AB266988	clone: QpjB112fl	97	mesophilic UASB sludge granules	X	X	X	X	0	X	X
AJ306771	clone SHA-42	88	1,2-Dichlorpropan mixculture	X	X	X	X	X	X	X
AB266990	clone: HgtB7fl.	98	mesophilic UASB sludge granules	X	X	X	X	X	X	X
EF034988	Clostridia	99	permafrost soil	0	0	X	X	X	X	X
AB267014	clone: YkB4fl.	87	mesophilic UASB sludge granules	X	0	X	X	X	X	X
EF053078	clone PEU-21	95	diethyl phthalate and phenol degrading UASB reactor	0	0	X	X	X	X	X
EU888006	Clostridia bacterium clone L33	99	leach bed reactor	0	X	0	0	0	X	X
EF613410	clone ZZ-S2F11	94	benzene-mineralizing consortium	0	X	X	X	X	X	X
Number of OTU's on DGGE gel				**15**	**9**	**9**	**9**	**9**	**13**	**15**

Table 5.31. Identification of phylotypes from archaeal community in the ASBR fed with methanol-containing synthetic wastewater.

Accession number	Closest relative	% sim.	Phylogeny	Metabolism	S	R	M1	M2	M3	M4	M5
CP000780	*Candidatus Methanoregula boonei 6A8*	83	M.microbiales	H_2+CO_2-Formate	X	X	0	0	0	0	0
AB370247	*Methanolobus sp. HigM*	83	M.sarcinales	Methylated c.	X	X	X	X	X	X	X
CP000300	*Methanococcoides burtonii DSM 6242*	84	M.sarcinales	Methylated c.	0	0	X	X	X	X	X
X51423	*Methanosaeta concilii*	96	M.sarcinales	Acetate	X	X	X	X	X	X	X
AB162774	*Methanolinea tarda*	88	M.microbiales	H_2+CO_2-Formate	X	X	X	X	X	X	X
AF120163	*Methanomethylovorans hollandica*	92	M.sarcinales	Methylated c.	X	X	X	X	X	X	X
Number of OTU's on DGGE gel					**8**	**7**	**5**	**6**	**5**	**7**	**7**

Table 5.32. Closest matches of phylotypes from archaeal community in the ASBR fed with methanol-containing synthetic wastewater (Environmental Database).

Accession number	Closest match	% sim.	Isolation source	S	R	M1	M2	M3	M4	M5
AF229776	uncultured archaeon TA03	84	anaerobic granular sludge	X	X	0	0	0	0	0
AY454755	U. Methanolobus sp. clone E_C06	85	estuary sediment	X	X	X	X	X	X	X
AB077216	clone:KuA6.	82	oil-contaminated groundwater	0	0	X	X	X	X	X
AB329663	clone: UASB26.	96	UASB granule	X	X	X	X	X	X	X
AB195851	uncultured archaeon	93	VFA degrading AB	X	X	X	X	X	X	X
AY780565	clone KB-1 2	94	chlorinated ethene-degrading culture	X	X	X	X	X	X	X
Number of OTU's on DGGE gel				**8**	**7**	**5**	**6**	**5**	**7**	**7**

5.8. Microbial Population Changes during Anaerobic Treatment of Isopropanol-containing Synthetic Wastewater in an ASBR

The reactor was firstly fed with a glucose-based synthetic wastewater in order to acclimatize the sludge. A rapid and successful start-up was observed for the reactor with COD removal efficiency over 95%. After the operation period with glucose, isopropanol was added to glucose-based synthetic wastewater at a concentration of 0.1 M at each cycle during the operation period with the solvent (Figure 5.61). The isopropanol concentration applied to the reactor was less than IC_{50} concentration determined in batch studies by taking into consideration of possible detrimental effect of isopropanol on the reactor.

The ASBR was operated with one cycle/day including the following four discrete steps: a feed step (0.5 h), a react step (22 h), a settling step (1 h), and a decant step (0.5 h). The reactor was operated with a SRT of 70 days and a HRT of 34 hours. VSS concentration in the reactor ranged between approximately 6000-7700 mg/L. At the beginning of the operation period of the reactor, COD removal efficiency of the reactor decreased to 68% after isopropanol addition to the synthetic wastewater and then increased to 90%. However, after day 46 a gradual deterioration in the reactor performance was observed with a COD removal efficiency falling to 70% (Figure 5.61). A sudden decline in the performance of the reactor was observed after day 52 with a COD removal efficiency of 67% at the end of the operation period (Figure 5.61). An example of the evolution of the COD during a cycle at the end of the operation period is presented in Figure 5.62.

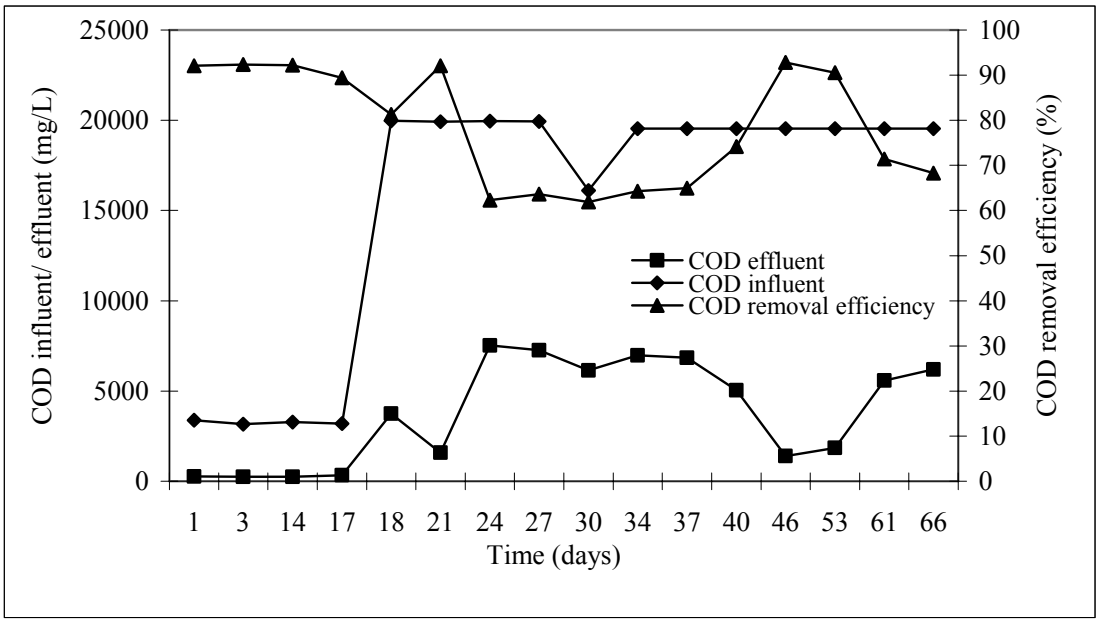


Figure 5.61. Performance of the ASBR fed with isopropanol-containing synthetic wastewater with time

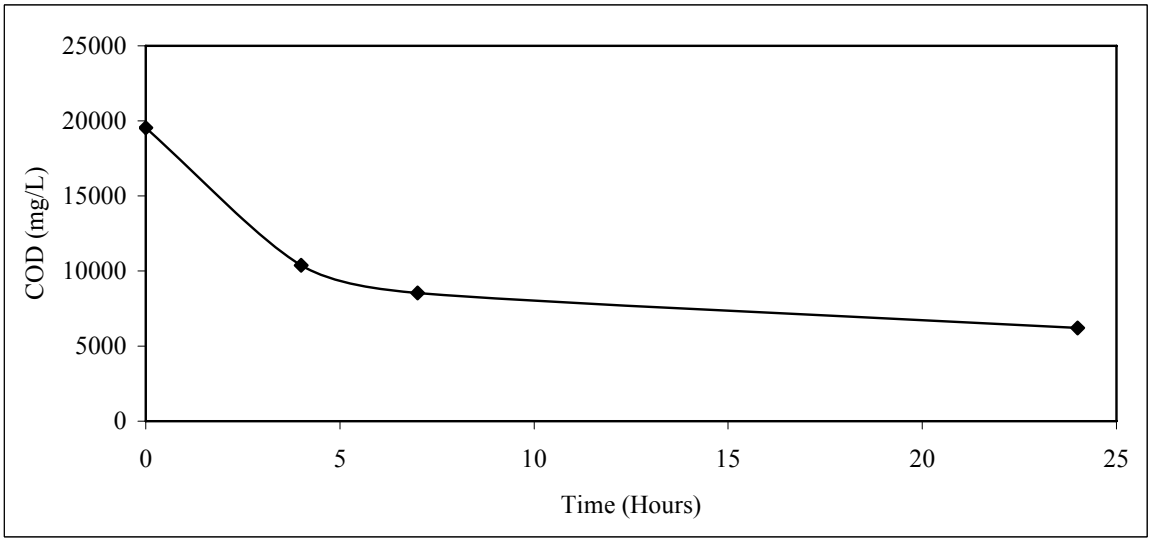


Figure 5.62. Changes in COD concentration during the cycle time at the end of the operation period of the ASBR fed with isopropanol -containing synthetic wastewater

5.8.1. FISH results of the ASBR reactor operated with isopropanol-containing synthetic wastewater

After feeding the anaerobic reactor with propanol containing synthetic wastewater, the percentage of active population in the reactor decreased from 84±3.2% to 32.1±2.4% and then to 27.4±2.4% at the end of the period of reactor operation. Table 5.33 shows the FISH results of the sludge taken from the ASBR fed with methanol-containing synthetic wastewater as a function of time. Since active population in the anaerobic reactor decreased over time with the effect of isopropanol, the FISH results were standardized against active total population (Table 5.34) in order to make a comparison between the sludge samples taken from the reactor over time. Figure 5.63 show standardized FISH results of the sludge taken from the ASBR fed with isopropanol-containing synthetic wastewater. At the end of the operation period, the most dominant methanogen was *Methanosaeata* among other methanogens. However, it should be mentioned that, the relative amount of the group decreased almost to half compared to glucose-fed reactor. The most affected methanogenic Archaea was *Methanomicrobiales***.**

Table 5.33. FISH results of the sludge taken from the ASBR fed with isopropanol-containing synthetic wastewater.

	Seed sludge		**Glucose-fed**		**Day 31**		**Day 53**		**Day 66**	
	%	**±**	**%**	**±**	**%**	**±**	**%**	**±**	**%**	**±**
Active Cells	80.0	4.0	84	3.2	32.1	2.4	30.8	2.3	27.4	2.4
Eubacteria	42.0	2.0	48	2.1	34.7	2.5	32.5	3.6	37.1	3.7
Archaea	62.0	1.0	54	2.2	61.4	2.9	65.6	3.7	60.7	4.1
Methanosaeta	32.0	0.8	34	3.4	50.5	3.1	50.2	2.4	52.6	4.0
Methanosarcina	8.0	1.5	17	1.3	14.5	2.1	12.9	2.7	12.7	2.0
Methanomicrobiales	14.2	1.6	22	1.2	2.1	1.1	2.7	1.3	2.1	1.7
Methanococcales	12.0	1.1	12	1.4	7.6	2.3	5.7	2.1	6.1	2.9
Methanobacteriales	17.0	1.3	21	1.4	28.6	3.6	29.4	3.4	28.4	4.2

Table 5.34. Standardized FISH results of the sludge taken from the ASBR fed with isopropanol-containing synthetic wastewater.

	Seed Sludge	**Glucose-fed**	**Day 31**	**Day 53**	**Day 66**
Methanosaeta	15.9	15.5	9.9	10.1	8.7
Methanosarcina	4.0	7.8	2.9	2.6	2.4
Methanomicrobiales	7.0	10	0.4	0.5	0.4
Methanococcales	5.9	5.5	1.5	1.1	1.0
Methanobacteriales	8.4	9.6	5.6	5.9	4.7

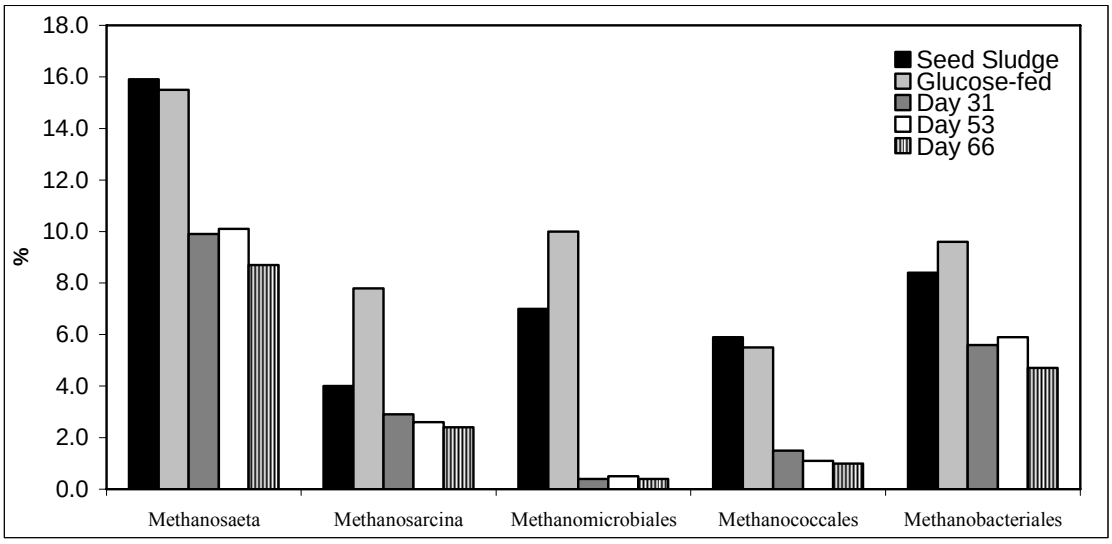


Figure 5.63. Standardized FISH results of the sludge taken from the ASBR fed with isopropanol-containing synthetic wastewater.

5.8.2. DGGE results of the ASBR reactor operated with isopropanol-containing synthetic wastewater

After reactor was fed with isopropanol containing synthetic wastewater, a decrease in the bacterial and Archaeal diversity was observed. Bacterial diversity decreased from 15 to 9 OTUs as archaeal diversity decreased from 8 to 7. According to Bacterial DGGE fingerprinting data, number of OTUs in the propanol-fed reactor sludges increased with time and then slightly decreased during the operating period (From 9 to 17 and then to 15). Archaeal diversity of the reactor samples showed same tendency like bacterial diversity; diversity oscillates from 7 to 8 then to 6 OTUs.

The data obtained from Bionumerics software analysis of DGGE gel photos were used to construct a relation analysis tree of samples. Bacterial communities of Propanol reactor form distinctive clusters at 55% in relation analysis with Dice coefficient. In Pearson coefficient analysis all samples from the reactor except P1 form a cluster at 71%. Bacterial community profile clustered at 40% both in Dice and in Pearson correlation efficient. The effect of relative abundance of similar OTUs was more noticeable in relation analysis of archaeal communities.

In both correlation analysis communities of reactor fed with propanol form distinctive clusters (96% for the Pearson coefficient and 65% for the Dice coefficient). The effect of OTU types present was more noticeable in relation analysis of archaeal communities. Archaeal community profiles clustered at higher point in Pearson coefficient analyses than Dice (55% for Dice, 78% for Pearson coefficients). Relation analysis obtained using Pearson correlation coefficient show relative OTU abundance is a more distinctive criterion than OTU types in Propanol reactor's Archaeal community.

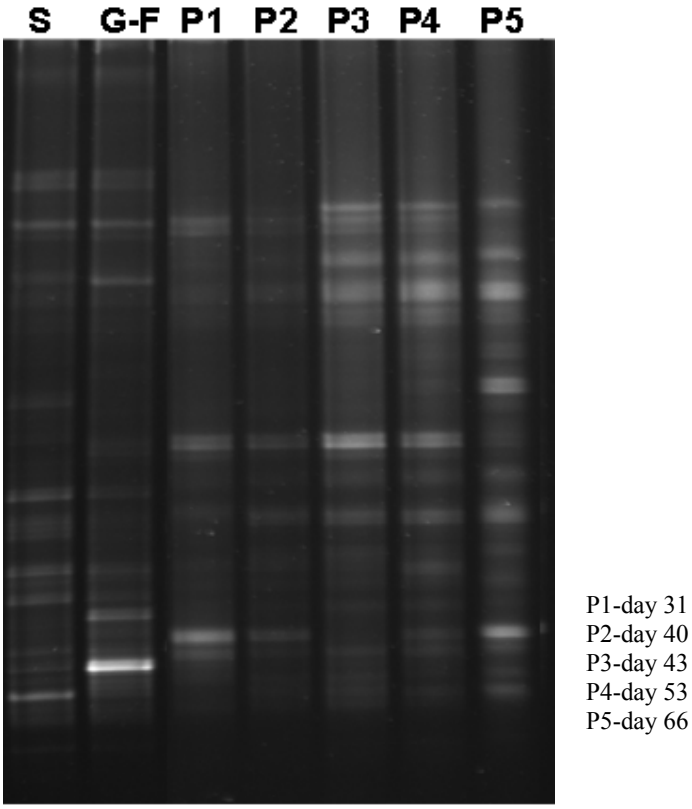


Figure 5.64. Analysis of bacterial community in the ASBR fed with isopropanol-containing synthetic wastewater by DGGE.

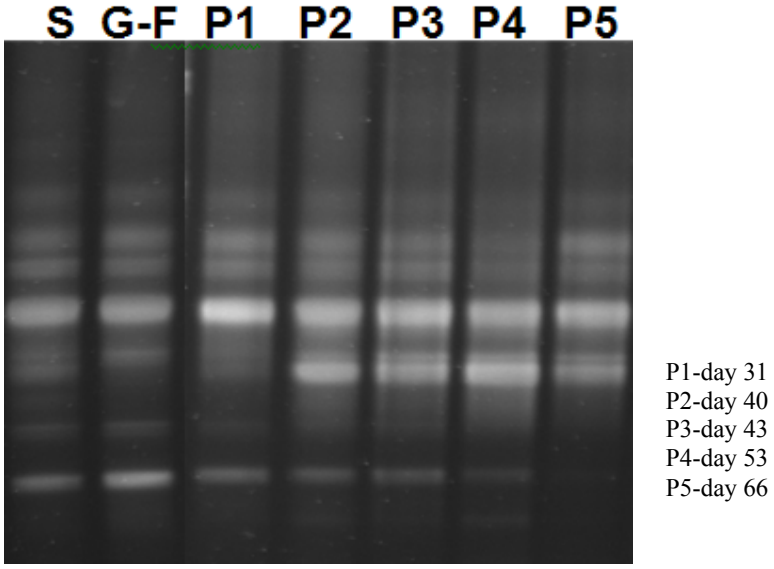


Figure 5.65. Analysis of Archaeal community in the ASBR fed with isopropanol-containing synthetic wastewater by DGGE.

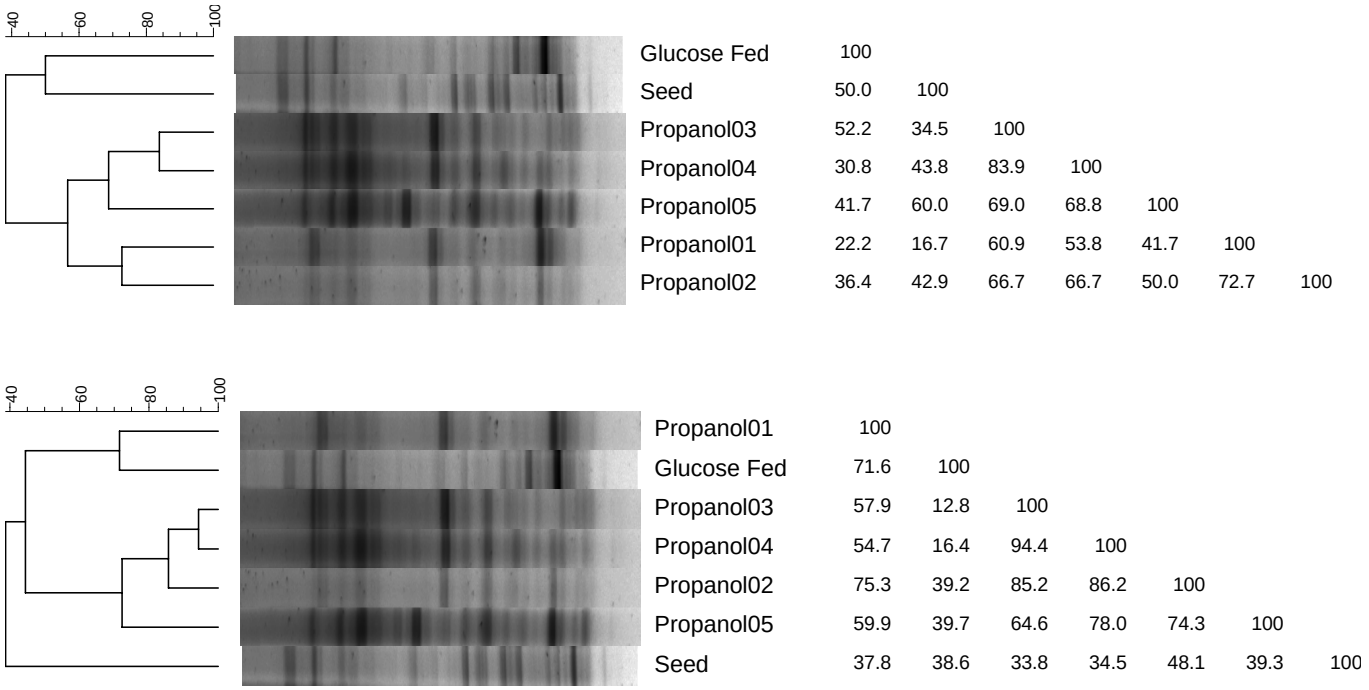


Figure 5.66. Phylogenetic tree constructed (based on Dice coefficient and Pearson coefficient) from the bacterial DGGE profiles in the ASBR fed with isopropanol-containing synthetic wastewater (P1-day 31, P2-day 40, P3-day 43, P4-day 53, P5-day 66).

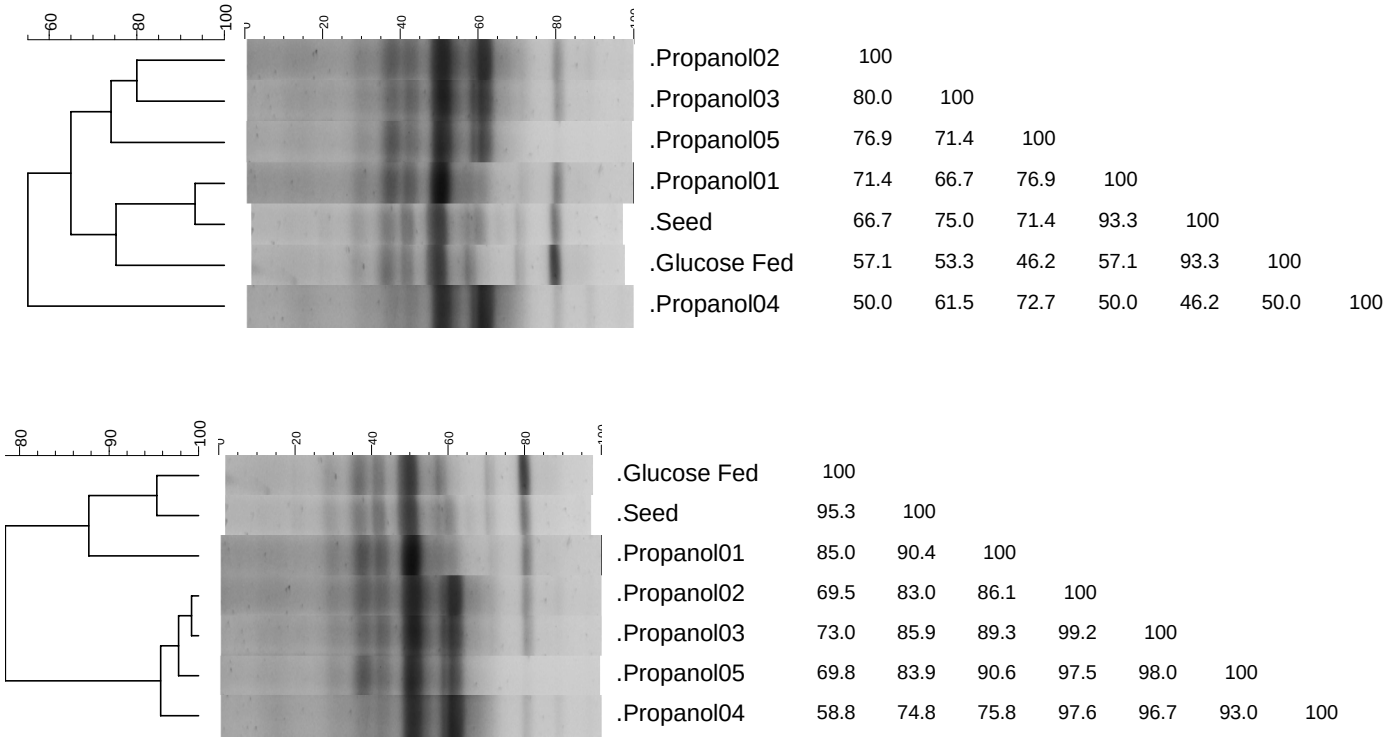


Figure 5.67. Phylogenetic tree constructed (based on Dice coefficient and Pearson coefficient) from the Archaeal DGGE profiles in the ASBR fed with isopropanol-containing synthetic wastewater (P1-day 31, P2-day 40, P3-day 43, P4-day 53, P5-day 66).

Total 10 bands were excised from bacterial DGGE gel of the sludge sample taken from propanol-fed reactor as 6 bands were excised from Archaeal DGGE gel. Bands were amplified and sequenced to assess the identity of community members. According sequence analysis data, *Proteobacteria*, *Delta* and *Gamma*, dominated reactor communities (4/10), and followed by *Firmicutes* by 3/10 of sequences according sequence analysis data. Sequences related to *Chloroflexi* and *Aquificae* were also found. Syntrophic proteobacteria were responsible in sulfate reduction (in case of *Alkalilimnicola ehrlichei* nitrate reduction) coupled with fatty acid oxidation, especially propionate, in presence of H_2 using microorganisms mainly hydrogenotrophic methanogens. In all reactors, bands with sequences related to syntrophic bacteria were found. Although *Firmicutes* mainly hosted by fermentative bacteria, different metabolic pathways are present within this group. *Desulfotomaculum reducens*, a well known sulfate reducer was present in seed sludge, glucose-fed control reactor and the samples taken from the propanol-fed reactor. A fermentative Clostridium species were present in Propanol reactor as a propionate and butyrate producing Firmicutes, *Acidaminococcus intestinalis*, was found in seed sludge, glucose-fed control reactor and the samples taken from the propanol-fed reactor. Both bacteria belonging to *Chloroflexi* and *Aquificae* present in both propanol and control reactor communities. The former mainly utilizes protein and limited sugars whereas the latter is responsible for sulfur compounds reduction with H_2 oxidation. The *Chloroflexi* have long been recognized as an evolutionary and environmentally significant group of bacteria (Hugenholtz et al., 1998; Sekiguchi et al., 2001, 2003). Cultivation-independent 16S rRNA studies suggest that many species of the *Chloroflexi* thrive in anaerobic dechlorinating enrichments (Chandler et al., 1998; Hugenholtz et al., 1998) and anaerobic digesters (Chouari et al., 2005). In another study, Sekiguchi and colleagues (1998) found that *Chloroflexi* clone sequences were predominant in clone libraries constructed using genomic DNA extracted from both mesophilic and thermophilic methanogenic sludge granules.

Archaeal communities in propanol reactors were dominated by *Methanosarcinales* group; *Methanosaeta* and *Methanosarcina*. One of the closest relative of *Methanosarcinales* group; *Methanolobus* spp. HigM was present in seed and glucose-fed reactor however, was not found to be in propanol-fed reactor. *Methanomethylovorans* spp. Z1, which is also from *Methanosarcinales* group was present in all samples. Both species

can use methylated compounds. H_2+CO_2-Formate using *Methanomicrobiales* was also confirmed in all reactors.

Table 5.35. Identification of phylotypes from bacterial community of the ASBR fed with isopropanol-containing synthetic wastewater

Accession number	Closest relative	% sim.	Phylogeny	Metabolism	S	G-F	P1	P2	P3	P4	P5
CP000612	*Dehalococcoides sp. BHI80-15*	88	Chloroflexi	H_2 Oxidation	X	X	0	X	X	X	X
CP000478	*Syntrophobacter fumaroxidans MPOB*	85	Deltaproteo.	VFA oxidation	X	0	0	0	X	X	X
AY651787	*Syntrophobacter sulfatireducens*	88	Deltaproteo.	VFA oxidation	X	X	0	0	X	X	X
CP000252	*Syntrophus aciditrophicus SB*	83	Deltaproteo.	VFA oxidation	X	X	0	X	X	X	X
CP001080	*Sulfurihydrogenibium sp. YO3AOP1*	88	Aquificae	H_2 oxidation sulfur compounds reduction	X	X	X	X	X	X	X
CP001078	*Clostridium botulinum E3 str. Alaska E43*	95	Firmicutes	Fermentation	0	0	0	0	X	0	X
BX248359	*Corynebacterium diphtheriae*	84	Actinobacteria	Fermentation	X	0	X	X	X	X	X
AB243673	*Longilinea arvoryzae*	91	Chloroflexi	Protein and sugar degradation	0	0	X	X	0	X	X
AF473835	*Acidaminococcus intestinalis*	87	Firmicutes	Amino acid degradation to VFA	0	X	X	X	X	X	X
CP000453	*Desulfovibrio vulgaris*	78	Deltaproteo.	Sulfate reduction acetate formation	X	0	0	0	0	X	X
Number of OTU's on DGGE gel					**15**	**9**	**9**	**13**	**14**	**17**	**15**

Table 5.36. Closest matches of phylotypes from bacterial community of of the reactor fed with isopropanol-containing synthetic wastewater (Environmental Database)

Accession number	Closest match	% sim.	Isolation source	S	G-F	P1	P2	P3	P4	P5
AB266990	bacterium EU6	90	toluene-degrading methanogenic consortium bacterium	X	X	0	X	X	X	X
AY426441	clone B16	93	UASB bioreactor treating paper mill wastewater	X	0	0	0	X	X	X
AB266988	clone: QpjB112fl	97	mesophilic UASB sludge granules	X	X	0	0	X	X	X
AJ306771	clone SHA-42	88	1,2-Dichlorpropan Mixculture	X	X	0	X	X	X	X
AB266990	clone: HgtB7fl.	98	mesophilic UASB sludge granules	X	X	X	X	X	X	X
EF034988	Clostridia	99	permafrost soil	0	0	0	0	X	0	X
AB267014	clone: YkB4fl.	87	mesophilic UASB sludge granules	X	0	X	X	X	X	X
EF053078	clone PEU-21	95	diethyl phthalate and phenol degrading UASB reactor	0	0	X	X	0	X	X
EU888006	Clostridia bacterium clone L33	99	leach bed reactor	0	X	X	X	X	X	X
DQ443887	clone LM5-8	85	sulfate-reducing bioreactor	X	0	0	0	0	X	X
Number of OTU's on DGGE gel				**15**	**9**	**9**	**13**	**14**	**17**	**15**

Table 5.37. Identification of phylotypes from archaeal community of the ASBR fed with isopropanol-containing synthetic wastewater

Accession number	**Closest relative**	**% sim.**	**Phylogeny**	**Metabolism**	**S**	**G-F**	**P1**	**P2**	**P3**	**P4**	**P5**
AY422331	*Methanogenic archaeon F1/B-2*	88	*M.sarcinales*	Acetate	X	X	X	0	0	0	0
CP000780	*Candidatus Methanoregula boonei 6A8*	83	*M.microbiales*	H_2+CO_2-Formate	X	X	X	X	X	X	0
AB370247	*Methanolobus sp. HigM*	83	*M.sarcinales*	Methylated c.	X	X	0	0	0	0	0
X51423	*Methanosaeta concilii*	96	*M.sarcinales*	Acetate	X	X	X	X	X	X	X
AB162774	*Methanolinea tarda*	88	*M.microbiales*	H_2+CO_2-Formate	X	X	X	X	X	X	X
EF174501	*Methanomethylovorans sp. Z1*	92	*M.sarcinales*	Methylated c.	X	X	X	X	X	X	X
Number of OTU's on DGGE gel					**8**	**7**	**7**	**7**	**8**	**5**	**6**

Table 5.38. Identification of phylotypes from archaeal community of ASBR fed with isopropanol-containing synthetic wastewater (Environmental Database).

Accession number	Closest matches	% sim.	Isolation source	S	G-F	K	P1	P2	P3	P4	P5
EU591674	clone MCSArc_H3	92	methanogenic reactor	X	X	X	X	0	0	0	0
AF229776	uncultured archaeon TA03	84	anaerobic granular sludge	X	X	X	X	X	X	X	0
AY454755	U. Methanolobus sp. clone E_C06	85	estuary sediment	X	X	0	0	0	0	0	0
AB329663	clone: UASB26.	96	UASB granule	X	X	X	X	X	X	X	X
AB195851	uncultured archaeon	93	VFA degrading AB	X	X	X	X	X	X	X	X
AY780565	clone KB-1 2	94	chlorinated ethene-degrading culture	X	X	X	X	X	X	X	X
Number of OTU's on DGGE gel				**8**	**7**	**6**	**7**	**7**	**8**	**5**	**6**

5.9. Comparison of Archaeal and Bacterial Community Dynamics in ASBRs Treating Selected Solvents

In order to determine reaction of prevelant microbial communities to different organic solvents such as methanol, toluene and isopropanol, the results of molecular studies were compared. Table 5.39 and Table 5.40 show the overall and standardized FISH results of the sludge taken from the ASBRs fed with different solvent-containing synthetic wastewater, respectively. As can be seen from the Figure 5.68, acetoclastic *Methanosaeta* species was the prevalent methanogens in the Archaeal community in the ASBRs fed with solvent containing wastewater. The remaining portion of *Archaea* in the reactors consisted mainly of hydrogenotrophic *Methanobacteriales*. *Methanomicrobiales* and *Methanococcales* which are hydrogenotrophic methanogens were the most effected group by the all selected solvents. It has been reported that *Methanococcales* group does not play an important role in anaerobic reactors (Raskin et al.,1995). However, it is not clear that why *Methanomicrobiales* group lost significantly activity in the solvent-fed reactors. Hydrogenotrophic methanogens play a crucial role in constantly eliminating hydrogen and using a limited number of small molecules such as CO2, formate, methanol, methylamines, and acetate to produce methane. The syntrophic association between substrate oxidizers, namely VFAs oxidizers, and hydrogen-scavenging methanogens is indispensable to sustain the overall process of anaerobic methane production. *Methanosarcina* was significantly affected by toluene and isopropanol whereas it was not affected from methanol because the group utilize methanol as substrate. Most severe effect of the solvents on amount of total methanogen was caused by isopropanol among others.

In a current study, anaerobic treatment of methanol-, acetone- and propanol-contaminated wastewater under psycrophilic conditions (15°C) was investigated. Sludge granules were obtained from laboratory-scale anaerobic bioreactors used to treat pharmaceutical-like and microbial diversity and diversity changes of the sludge samples were ascertained by applying 16S rRNA gene cloning and terminal restriction fragment length polymorphism (TRFLP) analyses (Enright et al., 2007b). In the study, the dominancy of hydrogenotrophic methanogens within the archaeal community in the sludge samples has been reported and community development was linked to both operating temperature and wastewater composition.

Table 5.39. Comparison of FISH results of the sludge taken from the ASBRs fed with different solvent-containing synthetic wastewater (day 66).

	Seed sludge		**Glucose-fed**		**Toluene**		**Methanol**		**Iso-propanol**	
	%	±	%	±	%	±	%	±	%	±
Active Cells	80.0	4.0	84	3,2	42.6	2.4	40.7	3.9	27.4	2.4
Eubacteria	42.0	2.0	48	2,1	40.5	2.8	20.1	2.7	37.1	3.7
Archaea	62.0	1.0	54	2,2	59.1	1.4	76.4	2.8	60.7	4.1
Methanosaeta	32.0	0.8	34	3,4	48.8	1.4	56.7	2.4	52.6	4.0
Methanosarcina	8.0	1.5	17	1,3	14.2	1.6	19.4	2.4	12.7	2.0
Methanomicrobiales	14.2	1.6	22	1,2	0	0	2.2	1.1	2.1	1.7
Methanococcales	12.0	1.1	12	1,4	5.4	1.1	6.4	1.4	6.1	2.9
Methanobacteriales	17.0	1.3	21	1,4	22.1	1.9	29.6	2.8	28.4	4.2

Table 5.40. Comparison of standardized FISH results of the sludge taken from the ASBRs fed with solvent-containing synthetic wastewater (day 66).

	Seed Sludge	**Glucose-fed**	**Toluene**	**Methanol**	**Isopropanol**
Methanosaeta	15.9	15.5	12.3	17.6	8.7
Methanosarcina	4.0	7.8	3.6	6.0	2.4
Methanomicrobiales	7.0	10	0.0	0.7	0.4
Methanococcales	5.9	5.5	1.4	2.0	1.0
Methanobacteriales	8.4	9.6	5.6	9.2	4.7

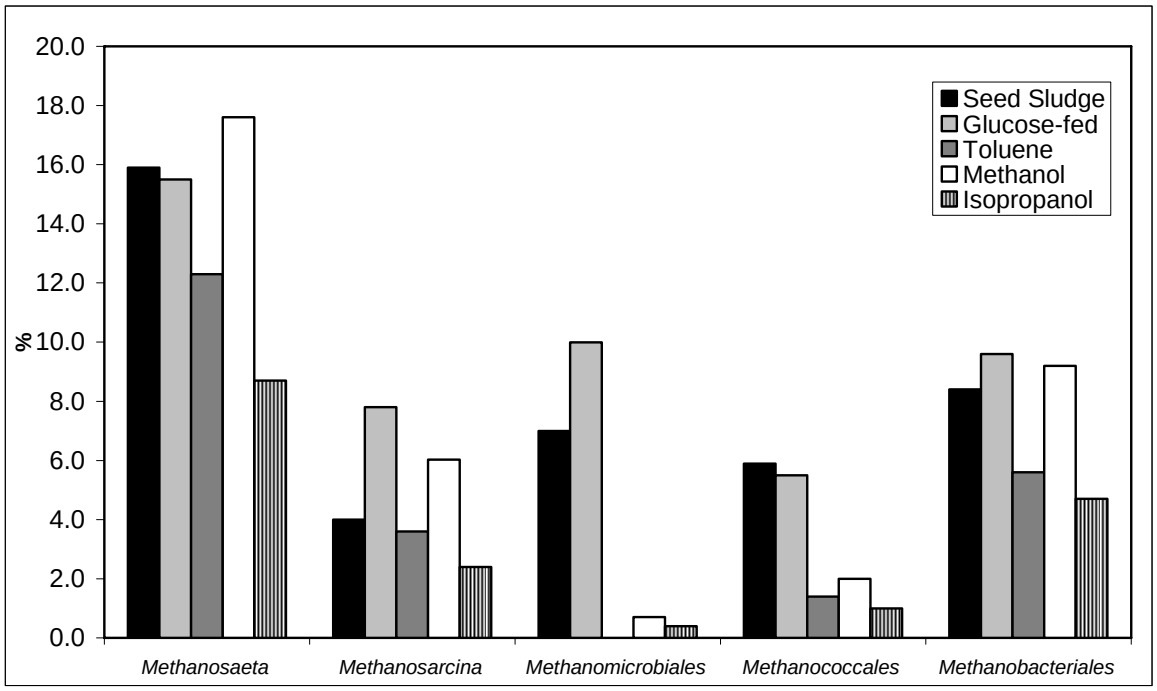


Figure 5.68. Comparison of standardized FISH results of the sludge taken from the ASBRs fed with solvent-containing synthetic wastewater.

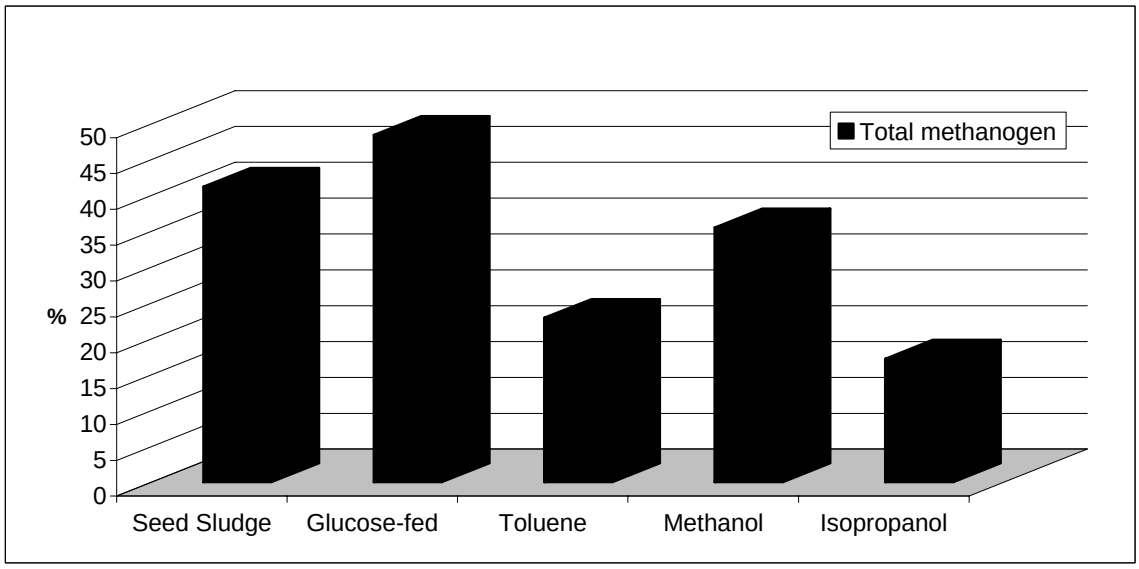


Figure 5.69. Comparison of standardized FISH results of the sludge taken from the ASBRs fed with solvent-containing synthetic wastewater.

When bacterial and Archaeal taxa identified by sequence analysis of excised bands from DGGE gel were analyzed, it can be said that microbial diversity changed significantly throughout the course of the experiment where the different wastewater compositions including organic solvents were fed to the anaerobic reactors. Consistent and reproducible changes in the relative intensity of bands representing different bacterial and Archaeal groups in the DGGE profiles strongly indicated that changes in the relative abundance of microbial composition occurred in response to the changing wastewater composition. When the reactor fed with solvents, a decrease and then an increase in the diversity was observed. According to bacterial DGGE fingerprinting data, number of OTUs in all reactor sludges increased until 4th sampling time then decreased slightly. Archaeal diversity of the reactors showed the same tendency (Figure 5.73).

Microbial diversity is more rapidly changing in bacterial community due to rapid life cycle of bacteria. As Figure 5.70 shows that different sampling times as well as different reactors differ from each other by presence and absence of bacterial bands. The change in abundance of a specidic OTU is more remarkable in Archael DGGE gel pictures since more stable community structure of Archaea clearly shows changes in abundance of specific OTU which makes Pearson Coefficient correlation analysis a suitable analysis type for stable communities.

Bacterial communities of all reactors form distinctive clusters. Propanol and methanol-fed reactors clustered around at 55% in relation analysis with Dice coefficient as toluene-fed reactor clustered around 65%. In Pearson coefficient analysis two clusters were observed. One composed of from the samples taken from methanol-fed reactor and toluene-fed reactors. Other cluster composed of propanol-fed reactors. Bacterial community profile clustered at 40% in Dice correlation coefficient, as profile cluster at 50% in Pearson correlation efficient. More distinctive cluster formation observed in Dice correlation coefficient analysis indicates the diversity of reactors (OTU types) is unique to each reactor. Cluster formation and similarity among same reactors is more observable in Archaeal community profile. In both correlation analysis communities of reactors form distinctive clusters (around 80% in Dice correlation, around 92% in Pearson correlation). Although communities of reactors cluster at higher percentages, Archaeal community profiles clustered around same point in both analyses (around 75%). This result may show that relative abundance of similar OTUs is

suitable criterion for the changes in same community as OTU type presence gives more accurate information when different communities considered.

As sequence analysis clearly show that methanol-fed reactor differ from other reactors by having unique OTUs in both bacterial and archaeal communities. It shows that microbial communities in the sludge samples taken from methanol-fed reactor differed than other two solvents. One of the reasons of this could be different anaerobic degradation pathway of methanol into a variety of substances which can be used directly by microorganisms. In archeal sequence analysis several sequences only encountered in methanol-fed reactor or not present in other solvent-fed reactors. Transformation of methanol into different methylated compounds affecting archaeal diversity directly since methylated compounds can be used directly by methylotrophic methanogens of *Methanosarcinale*s group. Appearences of unique OTUs in archaeal DGGE gel clearly indicate this event. Also Archaeal DGGE gel show that a decrease of abundance of this OTU (Band 2) related to *Methanomicrobiales* group which may indicate presence of a strong solvent interacts directly with these groups since this group do not present in methanol-fed reactor where effect of solvent observed significantly.

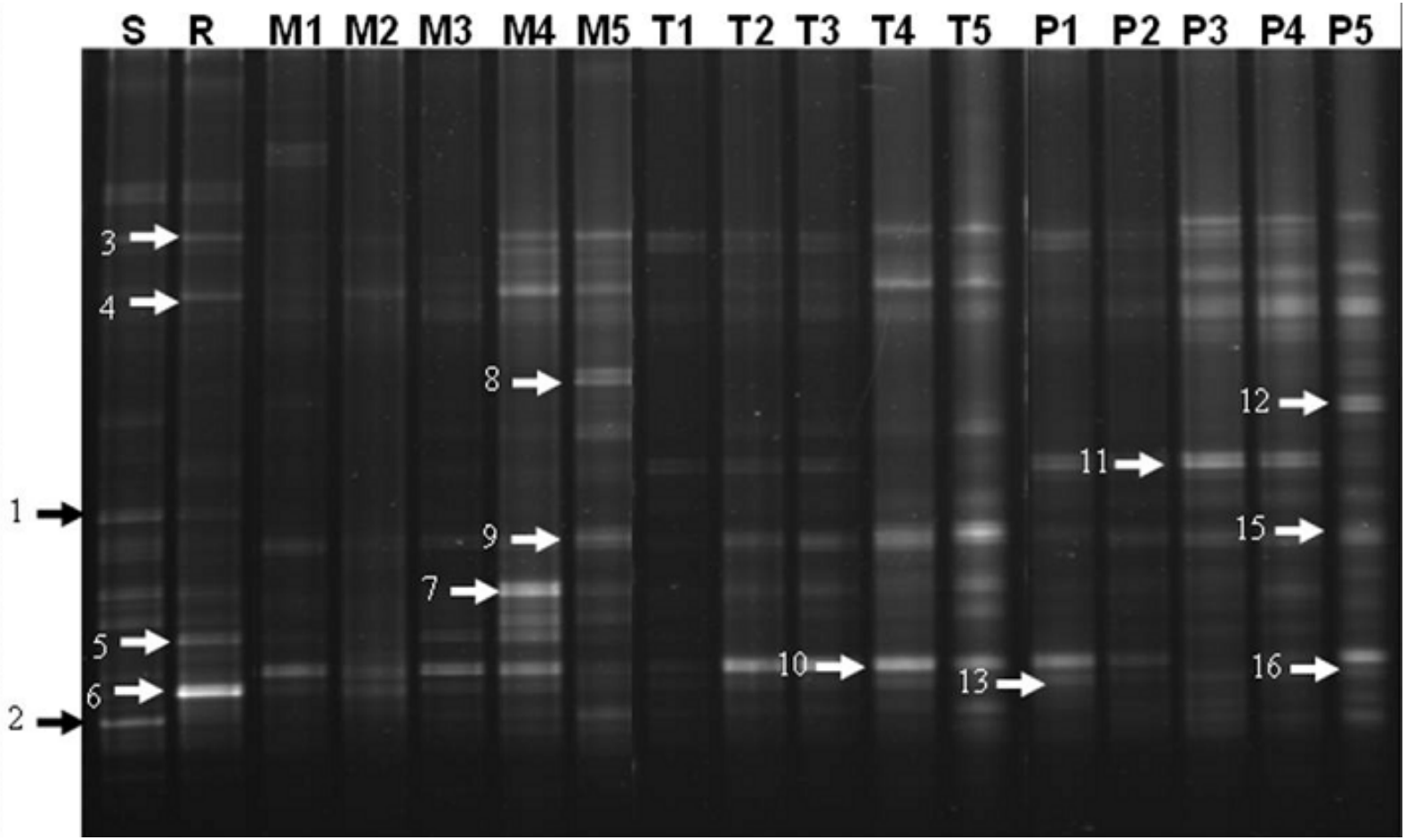


Figure 5.70. Analysis of bacterial community in the solvent-fed ASBRs by DGGE

Table 5.41. Identification of phylotypes from bacterial community of of the reactor fed with solvent-containing synthetic wastewater

Band no:	Closest relative	% sim.	Phylogeny	Metabolism	S	G-F	M1	M2	M3	M4	M5	T1	T2	T3	T4	T5	P1	P2	P3	P4	P5
1	*Dehalococcoides* sp. BHI80-15	88	Chloroflexi	H_2 Oxidation	X	X	0	0	0	0	0	0	0	0	X	X	0	X	X	X	X
2	*Syntrophobacter fumaroxidans* MPOB	85	Deltaproteo.	VFA oxidation	X	0	X	0	X	0	X	X	X	X	X	X	0	0	X	X	X
3	*Syntrophobacter sulfatireducens*	88	Deltaproteo.	VFA oxidation	X	X	X	X	0	X	X	X	X	X	X	X	0	0	X	X	X
4	*Syntrophus aciditrophicus* SB	83	Deltaproteo.	VFA oxidation	X	X	X	X	X	X	X	0	X	0	X	X	0	X	X	X	X
6-13-16	*Sulfurihydrogenibium* sp. YO3AOP1	88	Aquificae	H_2 oxidation sulfur compounds reduction	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	*Clostridium botulinum* E3 str. Alaska E43	95	Firmicutes	Fermentation	0	0	X	X	X	X	X	X	X	X	X	X	0	0	X	0	X
9-15	*Corynebacterium diphtheriae*	84	Actinobacteria	Fermentation	X	0	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	*Longilinea arvoryzae*	91	Chloroflexi	Protein and sugar degradation	0	0	X	X	X	X	X	X	X	X	X	X	X	X	0	X	X
11	*Acidaminococcus intestinalis*	87	Firmicutes	Amino acid degradation to VFA	0	X	0	0	0	X	X	X	X	X	0	X	X	X	X	X	X
12	*Desulfovibrio vulgaris*	78	Deltaproteo.	Sulfate reduction acetate formation	X	0	0	0	0	0	0	0	0	0	0	0	0	0	0	X	X
5	*Desulfonauticus submarinus*	85	Deltaproteo.	hydrogenotrophic, sulfate-reduction	0	X	X	X	X	X	X	0	0	0	0	0	0	0	0	0	0
Number of OTU's on DGGE gel					**15**	**9**	**9**	**9**	**9**	**13**	**15**	**8**	**9**	**10**	**14**	**12**	**8**	**9**	**10**	**14**	**12**

Table 5.42. Identification of phylotypes from bacterial community of of the reactors fed with solvents-containing synthetic wastewater (Environmental database)

Band no:	Closest match	% sim.	Isolation sources	Accession	S	G-F	M1	M2	M3	M4	M5	T1	T2	T3	T4	T5	P1	P2	P3	P4	P5
1	bacterium EU6	90	toluene-degrading methanogenic consortium bacterium	AB266990	X	X	0	0	0	0	0	0	0	0	X	X	0	X	X	X	X
2	clone B16	93	UASB bioreactor treating paper mill wastewater	AY426441	X	0	X	0	X	0	X	X	X	X	X	X	0	0	X	X	X
3	clone: QpjB112fl	97	mesophilic UASB sludge granules	AB266988	X	X	X	X	0	X	X	X	X	X	X	X	0	0	X	X	X
4	clone SHA-42	88	1,2-Dichlorpropan mixculture	AJ306771	X	X	X	X	X	X	X	0	X	0	X	X	0	X	X	X	X
6-13-16	clone: HgtB7fl.	98	mesophilic UASB sludge granules	AB266990	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	*Clostridia* DGGE gel band 9a	99	permafrost soil	EF034988	0	0	X	X	X	X	X	X	X	X	X	X	0	0	X	0	X
9-15	clone: YkB4fl.	87	mesophilic UASB sludge granules	AB267014	X	0	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	clone PEU-21	95	diethyl phthalate and phenol degrading UASB reactor	EF053078	0	0	X	X	X	X	X	X	X	X	X	X	X	X	0	X	X
11	*Clostridia bacterium* clone L33	99	leach bed reactor	EU888006	0	X	0	0	0	X	X	X	X	X	0	X	X	X	X	X	X
12	clone LM5-8	85	sulfate-reducing bioreactor	DQ443887	X	0	0	0	0	0	0	0	0	0	0	0	0	0	0	X	X
5	clone ZZ-S2F11	94	benzene-mineralizing consortium	EF613410	0	X	X	X	X	X	X	0	0	0	0	0	0	0	0	0	0
Number of OTU's on DGGE gel					15	9	9	9	9	13	15	8	9	10	14	12	8	9	10	14	12

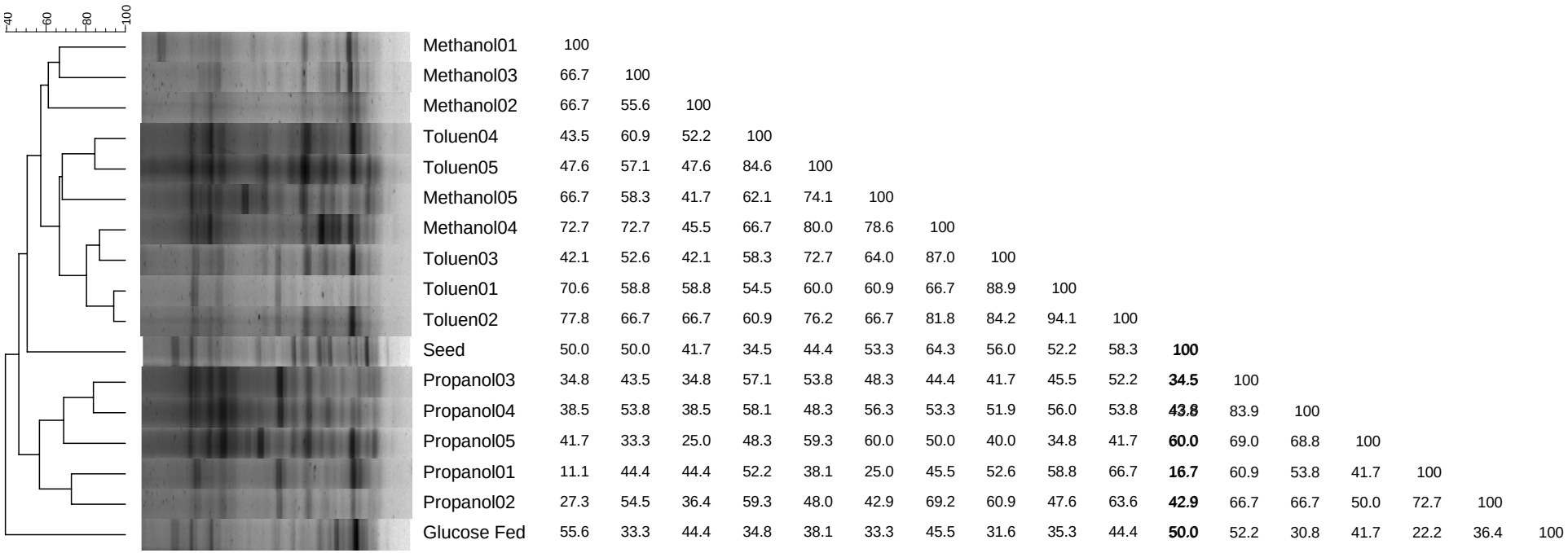


Figure 5.71. Phylogenetic tree constructed (based on Dice coefficient) from the Bacterial DGGE profiles from the reactor fed with solvent-containing synthetic wastewater.

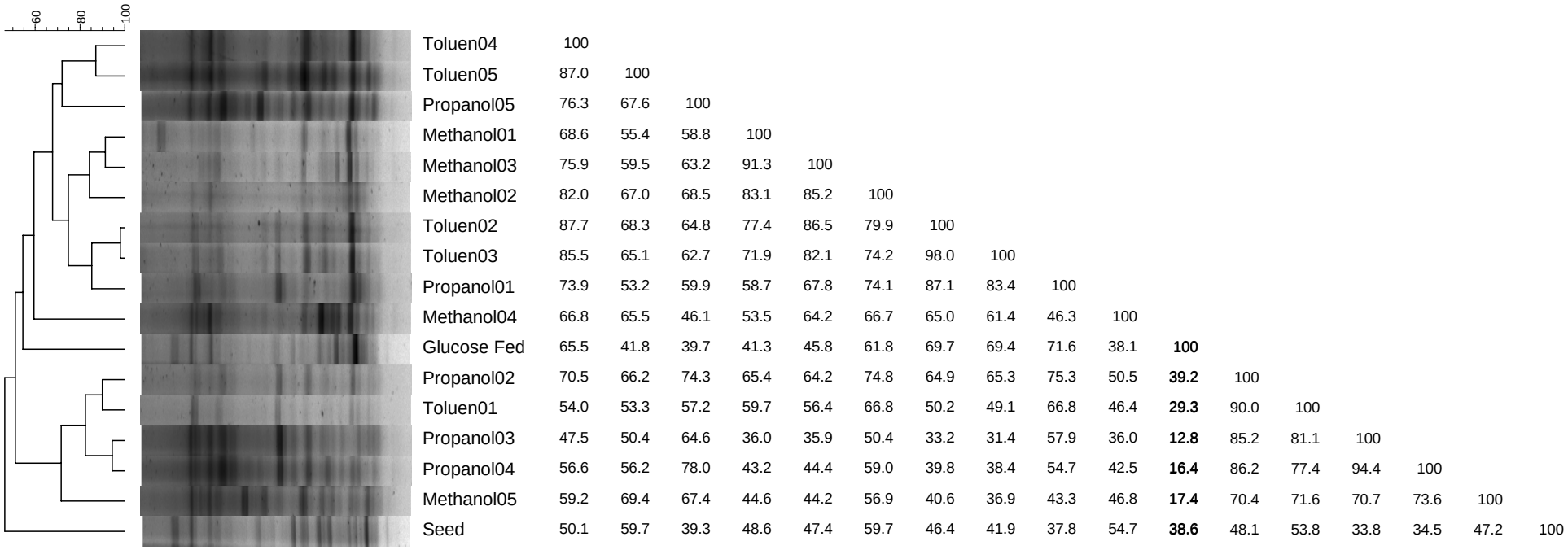


Figure 5.72. Phylogenetic tree constructed (based on Pearson coefficient) from the Bacterial DGGE Profiles from the reactor fed with solvent-containing synthetic wastewater.

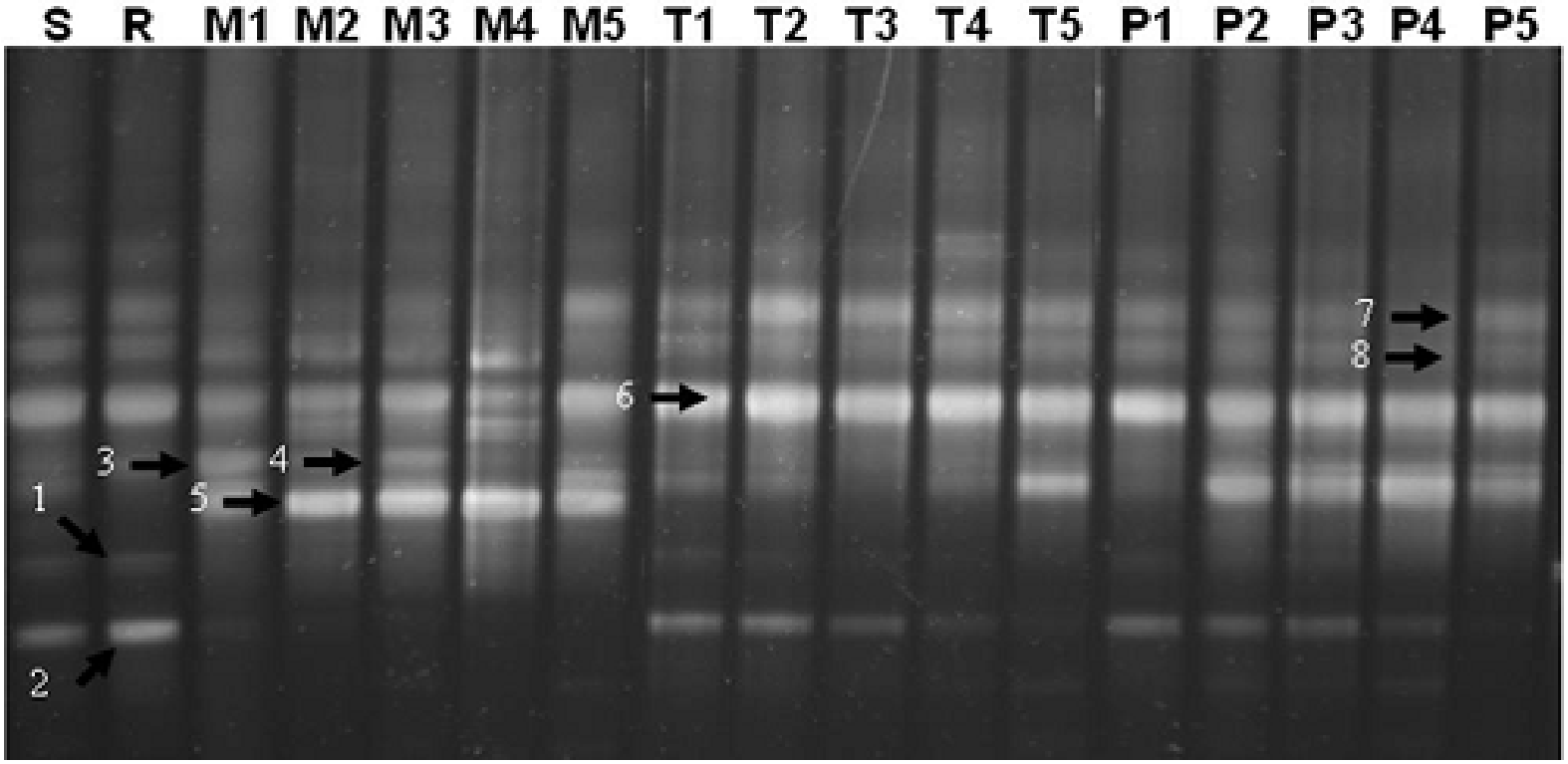


Figure 5.73. Analysis of archaeal community in the reactor fed with solvent-containing synthetic wastewater.

Table 5.43. Identification of phylotypes from archaeal community in the reactor fed with solvent-containing synthetic wastewater

Band no:	Closest relative	% sim.	Phylogeny	Metabolism	S	G-F	M1	M2	M3	M4	M5	T1	T2	T3	T4	T5	P1	P2	P3	P4	P5
1	*Methanogenic archaeon F1/B-2*	88	M.sarcinales	Acetate	X	X	0	0	0	0	0	X	0	0	0	0	X	0	0	0	0
2	*Candidatus Methanoregula boonei 6A8*	83	M.microbiales	H_2+CO_2-Formate	X	X	0	0	0	0	0	X	X	X	X	0	X	X	X	X	0
3-4	*Methanolobus sp. HigM*	83	M.sarcinales	Methylated c.	X	X	X	X	X	X	X	0	X	X	0	0	0	0	0	0	0
5	*Methanococcoides burtonii DSM 6242*	84	M.sarcinales	Methylated c.	0	0	X	X	X	X	X	0	0	0	0	0	0	0	0	0	0
6	*Methanosaeta concilii*	96	M.sarcinales	Acetate	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	*Methanolinea tarda*	88	M.microbiales	H_2+CO_2-Formate	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	*Methanomethylovorans sp. Z1*	92	M.sarcinales	Methylated c.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Number of OTU's on DGGE gel					**8**	**7**	**5**	**6**	**5**	**7**	**7**	**6**	**9**	**6**	**8**	**5**	**7**	**7**	**8**	**5**	**6**

Table 5.44. Closest matches of phylotypes from archaeal community in the reactor fed with solvent-containing synthetic wastewater (Environmental database)

Band no:	Closest Match	% sim.	Accession	Isolation source	S	G-F	M1	M2	M3	M4	M5	T1	T2	T3	T4	T5	P1	P2	P3	P4	P5
1	clone MCSArc_H3	92	EU591674	methanogenic reactor	X	X	0	0	0	0	0	X	0	0	0	0	X	0	0	0	0
2	uncultured archaeon TA03	84	AF229776	anaerobic granular sludge	X	X	0	0	0	0	0	X	X	X	X	0	X	X	X	X	0
3-4	U. Methanolobus clone E_C06	85	AY454755	estuary sediment	X	X	X	X	X	X	X	0	X	X	0	0	0	0	0	0	0
5	clone:KuA6.	82	AB077216	oil-contaminated groundwater	0	0	X	X	X	X	X	0	0	0	0	0	0	0	0	0	0
6	clone: UASB26.	96	AB329663	UASB granule	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	uncultured archaeon	93	AB195851	VFA degrading AB	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	clone KB-1 2	94	AY780565	chlorinated ethene-degrading culture	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Number of OTU's on DGGE gel					**8**	**7**	**5**	**6**	**5**	**7**	**7**	**6**	**9**	**6**	**8**	**5**	**7**	**7**	**8**	**5**	**6**

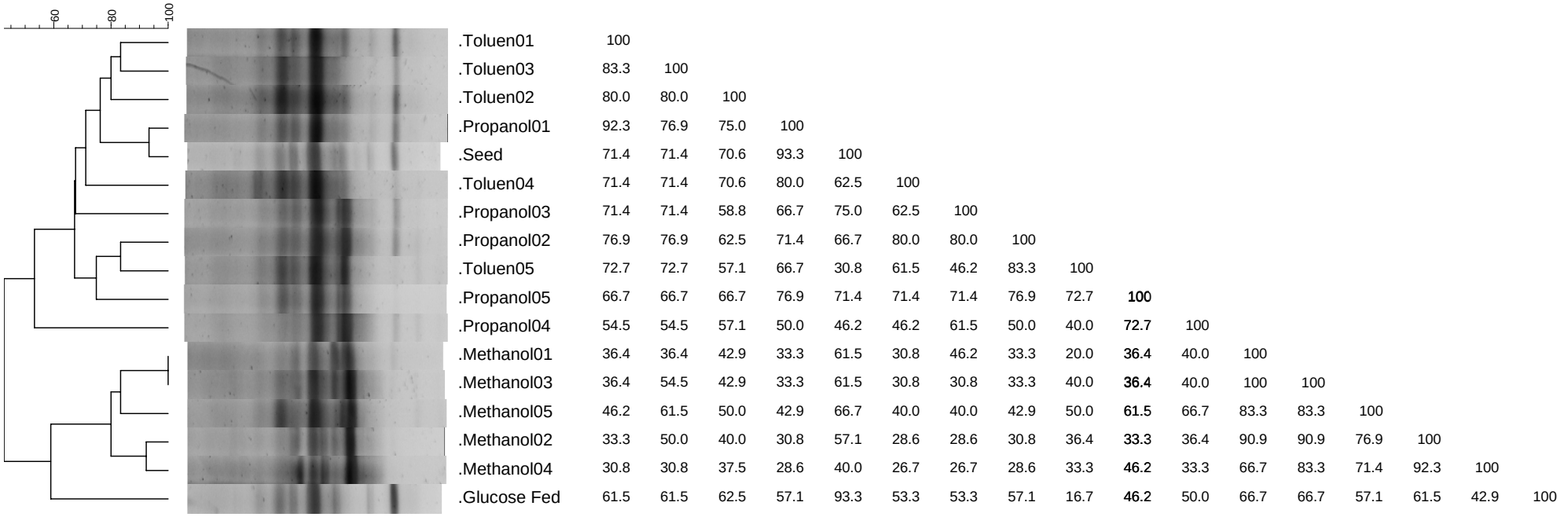


Figure 5.74. Phylogenetic tree constructed (based on Dice coefficient) from the Archaeal DGGE profiles in the reactors fed with solvent-containing synthetic wastewater.

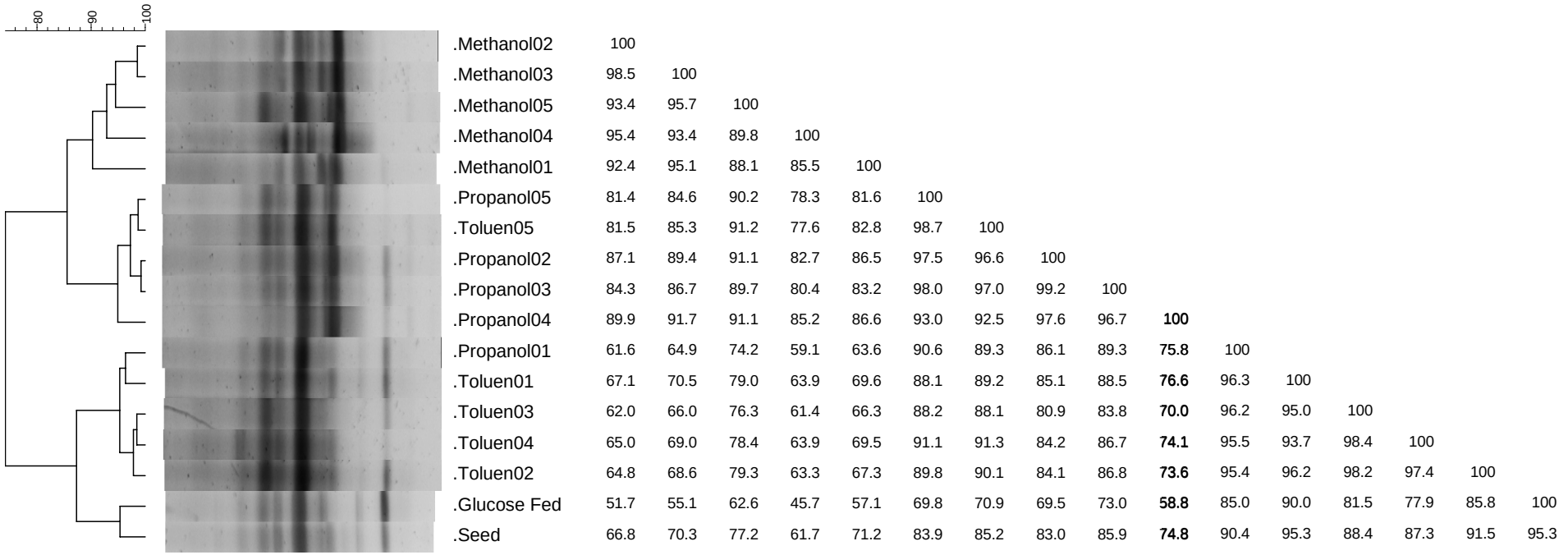


Figure 5.75. Phylogenetic tree constructed (based on Pearson coefficient) from the Archaeal DGGE Profiles in the reactors fed with solvent-containing synthetic wastewater.

6. CONCLUSIONS AND RECOMMENDATIONS

In the scope of this dissertation, anaerobic treatment of selected organic solvents including methanol, toluene and isopropanol on microbial diversity was investigated. A brief summary of the main findings is highlighted in the following sections:

- It has been shown that the anaerobic reactors inoculated with sludges having different quality and fed with same type of influent composition show significant variations in the performance even for a soluble glucose-based synthetic wastewater. Therefore, successful start-up and operation of anaerobic reactors requires a seed sludge with high methanogenic activity and a well-balanced microbial community including diverse bacterial species having different metabolic capability besides abundance of major methanogenic Archaeal groups.

- This study is the first report on the quantification of methanogenic *Archaea*, alfa-proteobacteria, beta-proteobacteria and gamma-proteobacteria using FISH in single phase and two phase anaerobic reactors. Findings realted to this section are:

  -FISH results showed that the phase separation has been succesfully maintained in the reactors (Acid reactor-44.2±2.6% Archaea, 56.5±2.0% eubacteria; Methane reactor- 88.7±1.4% Archaea, 28.1±4.2% bacteria; Single phase reactor- 57.1±0.8% Archaea, 49.6±1.1% eubacteria).

  -Gamma-proteobacteria was the predominant species compared to alfa and beta proteobacteria in the acid reactor whereas the relative ratio of the groups decreased significantly in the methane reactor.

  - In the acid reactor, dominancy of bacterial species over Archaeal methanogens was found. Abundance of acetoclastic *Methanosaeta* species in Archaeal population was also high (12.6% of the total community) in the sludge taken from the acid reactor.

- When effects of the selected organic solvents (IC_{50} concentration) on unacclimated anaerobic sludges taken from the reactors operated at different phases in single phase and two phase reactors have been evaluated, it has been found that

    - Vitality of the methanogens under methanol and isopropanol was higher in the sludge taken from the methane reactor compared to the single phase reactor. The situation was more evident for methanol.

    - Methanogens were not significantly affected by toluene in the sludge taken from the single phase reactor.

    - The most pronounced effect of the solvents tested on methanogenic *Archaea* in the sludge taken from the single phase reactor was caused by methanol and isopropanol.

    - Acetoclastic *Methanosaeta* were not significantly affected by toluene compared to the control reactor. However, *Methanosarcina* genus showed approximately 50% decrease in the toluene added SMA reactor compared to the control reactor.

    - *Methanobacteriales* were resistant to the solvents and remained as the major hydrogenotrophic methanogens.

- When the results of effects of selected solvents on methanogenic activity test and FISH studies for the sludges taken from single-phase and methane reactor are investigated, it can be seen that acetoclastic and methanogenic activity did not show a significant change for the sludges taken from different phases whereas FISH results of methanogens showed significant variations for the selected solvents. Vitality of the methanogens under IC_{50} concentrations of methanol and isopropanol was higher in the sludge taken from the methane reactor compared to the single phase reactor. The situation was more evident for methanol. On the other hand, methanogens showed a better resistance to toluene in the sludge taken from the single phase reactor.

- In the ASBR fed with toluene containing synthetic wastewater, the percentage of active population in the reactor decreased over the period of operation. At the beginning, the decrease in the active population did not reflect on the reactor performance. However, reactor performance failed following the decrease of the active population towards the end of the operation.

  - DGGE results showed that, the Archaeal community of the reactor at the end of the operation period with toluene was dominated by *Methanosaeta concilii* and *Methanomethylovorans* belonging to the *Methanosarcinales* group and *Methanolineatarda* belonging to the *Methanomicrobiales* whereas Methanogenic archaeon F1/B-2 (*Methanosarcinales*), *Methanoregula boonei* (*Methanomicrobiales*), *Methanolobus* spp. HigM (*Methanosarcinales*) were eliminated from the reactor.

  - FISH results confirmed the numerical dominance of the genus *Methanosaeta* over other methanogens in the anaerobic reactor exposed to toluene.

  - Although *Methanomicrobiales* were detected by DGGE, FISH studies showed that these species completely lost their activity in the ASBR fed with toluene.

- At the end of the operation with methanol, dominant species in the reactor sludge were *Methanosaeta* (17,6%), *Methanobacteriales* (9,2%), *Methanosarcina* (6,0%), respectively. *Methanosarcina* were also not affected from methanol.

    - The most affected methanogen in the sludge fed with methanol was *Methanomicrobiales*.

- At the end of the operation period with propanol, the most dominant methanogen was *Methanosaeata* among other methanogens in the ASBR. However, it should be mentioned that, the relative amount of the group decreased almost to half compared to the glucose-fed reactor. The most affected hydrogenotrophic methanogen from isopropanol was *Methanomicrobiales.*

Recommendations

In the view of the findings of this study, the following recommendations are made for future studies:

1. Elucidation of the anaerobic bioconversion routes of the solvents used in the present study may require the use of labelled substrates. To determine the metabolic relationship between the various intermediates, to distinguish between major and minor pathways of degradation, and to identify possible dead-end metabolites, the accumulation of label in the various compounds during isotope trapping experiments must be determined as a function of time.

2. The study supported the idea that both bacterial diversity and as important as methanogenic archaeal diversity are important in anaerobic reactors to maintain a stable reactor performance. It can be of interest to study bacterial population dynamics in anaerobic reactors consisting of different wastewaters using an integrative approach utilizing complementary molecular technologies like FISH-MAR or SIP combined with Q-PCR will be useful to find out their in situ functions.

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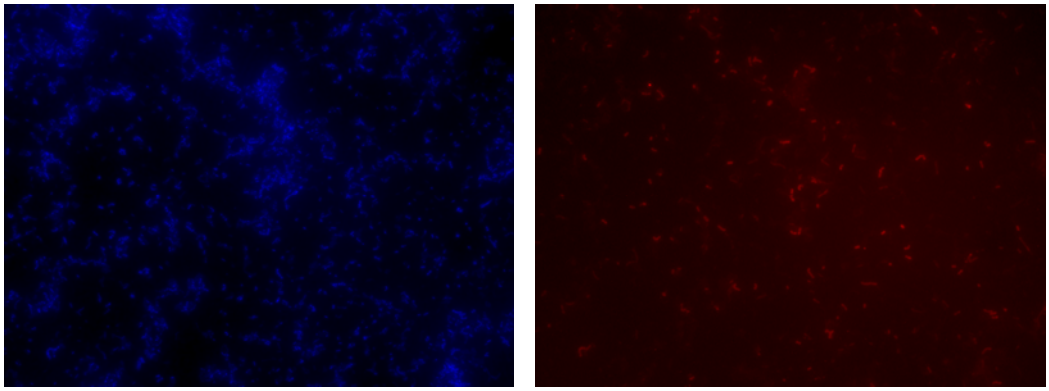
European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/ nucleotide.html)

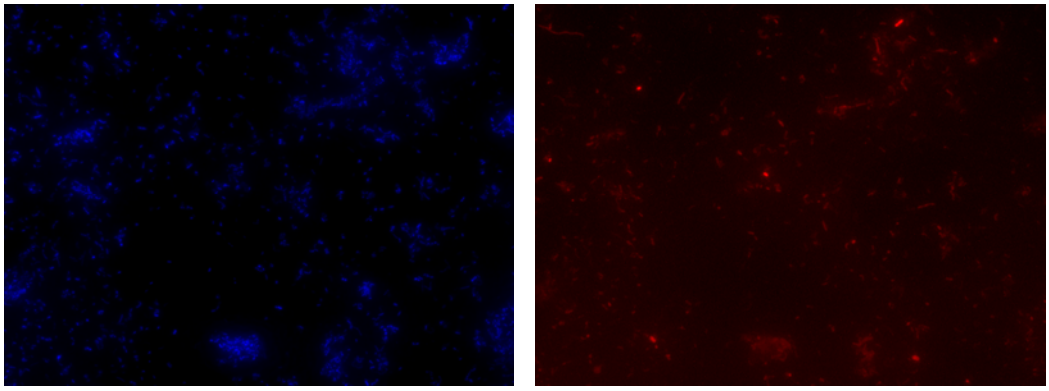
APPENDIX A

Selected FISH images belonging to Alfa, Beta, Gamma bacteria in the acid reactor

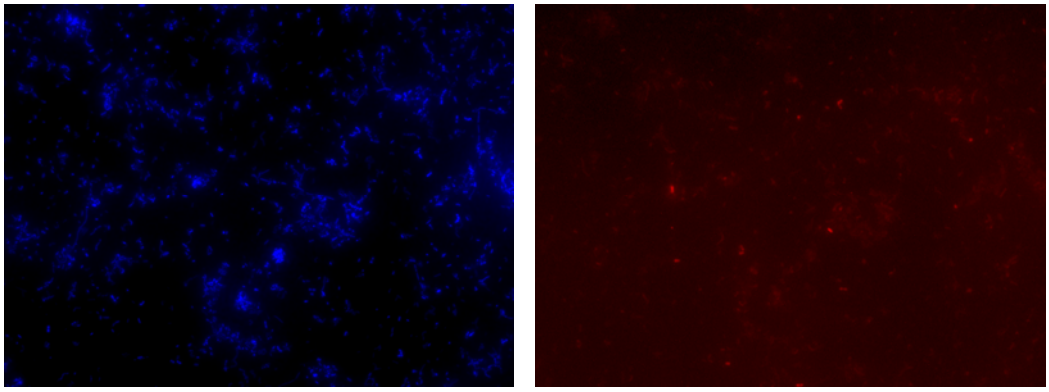
ALFA



BETA

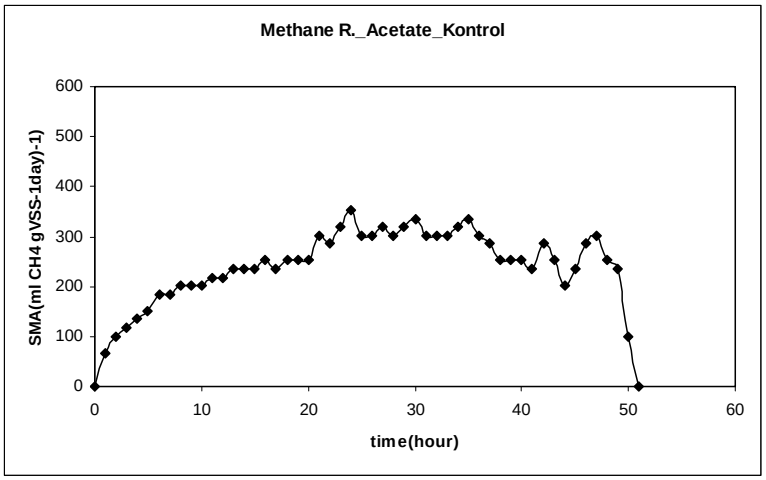


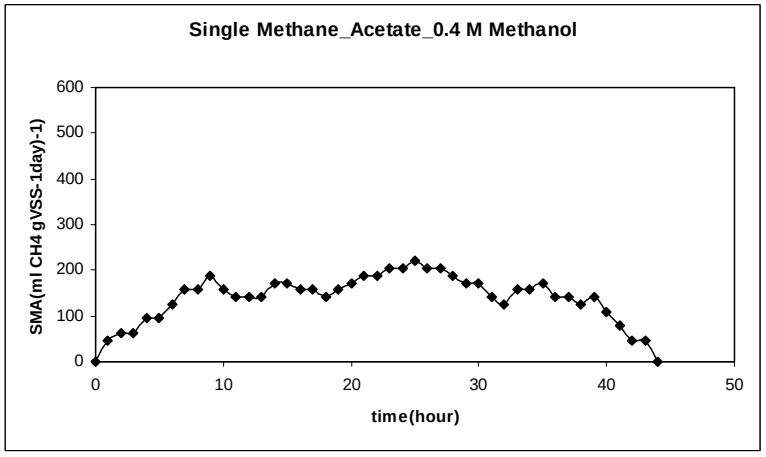
GAMMA

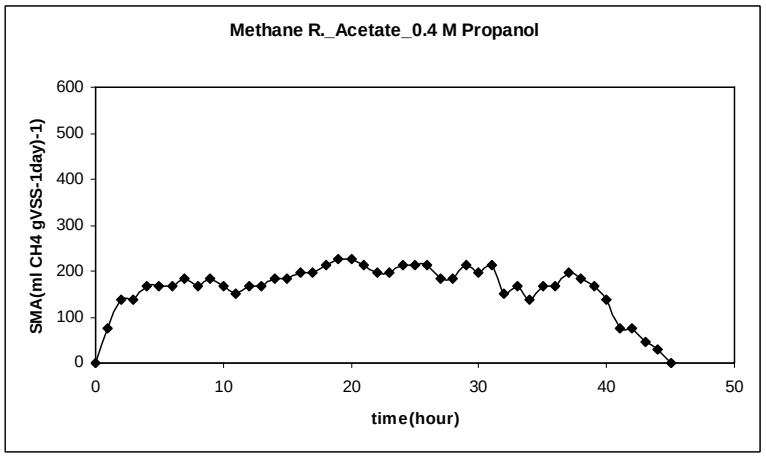


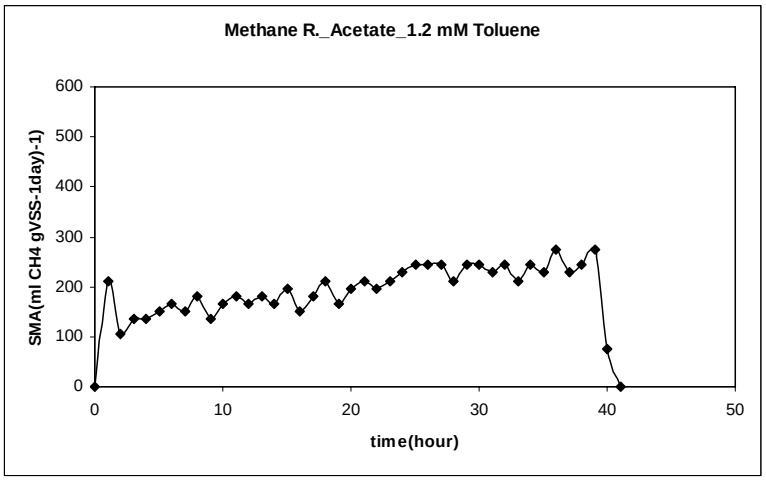
APPENDIX B

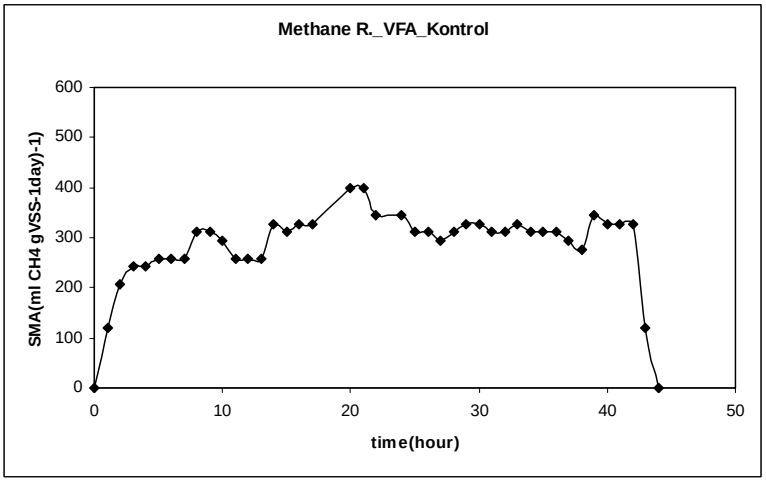
Effect of Selected Solvents on the Activity of Sludge taken from Methane Reactor

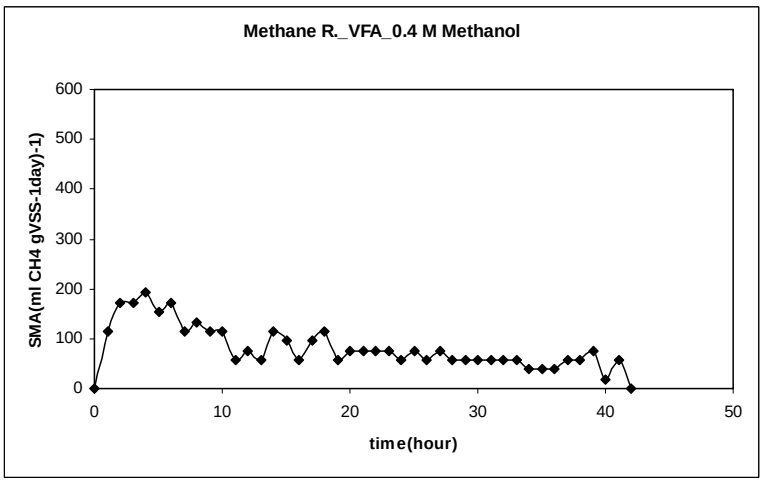


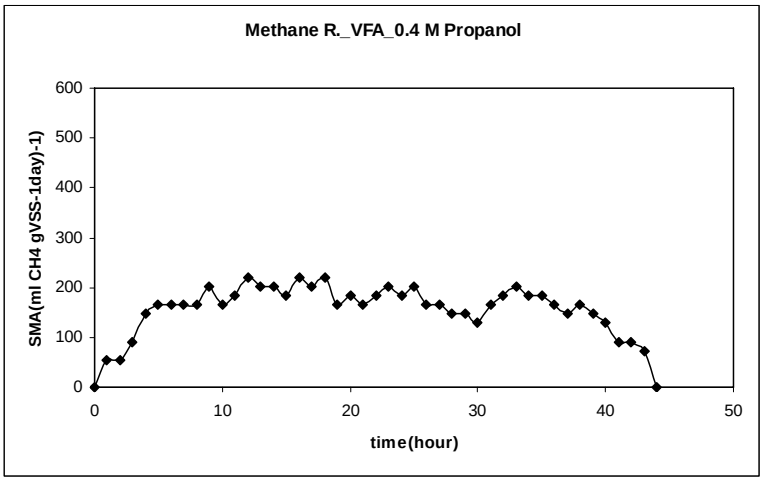


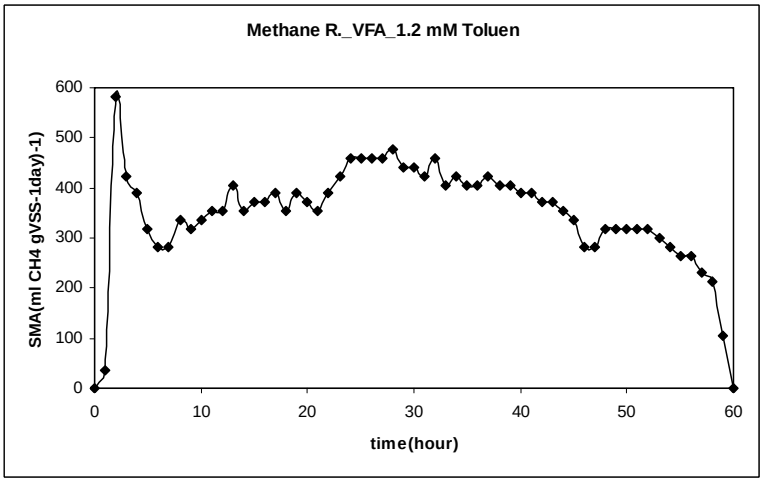



Methane R._Acetate_1.2 mM Toluene
SMA(ml CH4 gVSS-1day)-1)
0
100
200
300
400
500
600
0
10
20
30
40
50
time(hour)


Methane R._VFA_Kontrol
SMA(ml CH4 gVSS-1day)-1)
0
100
200
300
400
500
600
0
10
20
30
40
50
time(hour)

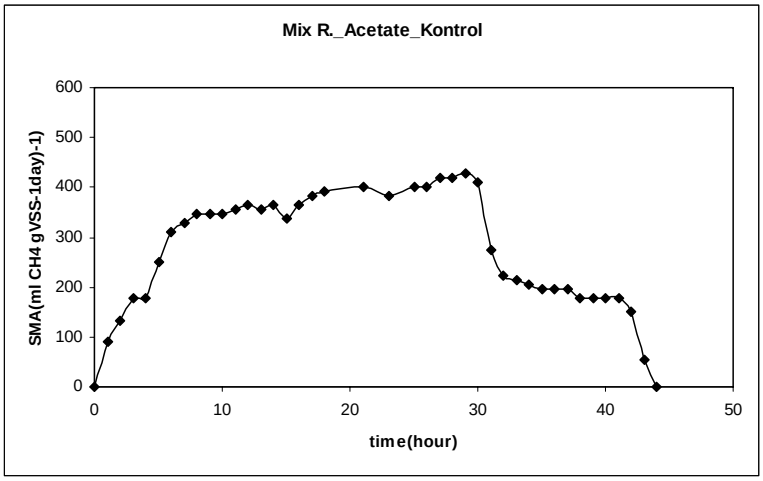

Methane R._VFA_0.4 M Methanol
SMA(ml CH4 gVSS-1day)-1)
0
100
200
300
400
500
600
0
10
20
30
40
50
time(hour)

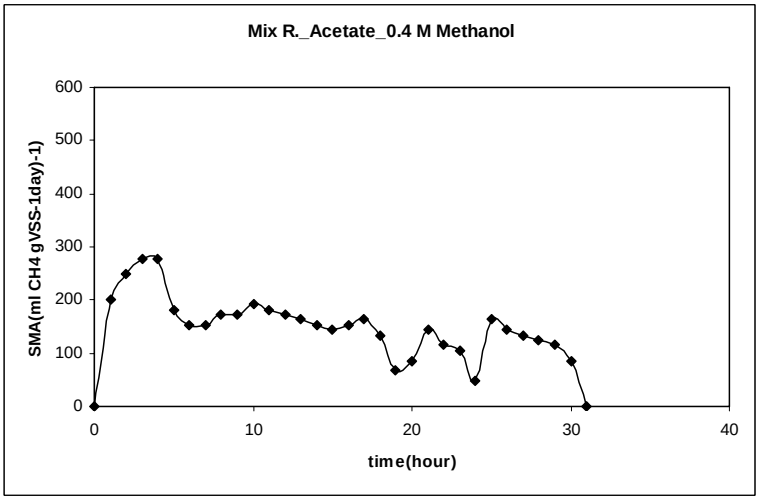

Methane R._VFA_0.4 M Propanol
SMA(ml CH4 gVSS-1day)-1)
0
100
200
300
400
500
600
0
10
20
30
40
50
time(hour)

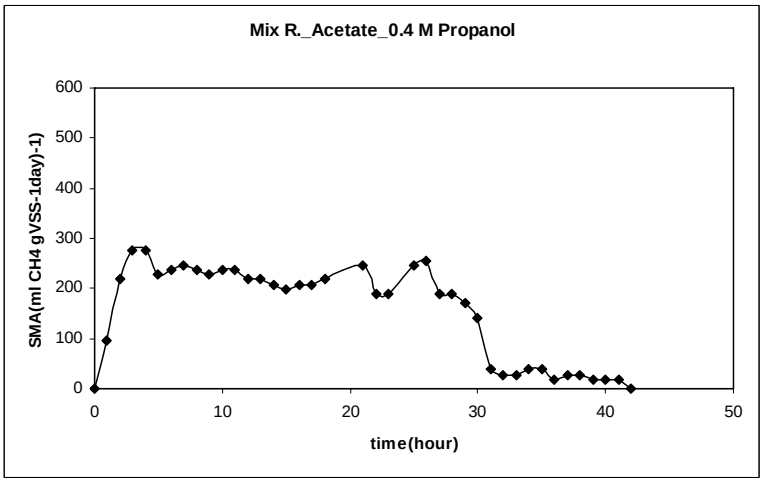

Methane R._VFA_1.2 mM Toluen
SMA(ml CH4 gVSS-1day)-1)
0
100
200
300
400
500
600
0
10
20
30
40
50
60
time(hour)

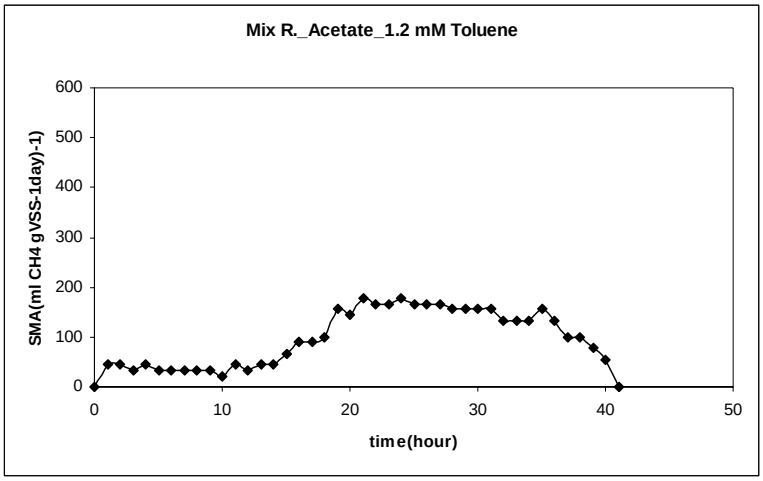
APPENDIX C

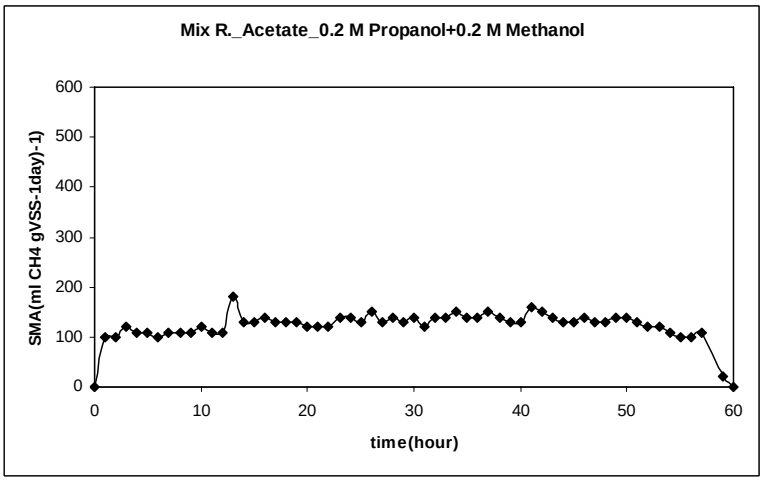
Effect of Selected Solvents on the Activity of Sludge taken from the Single Phase Reactor

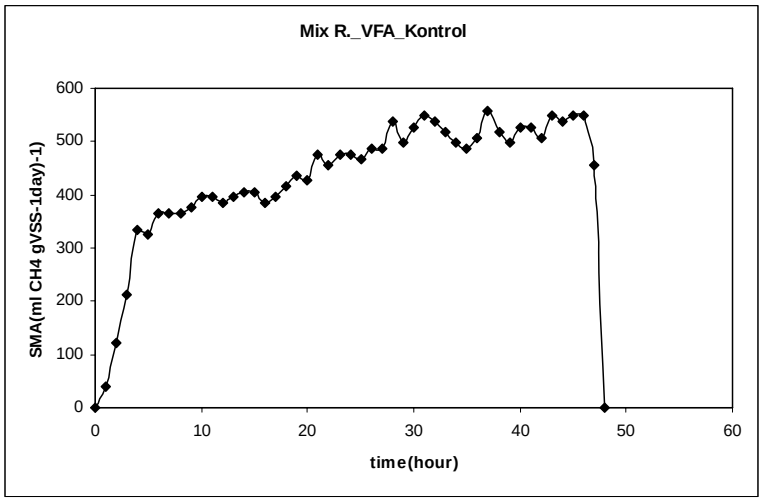


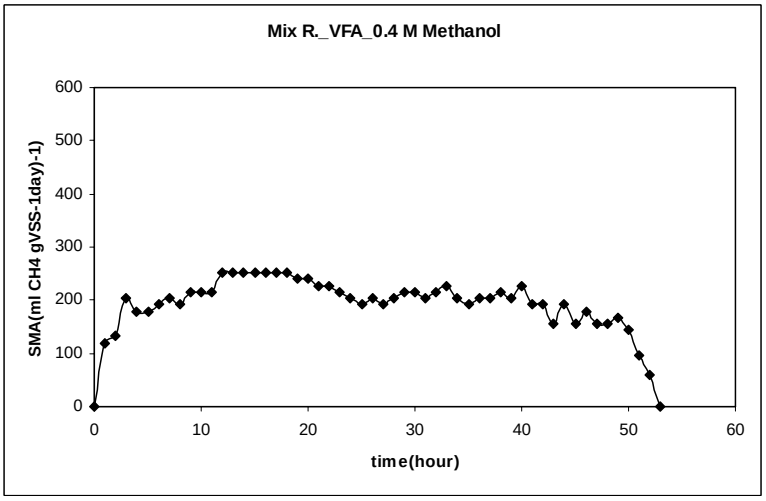


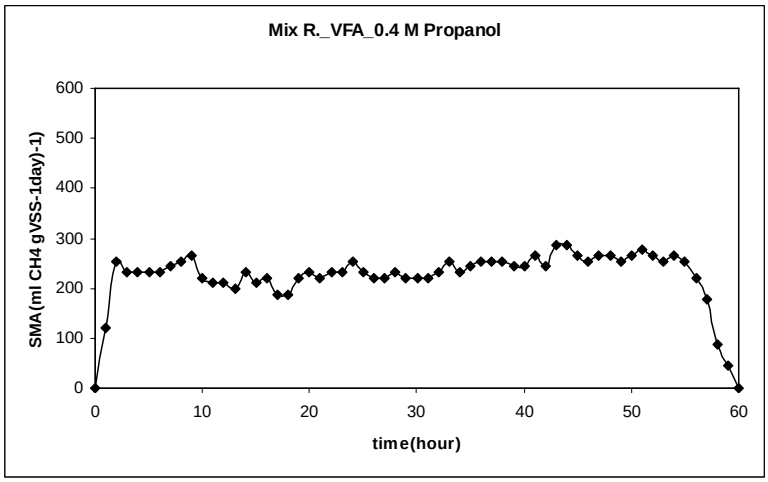


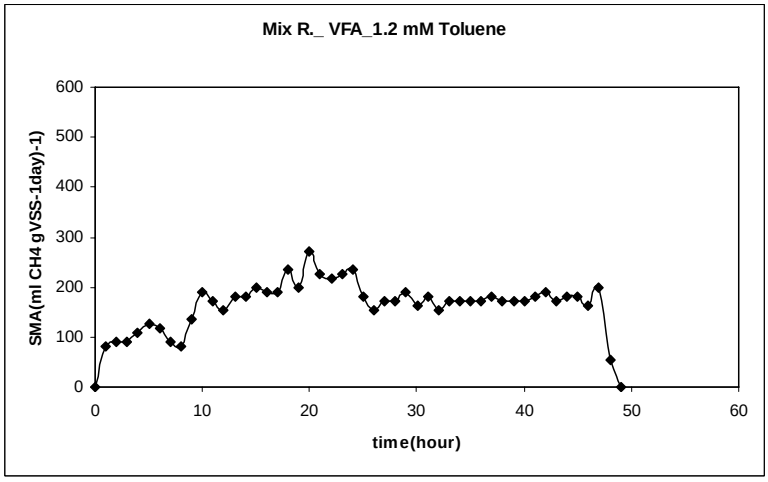

Mix R._Acetate_1.2 mM Toluene
SMA(ml CH4 gVSS-1day)-1)
0
100
200
300
400
500
600
0
10
20
30
40
50
time(hour)

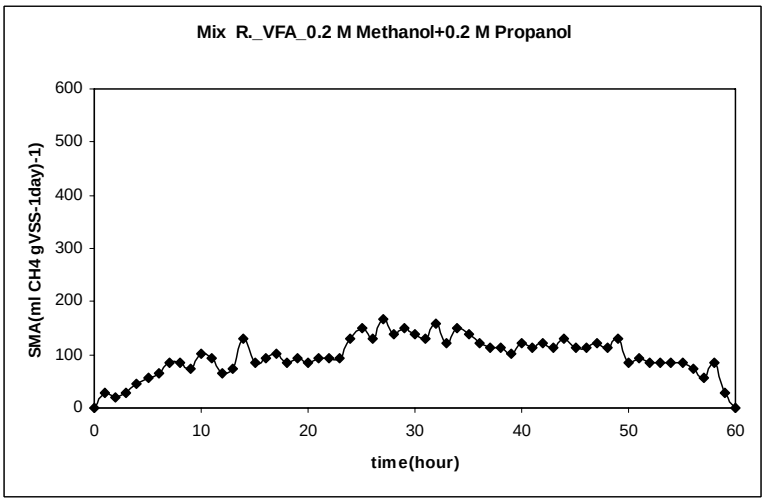

Mix R._Acetate_0.2 M Propanol+0.2 M Methanol
SMA(ml CH4 gVSS-1day)-1)
0
100
200
300
400
500
600
0
10
20
30
40
50
60
time(hour)


Mix R._VFA_Kontrol
SMA(ml CH4 gVSS-1day)-1)
0
100
200
300
400
500
600
0
10
20
30
40
50
60
time(hour)


Mix R._VFA_0.4 M Methanol
SMA(ml CH4 gVSS-1day)-1)
0
100
200
300
400
500
600
0
10
20
30
40
50
60
time(hour)

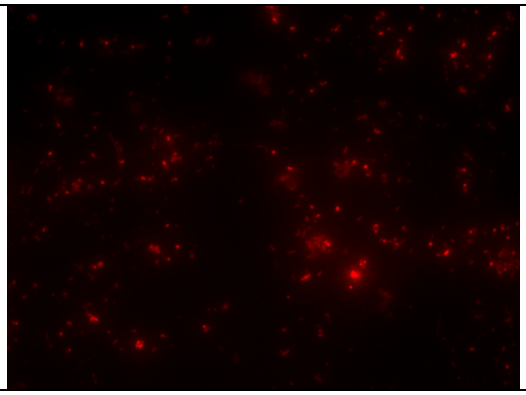
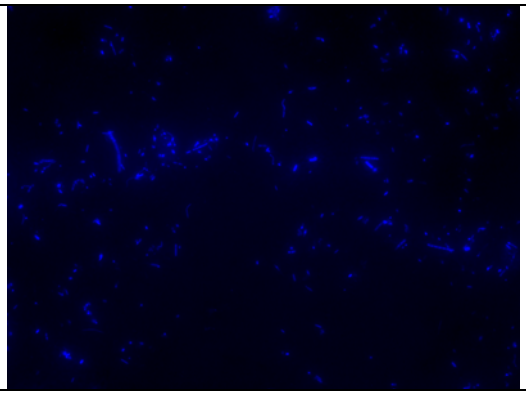
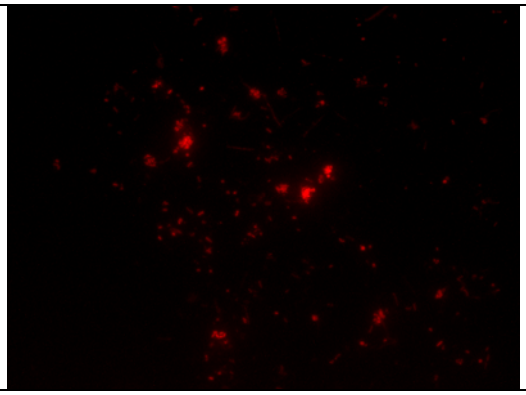
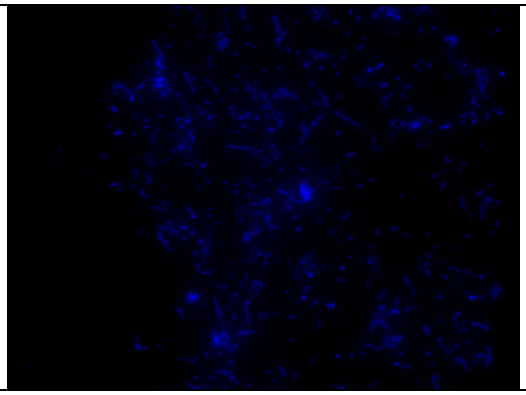
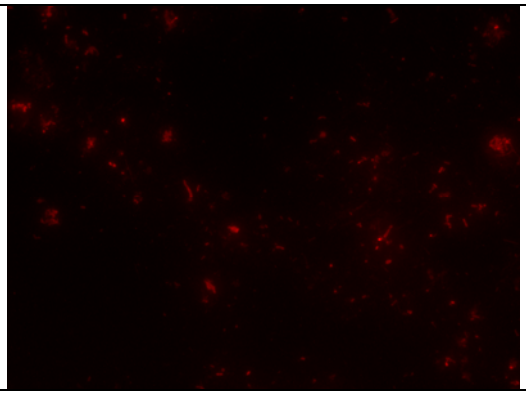
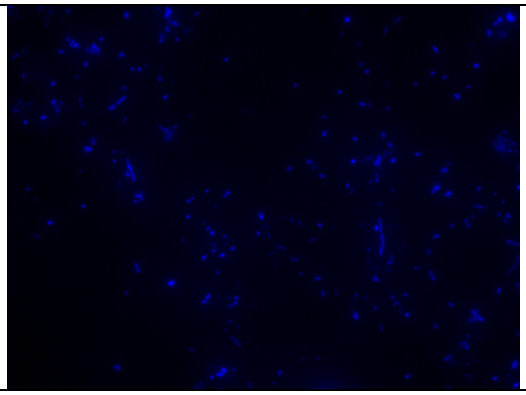

Mix R._VFA_0.4 M Propanol
SMA(ml CH4 gVSS-1day)-1)
600
500
400
300
200
100
0
0
10
20
30
40
50
60
time(hour)

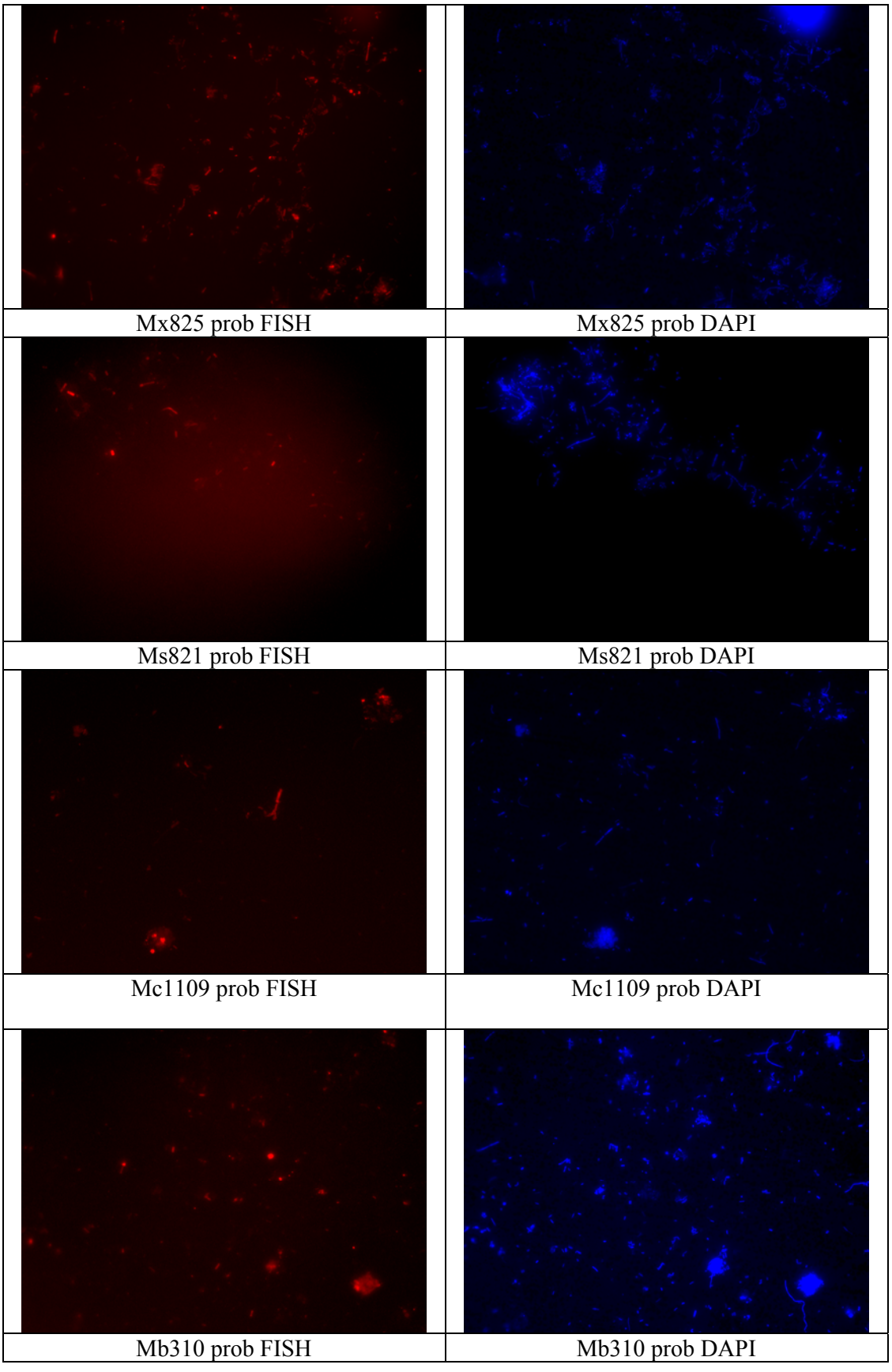

Mix R._ VFA_1.2 mM Toluene
SMA(ml CH4 gVSS-1day)-1)
600
500
400
300
200
100
0
0
10
20
30
40
50
60
time(hour)


Mix R._VFA_0.2 M Methanol+0.2 M Propanol
SMA(ml CH4 gVSS-1day)-1)
600
500
400
300
200
100
0
0
10
20
30
40
50
60
time(hour)

APPENDIX D

Selected FISH images from the ASBR reactor fed with toluene-containing synthetic wastewater

	
Universal prob FISH	Universal prob DAPI
	
Eubmix prob FISH	Eubmix probe DAPI
	
Arc915 prob FISH	Arc915 DAPI



Mx825 prob FISH | Mx825 prob DAPI

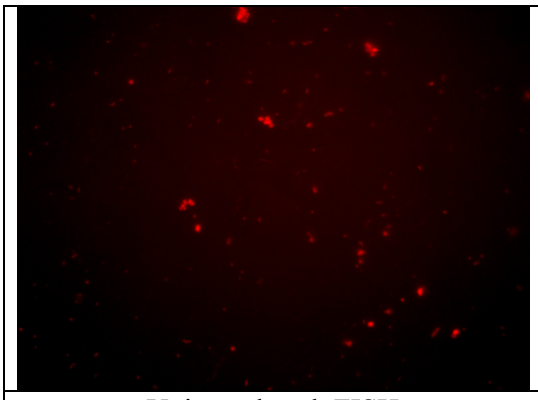
Ms821 prob FISH | Ms821 prob DAPI

Mc1109 prob FISH | Mc1109 prob DAPI

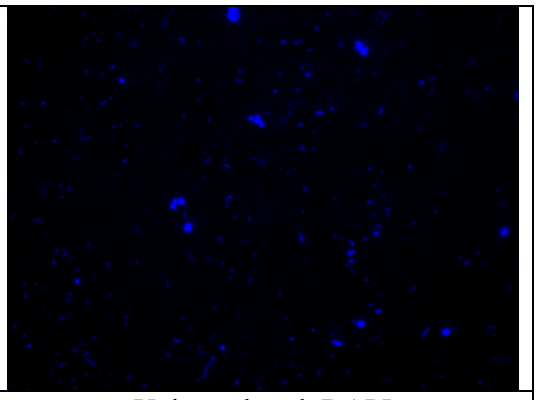
Mb310 prob FISH | Mb310 prob DAPI

APPENDIX E

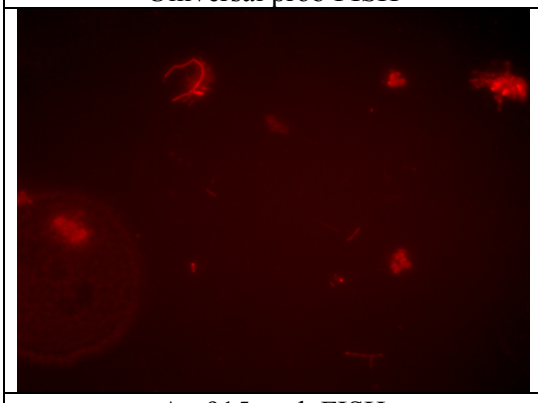
Selected FISH images from the ASBR reactor fed with methanol-containing synthetic wastewater



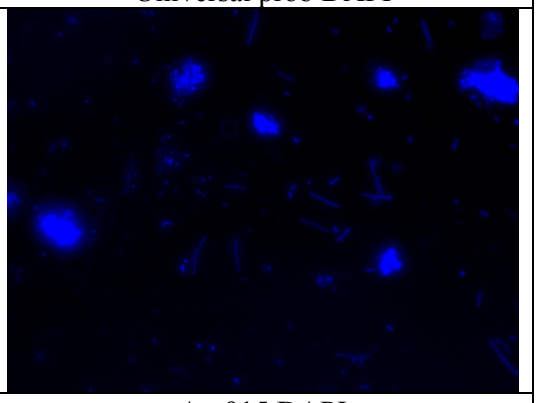
Universal prob FISH



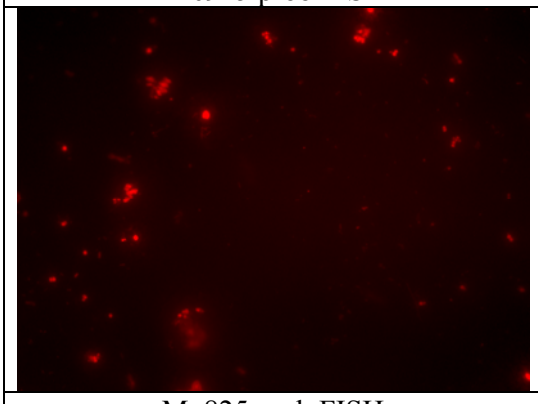
Universal prob DAPI



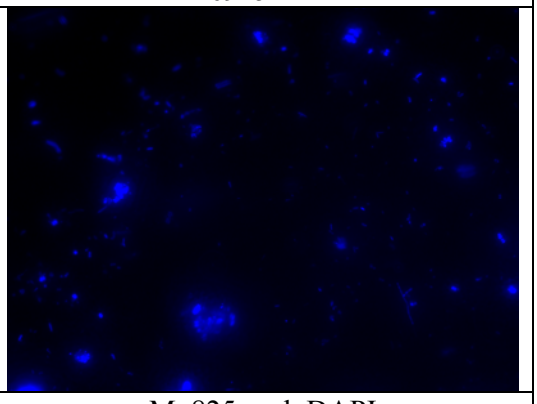
Arc915 prob FISH



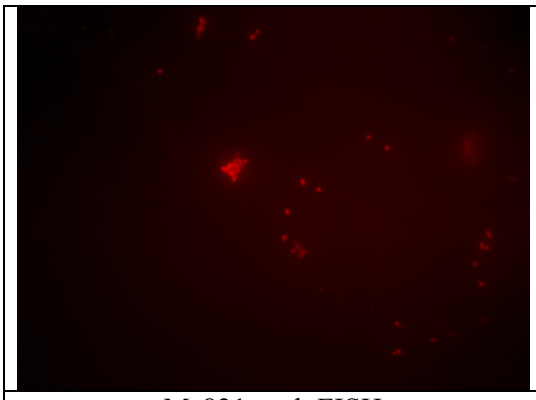
Arc915 DAPI



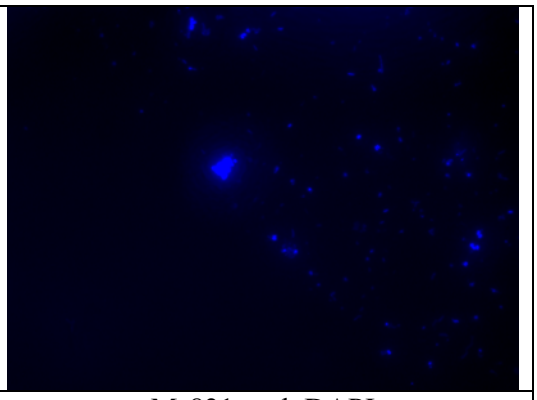
Mx825 prob FISH



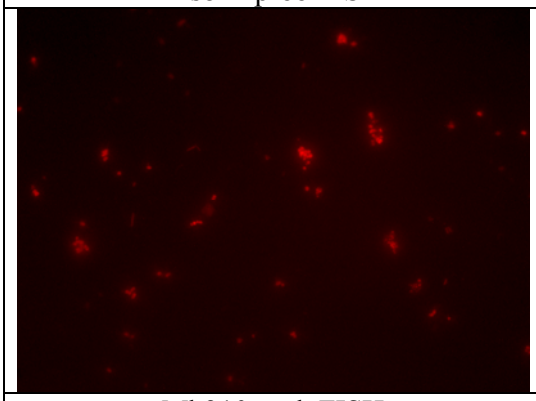
Mx825 prob DAPI



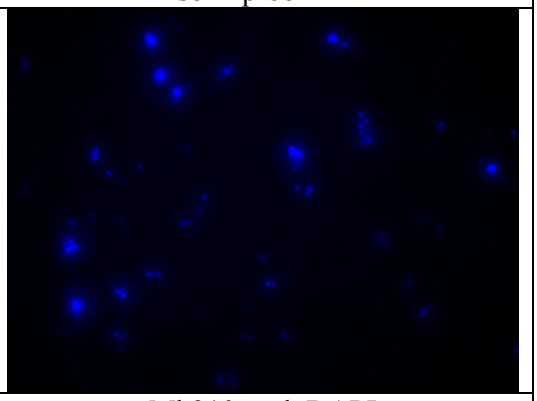
Ms821 prob FISH



Ms821 prob DAPI



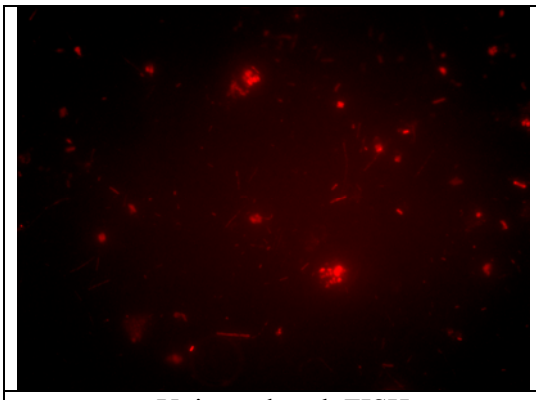
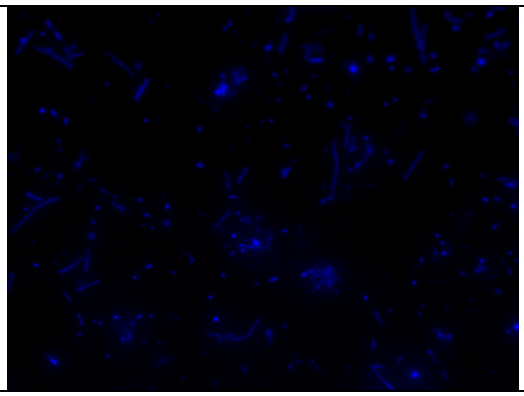
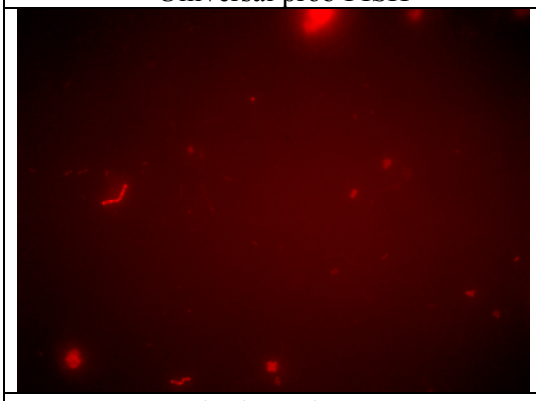
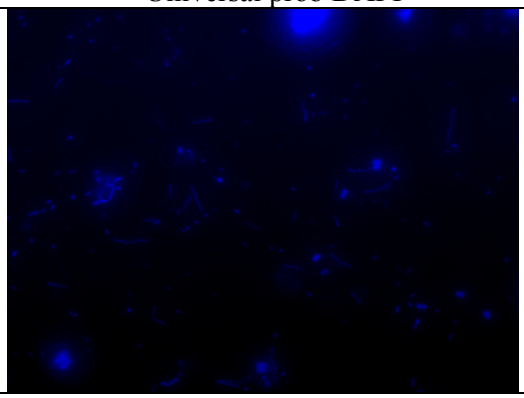
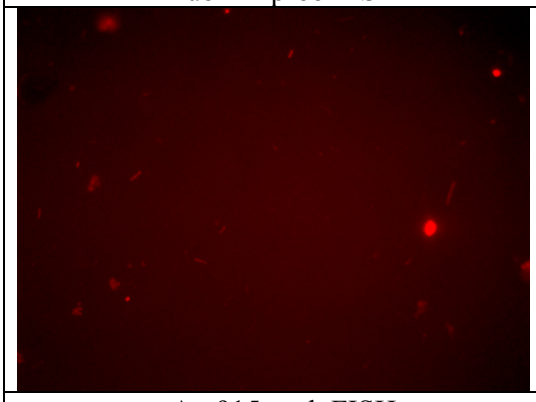
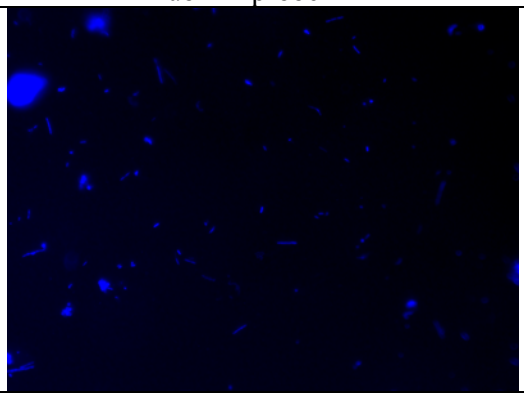
Mb310 prob FISH

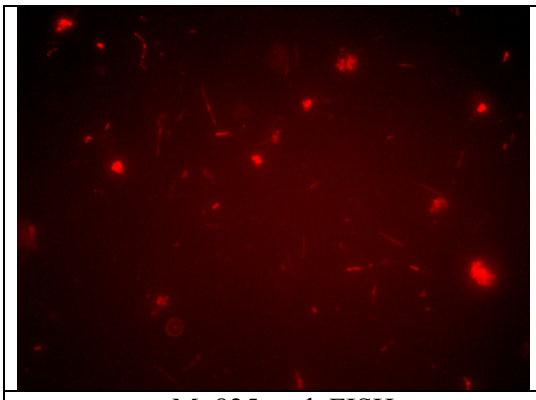


Mb310 prob DAPI

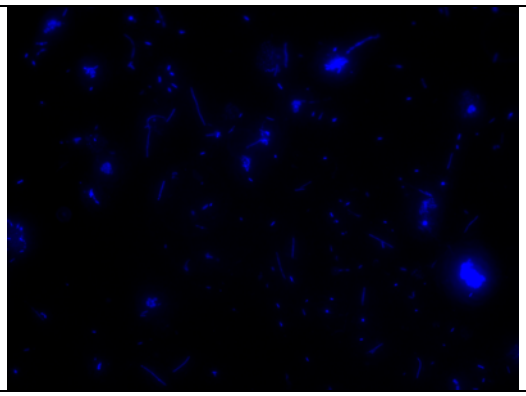
APPENDIX F

Selected FISH images from the ASBR reactor fed with isopropanol-containing synthetic wastewater

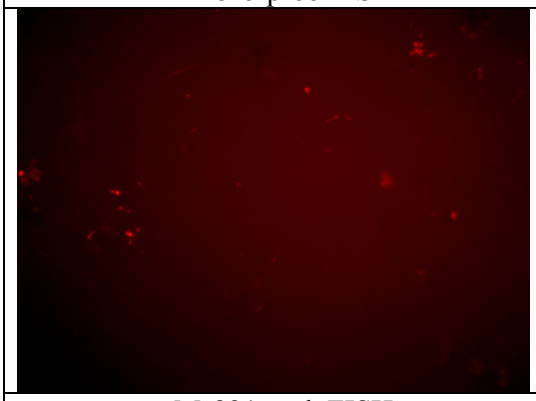
	
Universal prob FISH	Universal prob DAPI
	
Eubmix prob FISH	Eubmix probe DAPI
	
Arc915 prob FISH	Arc915 DAPI



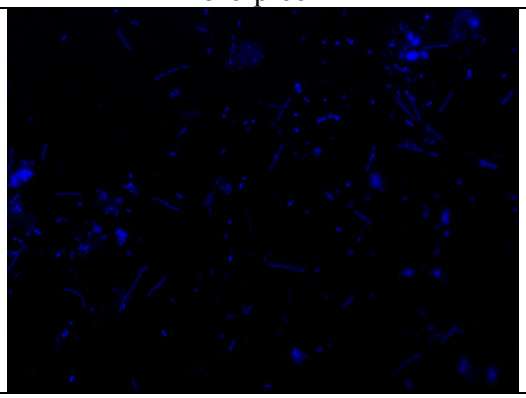
Mx825 prob FISH



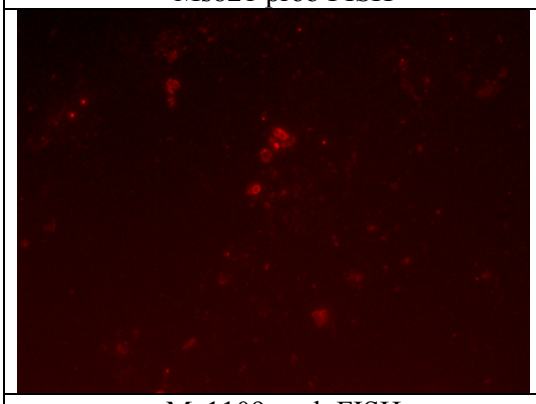
Mx825 prob DAPI



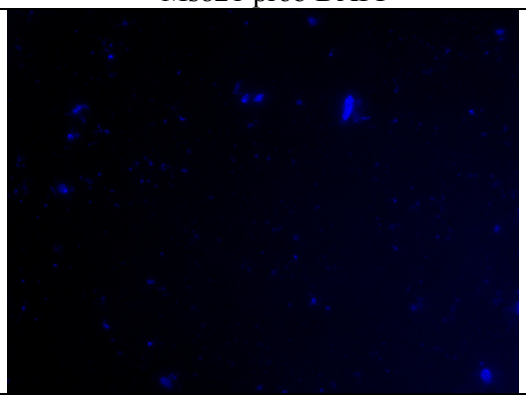
Ms821 prob FISH



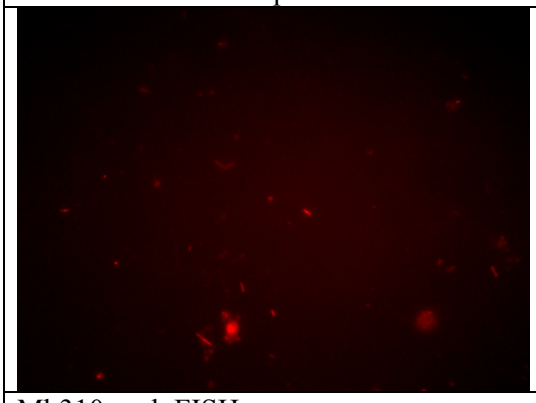
Ms821 prob DAPI



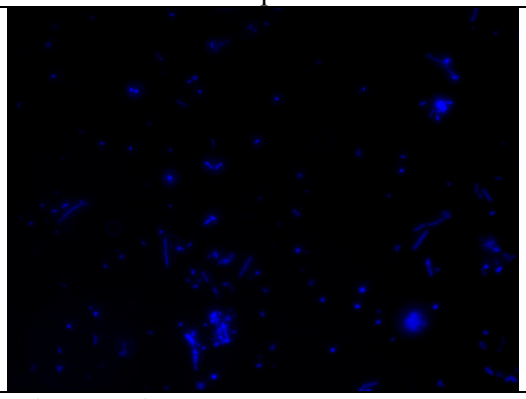
Mc1109 prob FISH



Mc1109 prob DAPI



Mb310 prob FISH



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