EVALUATION OF METHANOL EFFECT ON AN ANAEROBIC SLUDGE USING METHANOGENIC ACTIVITY MEASUREMENTS AND FLUORESCENT IN SITU HYBRIDIZATION

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in memory of Tarık Gözdereliler...

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ABSTRACT

Many kinds of solvents are discharged from industries such as refineries, paint and pharmaceutical manufacturers etc. Hundreds of organic and inorganic raw materials and many organic solvents used to dissolve the compounds are required in these processes. The most commonly used solvents are methanol, ethanol, acetone, and isopropanol. Moreover, methylene chloride, toluene, chloroform, chlorobenzene, chloromethane, cyanide, phenol, and benzene are also used. Defining effect of solvents on both microbial community structure and activity changes in anaerobic reactors can lead to improvements in the understanding of interactions in the bioreactors, thereby obtaining better reactor performance in terms of higher degradation capacity and biogas production.

In this study, a lab-scale anaerobic batch reactor inoculated with a seed sludge taken from a full-scale EGSB reactor was operated during 47 days as a preliminary-study. At the end of the operational period, sludge taken from the reactor was used to evaluate the effect of methanol on methanogenic activity and microbial community using SMA tests and FISH, respectively.

SMA test results showed that the maximum acetoclastic activity of methanol added sludge samples decreased from 437 mLCH4/gVSS.dto 252 mLCH4/gVSS.dcompared to control reactor sludge. In addition, maximum overall methanogenic activity was found to be 221 mLCH4/gVSS.din methanol added sludge samples, showing a 55% decrease when compared to control. According to FISH results, acetoclastic methanogens, *Methanosaeta* spp. and *Methanosarcina* spp. were found to be predominant methanogens in both sludges from the full-scale EGSB and lab-scale anaerobic batch reactors. However, predominance of acetoclastics methanogens tended to change to hydrogenotrophic methanogens, especially *Methanobacteriales* spp. under inhibitory effect of methanol. This shows that *Methanobacteriales* spp. is more resistant to stress conditions like inhibitory effect of a common organic solvent, i.e methanol.

ÖZET

Organik bazlı solventler ilaç, petrol, boya vb. endüstrilerin proseslerinde yoğun olarak kullanılmakta ve dolaysıyla atıksularında önemli miktarlarda bulunmaktadır. Başta kimyasal senteze dayalı ilaç endüstrisi olmak üzere, pek çok endüstride yüzlerce organik ve inorganik ham madde ve bu maddeleri çözmek için çok çeşitli solventler kullanılmaktadır. En yaygın olarak kullanılan solventler arasında metanol, etanol, aseton, ve isopropanol sayılabilir. Bunlara ek olarak, metilen klorür, toluen, kloroform, klorobenzen, siyanür, fenol ve benzen gibi solventler de bu proseslerde kullanılmaktadır Solventlerin mikrobiyal topluluğun yapısına ve aktivitesine etkilerinin tanımlanması ile solvent gideren anaerobik biyoreaktörlerdeki mikrobiyal etkileşimlerin anlaşılmasını ve bu reaktörlerden daha iyi performans ve biyogaz elde edilmesini mümkün kılabilecektir.

Bu çalışmada, tam ölçekli bir anaerobik EGSB reaktöründen alınan çamur ile aşılanan laboratuvar ölçekli ardışık kesikli bir anaerobik reaktör 47 gün boyunca işletilmiştir. Anaerobik reaktörden alınan çamurda sırasıyla SMA testleri ve FISH tekniği kullanılarak metanolün, metanojenik aktiviteye ve mikrobiyal kommuniteye inhibitör etkisi incelenmiştir.

Metanol etkisinin incelendiği SMA test sonuçları, kontrol reaktör çamuru örneğinin aktivitesi ile karşılaştırıldığında, maksimum asetoklastik metanojenik aktivitenin 437 $mLCH_4$/ gUAKM.gün'den 252 $mLCH_4$/ gUAKM.gün'e düştüğü gözlemlenmiştir. Ayrıca, maksimum toplam metanojenik aktivite değerleri göz önüne alındığında metanol eklenen çamur örneklerinin aktivitelerinde %55 düşüş gözlenmiştir. FISH sonuçlarına göre, *Methanosaeta* ve *Methanosarcina* türlerinin oluşturduğu asetoklastik metanojenler tam ölçekli anaerobik EGSB reaktör ve laboratuvar ölçekli ardışık kesikli reaktör çamurunda en baskın türler olarak belirlenmiştir. Fakat, metanolün inhibitör etkisi sonucunda asetoklastik metanojenlerden, hidrojenotrofik metanojenlere doğru bir kayma tespit edilmiştir. Özellikle *Methanobacteriales* türlerindeki artış, bu türlerin inhibitör varlığı gibi stres şartları altında daha dayanıklı olduklarını göstermektedir.

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Symbol	Explanation	Units used
AK	Acetate Kinase	
ASBR	Anaerobic Sequencing Batch Reactor	
AMP	Adenosine Monophosphate	
ATP	Adenosine Triphosphate	
COD	Chemical Oxygen Demand	mg/L
DNA	Deoxyribonucleic acid	
DGGE	Denaturating Gradient Gel Electrophoresis	
EDTA	Ethylenediaminetetraacetic Acid	
EGSB	Expanded Granular Sludge Bed	
FISH	Fluorescent in situ Hybridization	
HRT	Hydraulic Retention Time	hour
IC_{50}	Inhibitory concentration	mg/L
NRB	Nitrogen Reducing Bacteria	
PCR	Polymerase Chain Reaction	
PBS	Phosphate Buffer Solution	
PFA	Paraformaldehyde	
PTA	Phosphotransacetylase	
Q-PCR	Quantative Polymerase Chain Reaction	
RFLP	Restriction fragment length polymorphism	
RNA	Riboxynucleic Acid	
rRNA	Ribosomal RNA	
rDNA	Ribosomal DNA	
SMA	Specific Methanogenic Activity	mLCH4/gVSS.d
SRB	Sulphate Reducing Bacteria	
SRT	Sludge Retention Time	day
SS	Suspended Solids	mg/L

Symbol	Explanation	Units used
SSCP	Single Strand Conformation Polymorphism	
SO/XO	Substrate to Biomass Ratio	
TS	Total Solids	mg/L
TVS	Total Volatile Solids	mg/L
UASB	Upflow Anaerobic Sludge Blanket	
VFA	Volatile Fatty Acids	
VSS	Volatile Suspended Solids	mg/L
UAF	Upflow Anaerobic Filter	
CSTR	Continous Stirred Tank Reactor	

1. INTRODUCTION

Anaerobic treatment has been widely used especially for treatment of high strength industrial wastewaters such as pharmaceutical, pulp and paper, petrochemical, food and beverage alcohol distilleries, textile, dairy leachates, municipal wastewaters, solid wastes, agricultural wastes and manures. Anaerobic process has many benefits, including (1) reducing the quantity of solids to be land applied or landfilled, (2) decreasing the pathogen content and odor of the sludge and (3) producing methane gas which can be used as an alternative energy source. Although the general processes occuring in anaerobic biological wastewater treatment plants, such as hydrolysis, acidogenesis, acetogenesis, methanogenesis, are well understood, the complex microbial ecology of the biological sludge, symbiotic relationships and the effect of microbial diversity on performance of anaerobic digestion systems were poorly understood.

Inhibitory substances are one of the leading causes of anaerobic reactor failures since they are present in substantial consantrations in wastewaters and sludges. A wide range of inhibitors is responsible for the upset of anaerobic reactor systems. Organic solvent containing inhibitory wastewaters may affect activity and composition of methanogens, since the most sensitive step to inhibitory substanes through anaerobic digestion process is the methanogenesis.

Defining effect of organic solvents on both microbial community structure and activity changes can lead to improvements in the understanding of bioreactors treating wastewaters containing organic solvents, thereby obtaining better reactor performance in terms of higher degradation capacity with higher biogas production. Therefore, in this study, the inhibitory effect of methanol, which is an organic solvent and a main pollutant in some specific wastewaters, was evaluated in terms of specific methanogenic activity and microbial population dynamics using specific methanogenic activity test and fluorescent in situ hybridization studies, respectively.

2. FUNDAMENTALS OF ANAEROBIC DIGESTION

2.1. Anaerobic Treatment Process

Anaerobic wastewater treatment is considered the most cost-effective solution for organically polluted industrial waste streams (van Lier et al., 2001), and has gained interest due to increasing energy prices and more stringent legislation for the discharge of industrial wastewater, since 1970's (Lettinga et al., 1995). Anaerobic wastewater treatment systems can operate at different temperatures and convert a broad variety of wastes, such as food and beverage, pharmaceutical, pulp and paper, petrochemical (Macarie 2000), alcohol distilleries, dairy, textile, leachates. Anaerobic digestion is also used for municipal wastewaters, solid wastes, agricultural wastes and manures. Anaerobic treatment processes are known for the unique ability to convert highly objectionable wastes into useful products (McCarty 2001).

The process of anaerobic digestion results in lower energy release compared to other terminal electron accepting processes and therefore lower sludge yields. This feature of anaerobic digestion is a significant advantage since sludge management is an expensive component of biological treatment systems. Also low energy and sludge release imply that most of the energy in the original substrates is stored in the biological fuel, energy rich biogas. These features reduce operation costs of this process significantly and makes it a net energy producer (Lettinga, 1995). Although large reactor volumes and long retention times are needed in order to achieve high treatment efficieny in the system (McCarty, 1971), with the recent developments in our knowledge on anaerobic digestion and the quality of the equipments used in the system, much cost-effective reactor configurations and operations are being achieved.

2.2. Biochemistry and Microbiology of Anaerobic Digestion

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). In the first two phases of anaerobic digestion, organic pollutants are hydrolyzed and/or fermented into intermediate short-chain fatty acids (e.g., lactate, butyrate and propionate), which are further degraded to acetate and H_2/CO_2. In the last phase, acetate and H_2/CO_2 are converted into methane (Liu et al., 2001) (Figure 2.1).

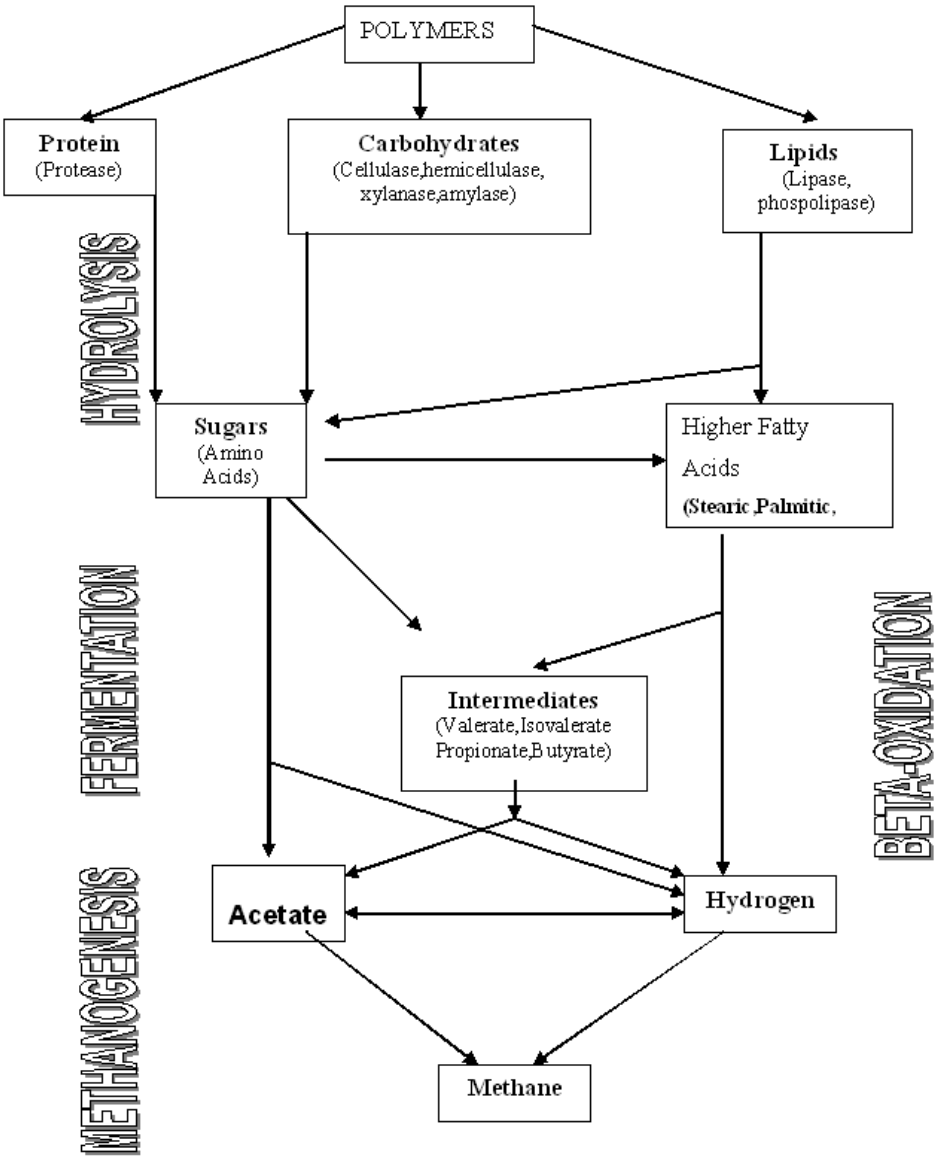


Figure 2.1. The breakdown of organic polymers (Stronach et al.,1986)

Several models have been developed to explain the biochemical steps in anaerobic digestion such as Three-stage Model (Gerardi, 2003), Six-stage Model (Lester et al., 1986) and Nine-stage Model (Harper and Pohland, 1986). According to Gerardi (2003), anaerobic digestion could be considered as a three-stage process:

1. Hydrolysis
2. Fermentation (Acidogenesis and Acetogenesis)
3. Methanogenesis

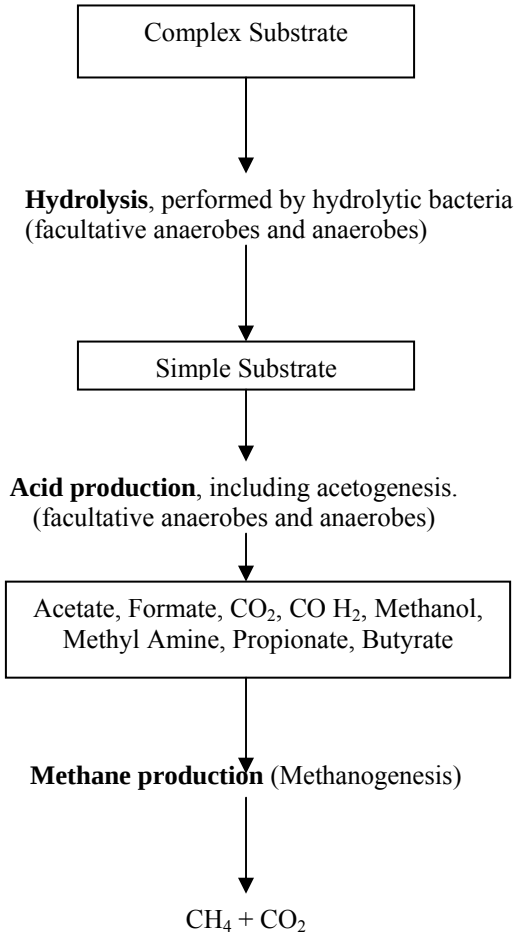


Figure 2.2. Three Stage Process Anaerobic Digestion (Gerardi, 2003)

In the six-stage model given by Stronach et al. (1986), biochemical reactions are classified into 6 parts which are given below:

1. Hydrolysis of organic polimers;
2. Fermentation of amino acids and sugars to hydrogen, acetate and short-chain VFA (volatile fatty acids) and alcohol;
3. Anaerobic oxidation of long chain fatty acids and alcohols;
4. Anaerobic oxidation of intermediary products such as volatile acids (propionate, butyrate,etc.);
5. Conversion of acetate into methane;
6. Conversion of hydrogen into methane.

Some authors report anaerobic degredation process in the Nine-stage Model (Harper and Pohland, 1986) have been listed as follows and shown diagrammatically in Figure 2.3.

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.

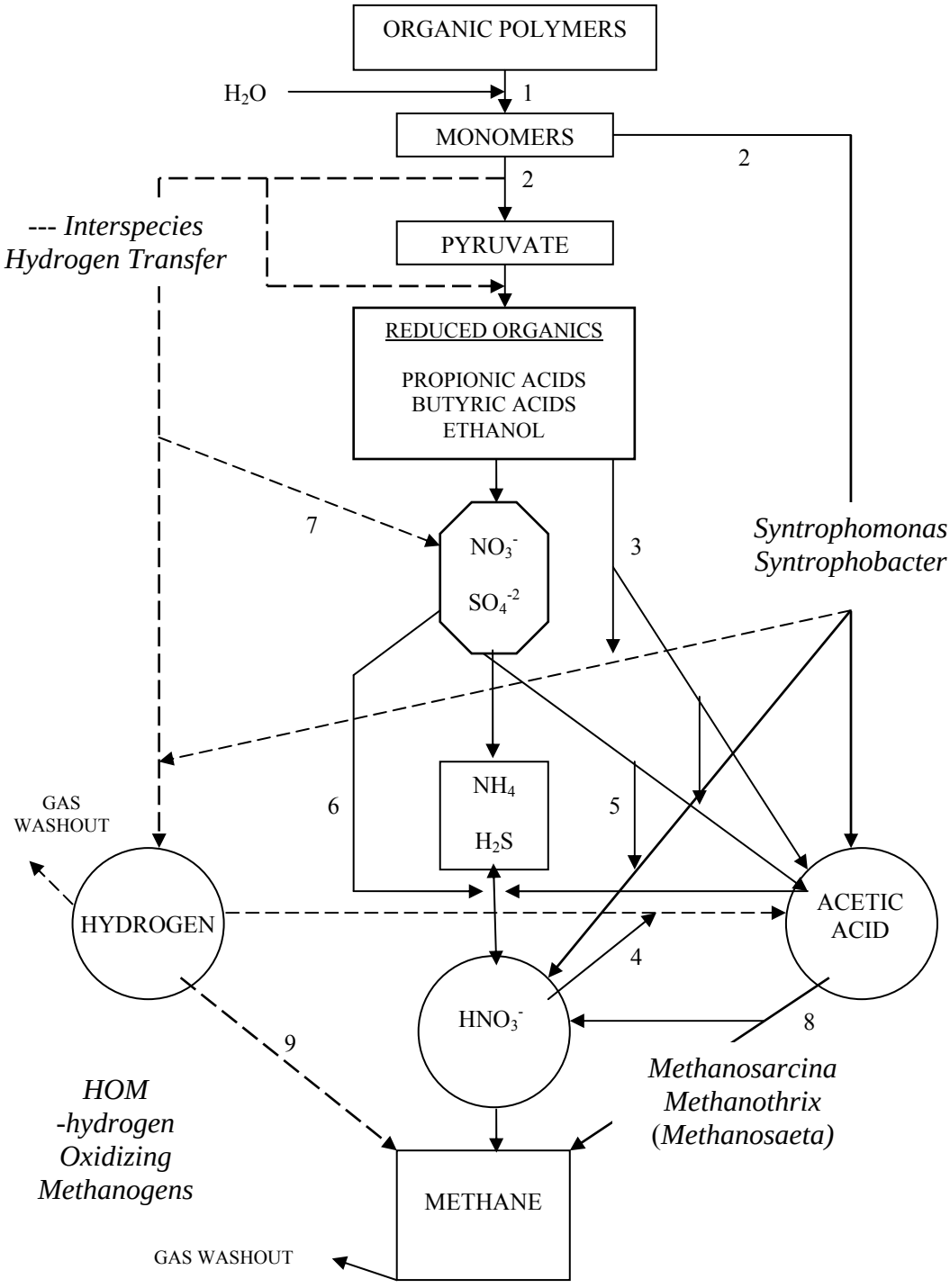


Figure 2.3. Substrate conversion patterns associated with the anaerobic digestion (Harper and Pohland, 1986).

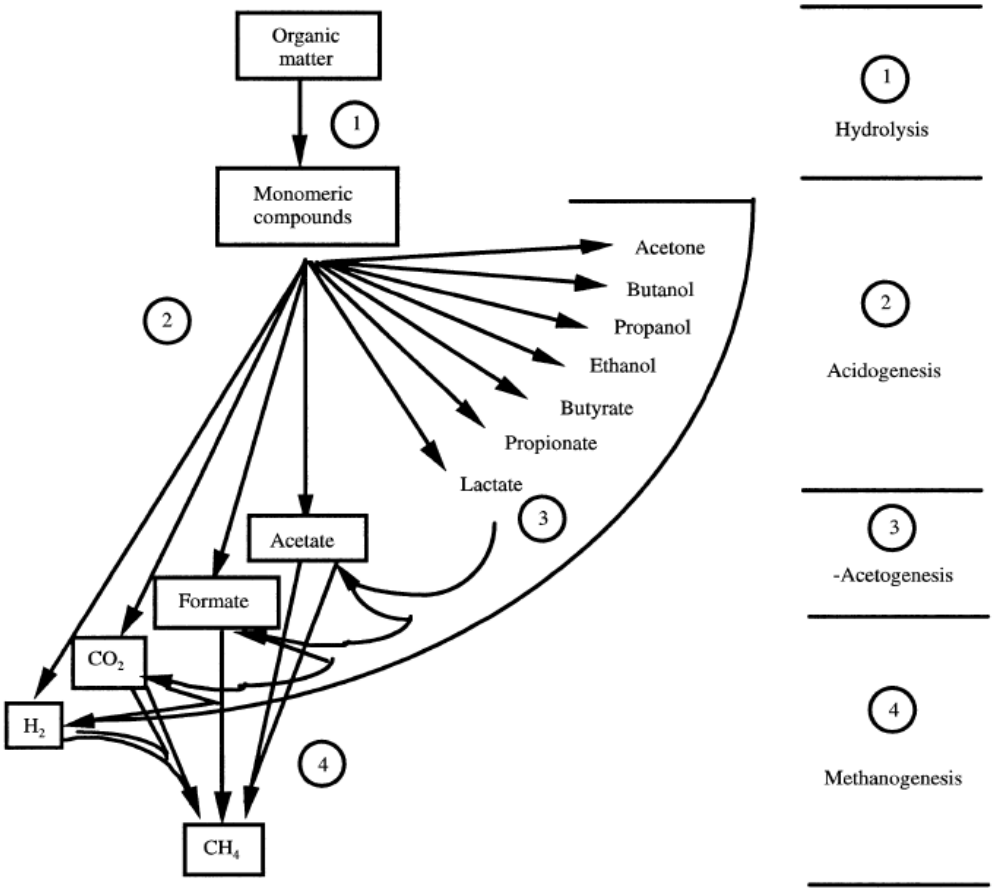


Figure 2.4. Schematic diagram showing anaerobic degradation of organic matter (Garcia et al., 2000)

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 acetolastic methanogens (Chynowth and Pullammanappallil 1996; Zinder et al., 1984). These microorganisms possess a unique biochemistry which enables them to derive metabolic energy from the methanogenic pathway (Whitman et al., 1992; Thauer, 1998). Most of the described species of methanogens are rather specialized. *Methanobrevibacter* spp. is only able to use $H_2 + CO_2$ for growth, whereas *Methanosaeta* spp.. only uses acetate as their energy substrate. *Methanosarcina sp*p. are more versatile; they can use H_2+CO_2, acetate, methanol, methylated amines and pyruvate for growth and methane production (Whitman et al., 1992; Jetten et al., 1992). As a consequence of the limited range of

substrates utilised by methanogens, the anaerobic breakdown of organic matter is carried out by communities of different physiological types of anaerobic bacteria (Stams 1994; Schink 1997; Stams and Oude Elferink 1997). Figure 2.4 illustrates the different phases of the anaerobic digestion process.

2.2.1. Hydrolysis

Complex wastes are required to be hydrolyzed into units as a first step to be taken up by the microbial cells. The hydrolysis of macromolecules such as lipids, proteins and carbohydrates under anaerobic conditions is carried out by specific extracellular enzymes, the reaction rates of which are influenced by pH, cell residence time and the waste constituents in the digester produced by hydrolytic bacteria.

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 since their activity produces the simpler substrates for the succeeding steps in the degradation sequence (Stronach et al., 1986). In the anaerobic digestion process, the hydrolytic activity relevant to each polymer is of paramount significance, since their activity produces simpler substrates for the succeeding steps in the degradation sequence (Stronach et al., 1986). It was stated that *Clostridium* 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 microorganisms are also capable of breaking down some intermediate products to simple volatile fatty acids (VFAs), carbon dioxide, hydrogen and ethanol (Eastman and Ferguson, 1981).

2.2.2. Fermentation (Acidogenesis and Acetogenesis)

Acidogenesis : The breakdown products such as amino acids, sugars and long chain fatty acids of the hydrolysis phase are converted to the intermediary products acetate, carbon dioxide and hydrogen by acid forming bacteria. It was reported that acetate is the most important compound produced in the fermentation of organic substrates with propionate production of secondary consequence (Sorensen et al., 1981).

Two groups of acid forming bacteria are known. The first group is acidogens or fermentative bacteria which are capable of metabolizing amino acids and sugars to the intermediary products, acetate and hydrogen. Important parameters affecting the formation of end product are temperature, pH and the composition of the influent feed. The catabolism of these organic compounds is carried out by a large number of both obligatory and facultatively anaerobic microorganisms and the process utilizes single amino acids, pairs of amino acids or a single amino acid with a non-nitrogenous compound. Single amino acids are converted by clostridia, mycoplasmas and streptococci while butanol, butyric acid, acetone and iso-propanol are generally produced by the bacteria of the genera *Clostridum* and *Butyribacterium* under anaerobic conditions. (i.e *Clostridium butyricum* produces butyrate, *Costridium acetobutylicum* mainly acetone and butanol and *Clostridium butylicum* produces butanol in addition to hydrogen, carbon dioxide and iso-proponol)

Acetogenesis : The second group of acid forming bacteria is the obligate hydrogen producing acetogenic bacteria, which produce acetic acid, carbon dioxide and hydrogen from propionate, butyrate and other higher fatty acids by the β-oxidation process. Fatty acids having more than two carbons lose one molecule at each reaction till all fatty acids are converted to acetate molecules. Acetic acid producing bacteria are *Methanobacterium bryantii*, *Desulfovibrio Syntrophobacter wolinii* (responsible for acetic acid production from propionic acid) (Malina et al., 1992; Stronach et al., 1986), *Syntrophomonas wofei* (responsible for acetic acid production from butyric, caproic and valeric acids), *Syntrophus buswellii* (Malina et al., 1992; Gujer et al., 1983)

2.2.3. Methanogenesis

Methanogenesis is a common and important process in many natural and engineered anaerobic environments, suc as, anaerobic digesters (Raskin et al., 1994), cattle rumen (Miller et al., 1986), rice fields (Joulian et al., 1998), oil wells (Ollivier et al., 1997), landfills (Fielding et al., 1988), and a range of extreme habitats (Garcia et al., 2000). It plays an important role in anaerobic treatment of organic wastes, formation of biogas as an alternative source of energy (Cicerone and Oremland, 1988). Methanogenesis is defined as the rate-limiting step in the whole anaerobic digestion process due to the slow growth rate of the methanogens comparing with acidogens (Malina et al.,1992; Noike et al., 1985; Speece, 1983). Therefore, the performance of anaerobic reactors and the quality of the effluent depend on the activity of methanogens.

Methane production in the anaerobic digestion is carried out by a group of strictly anaerobic *Archaea* called the methanogens. Methanogens convert the end products of the previous step into methane and carbon dioxide 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,and only bicarbonate and protons act as terminal electron acceptors (Garcia et al., 2000; De Bok et al., 2004; Stams et al,. 2006). A detailed explanation will be given for Methanogenic Archaea in the following sections of this chapter.

It has been reported that at least ten substrates can be converted to methane by pure cultures of methanogens. Three classes of compounds including CO_2-type substrates, methyl substrates and acetate are listed in Table 2.1.

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

I.CO_2-type substrates
Carbon dioxide (with electrons derived from H_2, certain alcohols, or pyruvate)
Formate
Carbon monoxide
II.Methyl substrates
Methanol
Methylamine
Dimethylamine
Trimethylamine
Methylmercaptan
Dimethylsulfide
III.Acetotrophic substrate
Acetate

CO_2-type substrates including CO_2, formate and carbon monoxide are reduced to methane by bacteria Even though the reduction of carbon dioxide to methane is generally hydrogen dependent, other substrates in this class can supply the electrons for CO_2 reduction.

$$CO_2+4H_2 \rightarrow CH_4+2H_2O \qquad \Delta G^\circ = -131kJ \qquad (3.1)$$

Methyl group substances which are the second class of methanogenic substrates are converted to methane by two conversion mechanisms. the formation of methane by reducing methyl group substances using an external electron donor such as H_2 is the first mechanism. In the conversion equations methanol (CH_3OH) is used as a model methyl substrate.

$CH_3OH+H_2 \rightarrow CH_4+H_2O$ $\Delta G°= -113kJ$ (3.2)

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.

$4CH_3OH \rightarrow 3CH_4+CO_2+2H_2O$ $\Delta G°= -319kJ$ (3.3)

The final methanogenic substrate is acetate. The conversion mechanism of acetate to methane and carbon dioxide called the acetotrophic reaction. It has been stated that 70% of the methane produced is derived from the acetic acid and the remaining 30% is produced from the reduction of CO_2 (Pavlostathis and Gomez, 1991).

$CH_3COO^-+H_2O \rightarrow CH_4+HCO_3^-$ $\Delta G°= -31kJ$ (3.4)

Each of the above reactions are exergonic and can be used to synthesize ATP. Concerning carbon for cellular biosynthesis, CO_2 is the precursor for all cellular components when growing on CO_2+H_2. If methanogenic substrates are acetate or methylated compounds, these compounds are also used in the organic cell components with the fixation of some CO_2.

2.3. Characteristics of Methanogens

Methanogens are microorganisms that produce methane as the end product of their anaerobic respiration. All methanogens are strictly anaerobic archaea belonging to the *Euryarchaeota*. They are a large and diverse group, all of which are obligate methane producers that obtain most of their energy from methanogenesis.

Methanogens have been cultivated from a wide variety of anaerobic environments. In addition to temperate habitats, they are also common in environments of extreme temperatures, salinity, and pH. The common methanogenic habitats include marine sediments, freshwater sediments, flooded soils, human and animal gastrointestinal tracts, termites, anaerobic digestors, landfill, geothermal systems, and heartwood of trees.

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

Morphologically, the methanogens exhibit a wide variety of shapes and sizes, including rods, regular and irregular cocci, long-chained rods, spirilla, sarcina and irregular unusual flattened plates. Motility is sometimes present. Some species can aggregate in clusters. Several species of *Methanosarcina* and *Methanosaeta* contain gas vacuoles. The

Gram reaction can be positive or negative even within members of the same genus. (Garcia et al., 2000).

Methanogens have unique cell membrane lipid and lack a rigid cell wall. They are capable of degrading substrates such as organic wastes and produce methane by their specialized coenzymes. Coenzymes that are unique to methane forming microorganisms are coenzyme M and the nickel containing coenzymes. Coenzyme M is used to reduce CO_2 to methane. The nickel-containing coenzymes are important hydrogen carriers in methanogens.

Even though, the methanogens are very diverse, they are only capable of utilizing a small number of substrates. The substrates are limited to three major types including CO2, methyl-group containing compounds, and acetate . Most organic substances, i.e, carbohydrates and long-chain fatty acids and alcohols, are not appropriate substrates for methanogens (Table 2.3). These compounds must first be processed by anaerobic bacteria or eukaryotes to produce the substrates used by the methanogens. Thus, in most methanogenic environments, most of the energy available for growth is utilized by these nonmethanogenic organisms.

Table 2.3. Methanogenic orders (Karakashev et al. 2005)

Order	**Physiology**
Methanopyrales	Hydrogenotrophic; hyperthermophilic
Methanobacteriales	Hydrogenotrophic; mesophilic or thermophilic
Methanococcales	Hydrogenotrophic; mesophilic or thermophilic
Methanomicrobiales	Hydrogenotrophic; mesophilic
Methanosarcinales	Strict aceticlastic (*Methanosaetaceae*), aceticlastic or hydrogenotrophic (*Methanosarcinaceae*); mesophilic or thermophilic

Most methanogens are hydrogenotrophs that can reduce CO_2 to methane with H_2 as the primary electron donor. Many hydrogenotrophic methanogens are also able to use formate as the major electron donor. Besides, some hydrogenotrophic methanogens can

also use secondary alcohols, such as 2-propanol, 2-butanol, and cyclopentanol, as electron donors. A small number of methanogens can also use ethanol.

Methyl-group containing compounds, including methanol, methylated amines (monomethylamine, dimethylamine, trimethylamine, and tetramethylammonium), and methylated sulfides (methanethiol and dimethylsulfide) are other types of substrates that are used by methanogenic Archaeae. Methanogens that are able to use methylated compounds, ormethylotrophicmethanogens, are limited to the order *Methanosarcinales*, except for *Methanosphaera* species, which belong to the order *Methanobacteriales*.

The third type of substrate that is used by methanogens is acetate. Acetate is a major intermediate in the anaerobic food chain, and as much as 70% of the biologically generated methane is derived from acetate. Surprisingly, only two genera are known to use acetate for methanogenesis: *Methanosarcina* and *Methanosaeta*. They carry out an aceticlastic reaction that splits acetate, oxidizing the carboxyl-group to CO_2 and reducing the methyl group to CH_4. *Methanosarcina* is a relative generalist that prefers methanol and methylamine to acetate, and many species also utilize H_2. *Methanosaeta*, which uses only acetate, is a superior acetate utilizer in that it can use acetate at concentrations as low as 5–20μM, while *Methanosarcina* requires a minimum concentration of about 1 mM. The difference of acetate affinity is probably due to 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, while *Methanosaeta* uses the high-affinity adenosine monophosphate (AMP)– forming acetyl-CoA synthetase.24–27 Moreover, based on their genome sequences, these two genera probably have different modes of electron transfer and energy conservation, even though the main steps in the methanogenesis pathway are likely to be similar.

Table 2.4. Typical organisms in methanogenesis reactions (Liu et al.,2008, Whitman et al.,2006, Zinder et al., 1993)

Reaction	Organisms
I. CO_2-type	
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	Most methanogens
$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	Many hydrogenotrophic methanogens
CO_2 + 4 isopropanol→CH4 + 4 acetone + 2 H_2O	Some hydrogenotrophic methanogens
$4\ CO + 2H_2O \rightarrow CH_4 + 3\ CO_2$	*Methanothermobacter* and *Methanosarcina*
II. Methylated C1 compounds	
$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	*Methanosarcina* and other methylotrophic methanogens
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	*Methanomicrococcus blatticola* and *Methanosphaera*
$2\ (CH_3)_2\text{-}S + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ H_2S$	Some methylotrophic methanogens
$4\ CH_3\text{-}NH_2 + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_3$	Some methylotrophic methanogens
$2\ (CH_3)_2\text{-}NH + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_3$	Some methylotrophic methanogens
$4\ (CH_3)_3\text{-}N + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_3$	Some methylotrophic methanogens
$4\ CH_3NH_3Cl + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4Cl$	Some methylotrophic methanogens
III. Acetate	
$CH_3COOH \rightarrow CH_4 + CO_2$	*Methanosarcina* and *Methanosaeta*

2.3.1. Taxonomy of Methanogens

Woese et al. (1990) proposed a new classification for living organisms, dividing life on earth into three major domains: Bacteria, Archaea and Eukarya. (Figure 2.5)

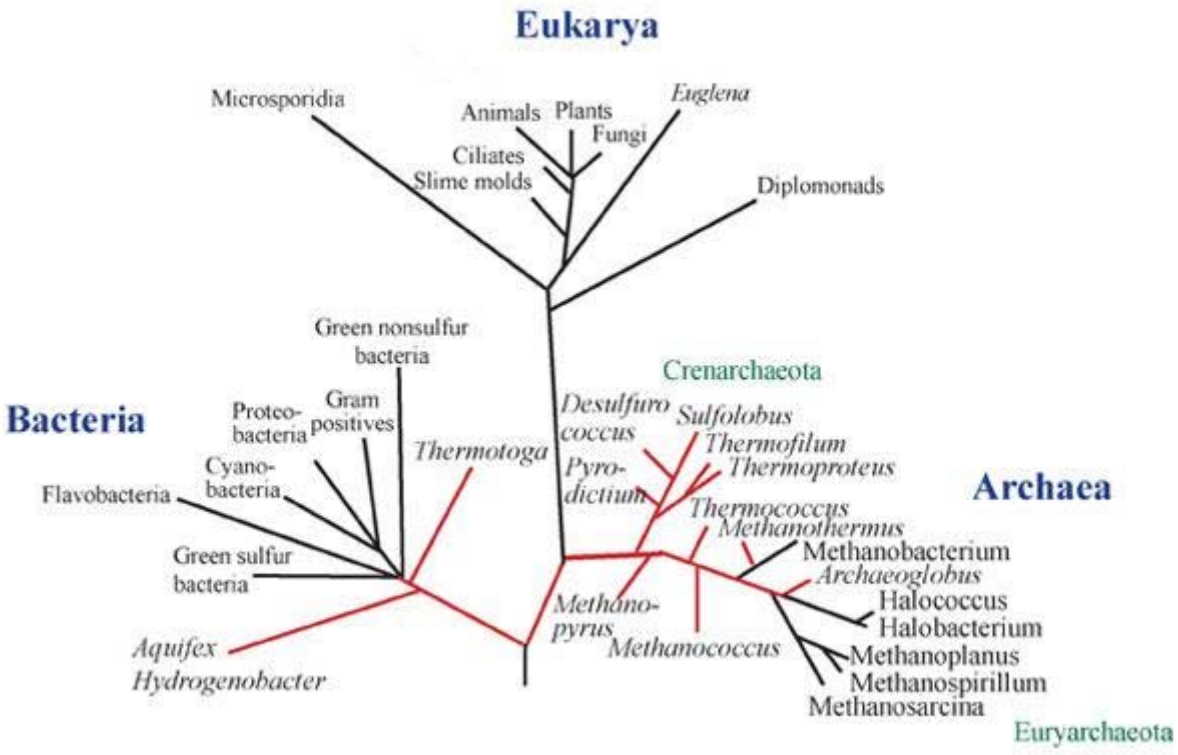


Figure 2.5. Universal phylogenetic tree (Woese et al.,1990)

The unique phylogenetic status and evolutinary divergence of Archaea suggest that they should exhibit wide physiological diversity. However traditional culture-based studies have led to belief that opposite was the case. Two major lineages of Archaea are Crenarchaeota and Euryarchaeota (Figure 2.6). The first kingdom, Crenarchaeota derived from being phylogenetically close to ancestor or source of Archaea (Woese et al. 1990). It was believed to include only sulphur-dependent extreme thermophiles. The Euryarchaeota is a heterogenous group comprimising a broad spectrum of organisms with varied patterns of metabolism from different habitats. It includes extreme halophiles, methanogens, and some extreme thermophiles so far. Moreover, a third archaeal kingdom has been discovered which is reported isolation of several archaeal sequences evolutinary distant from all Archaea known to date by Barns et al. 1994 and then in 1996. The new group was placed on phyologenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota.

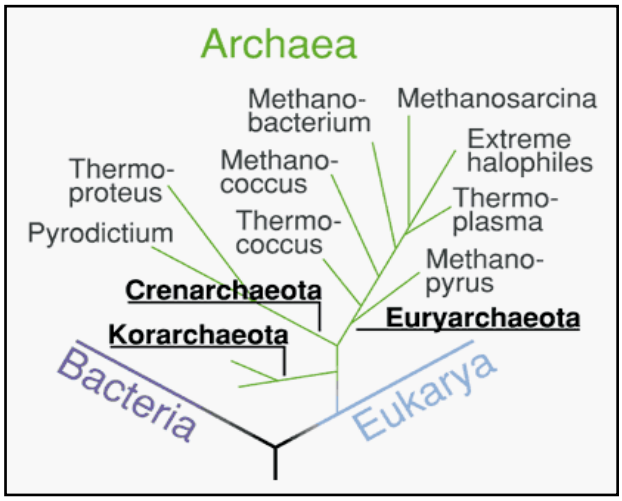


Figure 2.6. Major lineages of *Archaea*: Crenarchaeota, Euryarchaeota and Korarchaeota (Madigan et al., 2002)

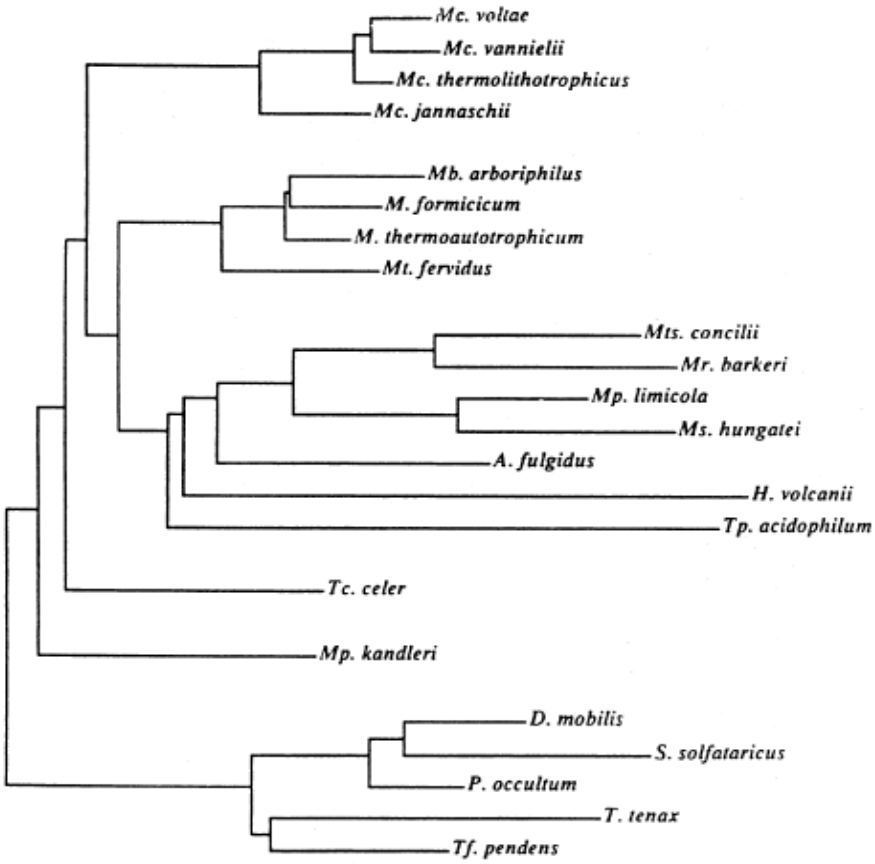


Figure 2.7. Representative phylogenetic tree for *Archaea*, based upon 16S rRNA sequences. The scale bar measures five nucleotides changes per 100 residues.

Phylogenetically, methanogens are Archeaobacteria, 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 (De Rosa and Gambacorta, 1988; Jones et al., 1987; Langworthy, 1985), a lack of peptidoglycan containig muramic acid (Kandler and Hippe, 1977), a distinctive ribosamal RNA sequences (Balch et al.,1979; 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 are classified into five orders within the kingdom *Archaeobacteria*: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanopyrales* (Figure 2.8). Organisms from different orders have less than 82% 16S rRNA sequence similarity. Methanogens belonging to different orders also possess different cell envelope structure, lipid composition, substrate range, and other biological properties.

Members of the order *Methanobacteriales* generally produce methane using CO_2 as the electoron acceptor and H_2 as the electron donor. Some species can also use formate, CO, or secondary alcohols as electron donors. The genus *Methanosphaera* can only reduce methanol with H_2. In most genera, the cells are short to long rods with a length of 0.6–25μm. They often form filaments up to 40μm in length. They are widely distributed in anaerobic habitats, such as marine and freshwater sediments, soil, animal gastrointestinal tracts, anaerobic sewage digestors, and geothermal habitats (Liu et al., 2008). Order of *Methanobacteriales* is divided into two families, *Methanobacteriaceae* and *Methanothermaceae*. The family *Methanobacteriaceae* contains three mesophilic genera, *Methanobacterium*, *Methanobrevibacter*, and *Methanosphaera*, and one extremely thermophilic genus *Methanothermobacter*. The family *Methanothermaceae* is represented by one hyperthermophilic genus, *Methanothermus*, which has only been isolated from thermal springs.

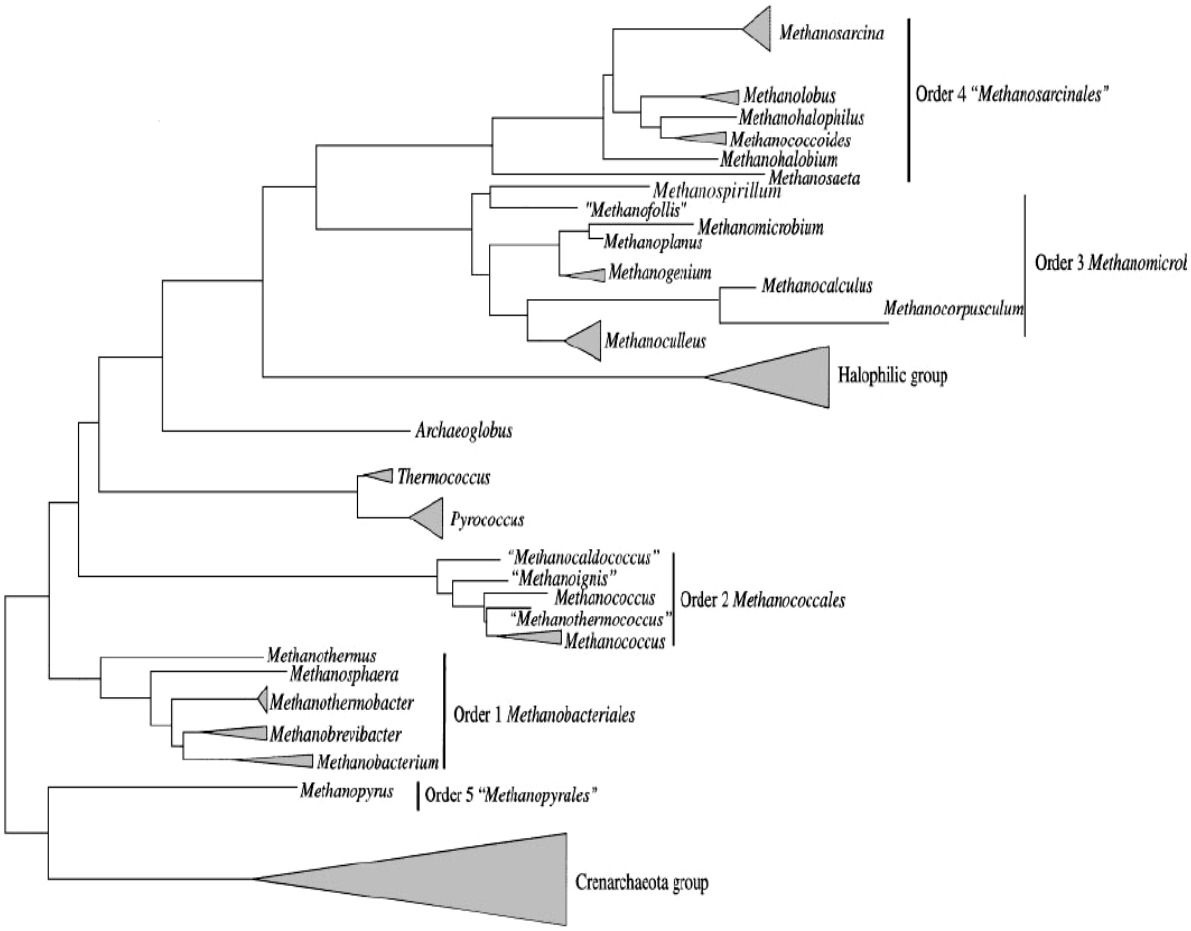


Figure 2.8. Updated phylogeny of methanogens, in the domain *Archaea.*
(Garcia et al., 2000)

Methanococcales are an order of coccoid, marine methanogens which are slightly halophilic, and most are chemolithotrophic. They produce methane using CO_2 as the electron acceptor and H_2 or formate as the electron donor. The cells are irregular cocci with a diameter of 1–3μm. The order of *Methanococcales* has been divided into two families distinguished by their growth temperatures, *Methanocaldococcaceae* and *Methanococcaceae.*

Members of the order *Methanomicrobiales* are methanogens that use CO_2 as the electron acceptor and H_2 as electron donor. Most species can use formate, and many species also use secondary alcohols as alternative electron donors. Their morphology is diverse, including cocci, rods, and sheathed rods. They are widely distributed in anaerobic habitats, including marine and freshwater sediments, anaerobic sewage digestors, and

animal gastrointestinal tracts. The order of *Methanomicrobiales* is divided into three families, *Methanomicrobiaceae*, *Methanospirillaceae*, and *Methanocorpusculaceae*. Order *Methanosarcinales* have the widest substrate range among methanogens. Most of them can produce methane by disproportionating the methylgroup containing compounds or by splitting acetate. Some species can reduce CO_2 with H_2, but formate is not used as an electron donor. Their cellular morphologies are diverse, including cocci, pseudosarcinae, and sheathed rods. All cells are nonmotile. They are widely distributed in marine and freshwater sediments, anaerobic sewage digestors, and animal gastrointestinal tracts. The order of *Methanosarcinales* is divided into two families, *Methanosarcinaceae* and *Methanosaetaceae*.

The order of *Methanopyrales* is represented by only one species, *Methanopyrus kandleri*. Cells reduce CO_2 with H_2 for methanogenesis. They are rod-shaped. *M. kandleri* is hyperthermophilic with a growth temperature range of 84–110°C. It inhabits marine hydrothermal system.

2.4. Molecular Methods used in Microbial Ecology

The development of molecular techniques using nucleic acids has led to many new findings in microbial ecology (Amann et al., 1995). Microbial diversity studies were limited in the past by the lack of methodological tools, but the availability of the new molecular methods, such as 16S rRNA gene cloning and sequencing (Urakawa et al., 2000), fluorescent in situ hybridization (FISH) (Amann, 1995; Amann et al., 1995), and denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; Muyzer and Smalla, 1998), has made it possible to investigate the dynamics of the composition and structure of microbial populations and communities in natural and engineered ecosystems, the phylogenetic relationships, and the impact of environmental or specific factors such as pollution by xenobiotics on microbial diversity (Morris et al., 2002). Furthermore, 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).

The classical approach for identification of viable microorganisms in environmental samples is plate counting on agar medium (Edlund and Jansson, 2006). It would appear that only between 0.5% and 10% prokaryote diversity has been identified due to the small size and the absence of distinguishing phenotypic characters of prokaryotic organisms, and the fact that most of these organisms cannot be cultured which are the most important factors that limit the evaluation of prokaryotic biodiversity (Torsvik et al., 2002). Almost 99% of all microorganisms in nature can not be isolated and classified based on physiological and biochemical features mainly due to the previously mentioned limitations of cultivation (Muyzer, 1999). Studies based on cultivation methods could not reveal the appropriate microbial diversity due to the high selectivity of these traditional methods. Such cultivation based approaches were subject to restrictions and biases leading to a distorted representation of the true community composition (Amann et al., 1995).

However, nucleic-acid based methods allow microbial community characterization without cultivation (Hofman-Bang et al., 2003). Techniques such as reassociation analysis of DNA (Torsvik et al., 1996), denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; Teske et al., 1996), and restriction fragment length polymorphism (Moyer et al., 1994) have yielded insight into bacterial diversity and community composition. In addition, phylogenetically based oligonucleotide hybridization techniques permit not only the monitoring of individual but also a quantification of phylogenetic groups their abundance in natural habitats (Amann et al., 1995). In situ hybridization with rRNA-targeted fluorescent oligonucleotide probes not only permits the identification and quantification of individual cells, but also analyse bacterial community composition in several environments (Llobet-Brossa et al., 1998). The ability to determine microbial diversity at a high-resolution level (groups, species and strains) without the need for cultivation will further our understanding of several issues and lead to new findings in the field of microbial ecology.

Table 2.5. 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	Sensitive quantitative amplification suitable for high-throughput over a wide dynamic range.	Primers developed from known sequences and can be biased.
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.

There is no doubt that the analysis of rRNA gene revolutionized microbial ecology and expanded our knowledge of microbial phylogeny. This concept was developed 30 years ago (Woese and Fox, 1977). Since the pioneering work of Carl Woose, the rRNA has become the most commonly used molecule for phylogenetic analysis. rRNA or the corresponding rDNA are particularly suitable as evolutionary chronometers (Stahl et al., 1988) Using 16s rDNA or rRNA is currently the most common approach for community analysis (Dahllöf, 2002). Studies based on the rRNA gene (rDNA) or the rRNA has become common to investigate community diversity. The rapidly growing rDNA sequence data bank, accessible via the internet, now makes it possible to compare sequences from across the world (Dahllöf, 2002).

A schematic diagram of different molecular methods for studying microbial diversity is given in Figure 2.9. We can define these methods as PCR based methods and non PCR based methods. All PCR based methods use PCR amplification products. Some of these methods are random sequencing in clone libraries, methods based on separation by electrophoresis like DGGE/TGGE, SSCP and T-RFLP. Flourescent in situ hybridization(FISH) is one of the most commonly used technique in non PCR based methods. The other important non PCR based method is the DNA reassociation.

2.4.1. PCR-based molecular methods

Polymerase chain reaction (PCR) is the first step for these methods. 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). However, PCR method has biases, especially in the amplification step. In consequence, all techniques that are based on PCR (cloning, pattern analysis and sequencing) will be affected by the biases introduced by PCR (Dahllöf, 2002).

Real Time PCR (Q-PCR): Real-time- or quantitative PCR is based on the continuous monitoring of changes of fluorescence in the PCR tube during PCR. In contrast to the conventional endpoint detection PCR, quantification occurs during the exponential phase of amplification (Malinen et al. 2003). Thus, the bias often observed in the PCR template-to-product ratios can be largely aoided (Suzuki & Giovannoni 1996).

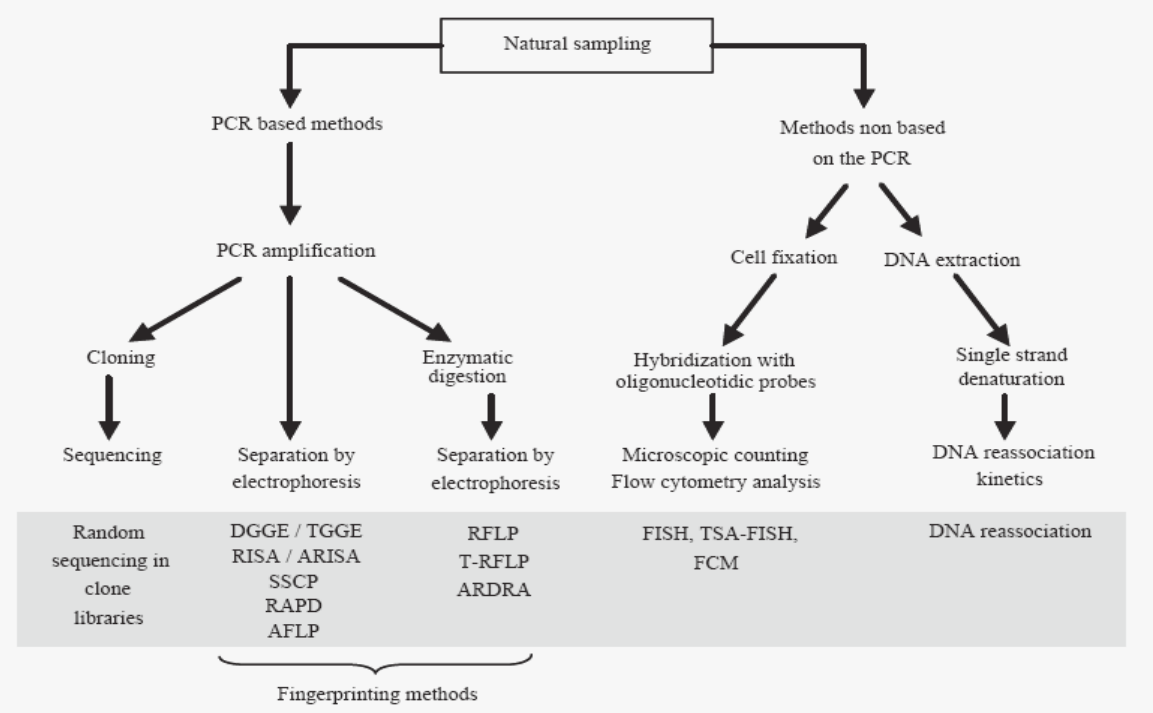


Figure 2.9. Diagram of the different molecular approaches for assessing the genetic diversity of microbial communities. (Doriga et al., 2005)

Pattern Analysis and Denaturing Gradient Gel Electrophoresis (DGGE): Pattern analysis or fingerprinting is often carried out by evaluating banding patterns of PCR products on gels (Dahllöf, 2002). Several fingerprinting techniques, such as DGGE, TGGE, restriction fragment length polymorphism (RFLP), and single strand conformation polymorphism (SSCP), 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).

DGGE is nowadays a routine technique that is used to asses the diversity of microbial communities, to monitor their dynamics (Muyzer, 1999; Muyzer and Smalla, 1998) and to screen clone libraries. This method can be used to obtain qualitative and semi-quantitative estimations of biodiversity. Also, the DGGE pattern obtained provides a rapid identification of the predominant species in the microbial community.

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.

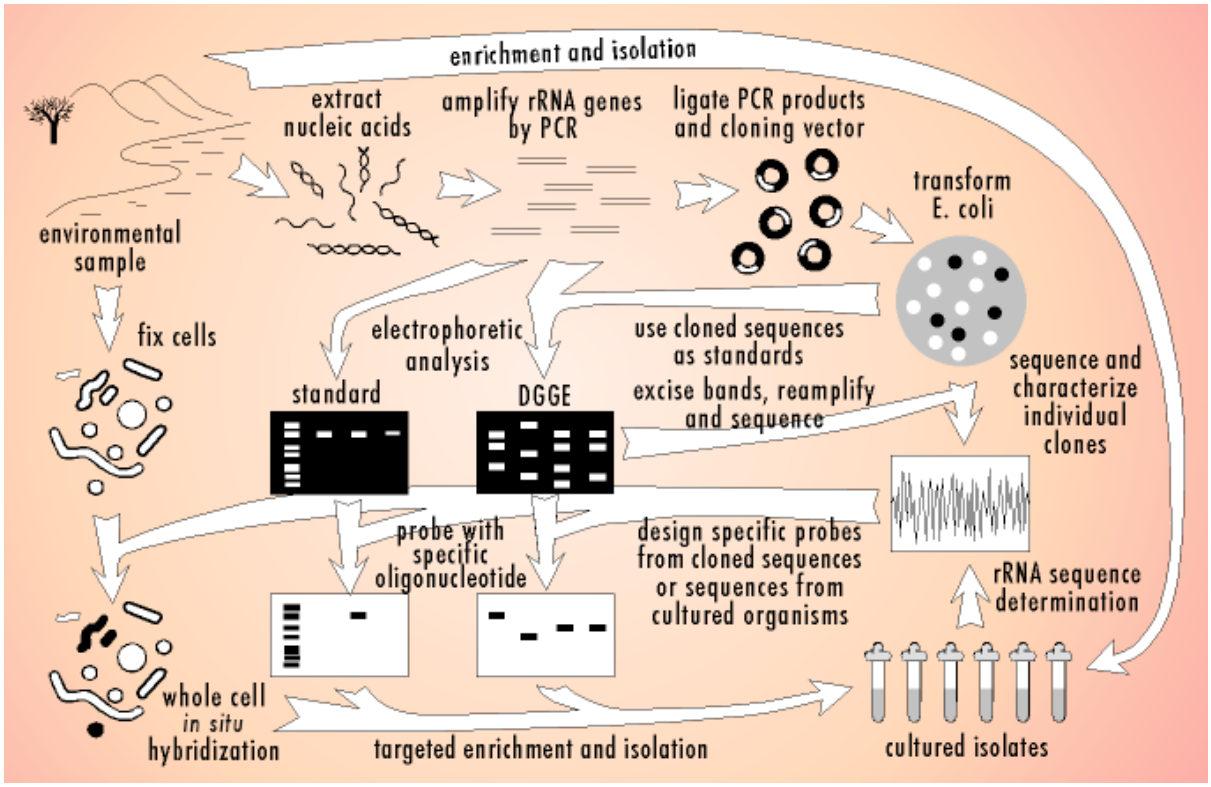


Figure 2.10. Summary of overall methodologies used in the phylogenetic studies (Head et al., 1998)

Molecular Cloning: Molecular cloning is at the base of most genetic engineering procedures and has greatly facilitated the analysis of any genome. The aim of molecular cloning is to isolate large quantities of specific genes or chromosomal fragments in pure form (Madigan et al., 2003). It also identify the members of a community from

environmental samples. Cloning can produce large amounts of DNA segments originally isolated from environmental samples. Analysis of 16S rRNA clone library to assess microbial diversity and populations in natural environments is an important approach (Giovanni et al., 1990).

2.4.2. Non-PCR Based Molecular Methods

DNA re-association analysis: This technique is used for whole DNA comparisons between two communities, or for studying the sequence variety of a single community. In this technique total DNA is extracted and purified. The DNA of one community may be radioactively labelled and used as a template, when comparing two communities. Crosshybridization between two DNA samples is then carried out, and the degree of similarity is monitored. This technique has been used to evaluate biodiversity in aquatic communities (Torsvik et al., 1990, Øvreås et al., 1998).

Fluorescent in situ Hybridization (FISH) **:** FISH is based on the microscopic analysis of already defined (at least its SSU rRNA gene sequence) groups of bacteria by a fluorogenic oligonucleotide (or probe) targeting SSU rRNA molecules inside cells (Giovannoni et al., 1988; DeLong et al., 1989; Amann et al., 1990). 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. These probes are generally 15–25 nucleotides in length and are labelled covalently at the 5'end with a fluorescent dye. After washing steps, specifically stained cells are detected by epifluorescence microscopy or flow cytometry. The determination of composition and number of bacteria can be achieved by rRNA-targeted oligonucleotide probes without cultivation, directly in their natural environment . rRNA gene fragments was used as phylogenetic stains firstly in 1989 (DeLong et al., 1989). Since the pioneering study of DeLong, fluorescent in situ hybridization technique has become a common tool for identification of microorganisms in environmental samples (Amann et al., 2001). 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., 1997;

Ravenschlag et al., 2001). The signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. This allows a quantification of rRNA concentrations both in single cells and in the environment (Murray et al., 1999; Pernthaler et al., 2001; Poulsen et al., 1993). Raskin et al. (1994a) evaluated the methanogenic group composition in anaerobic digesters by oligonucleotide probe hybridization. Several studies (Araujo et al., 2000; Imachi et al., 2000; Merkel et al., 1999; Sekiguchi et al., 1999; Tagawa et al., 2000; Upton et al., 2000; Wu et al., 2001) include FISH results using these same oligonucleotides but the experimental conditions are variable. These probes are still reasonably accurate to target most of the defined phylogenetic groups of methanogenic Archaea.

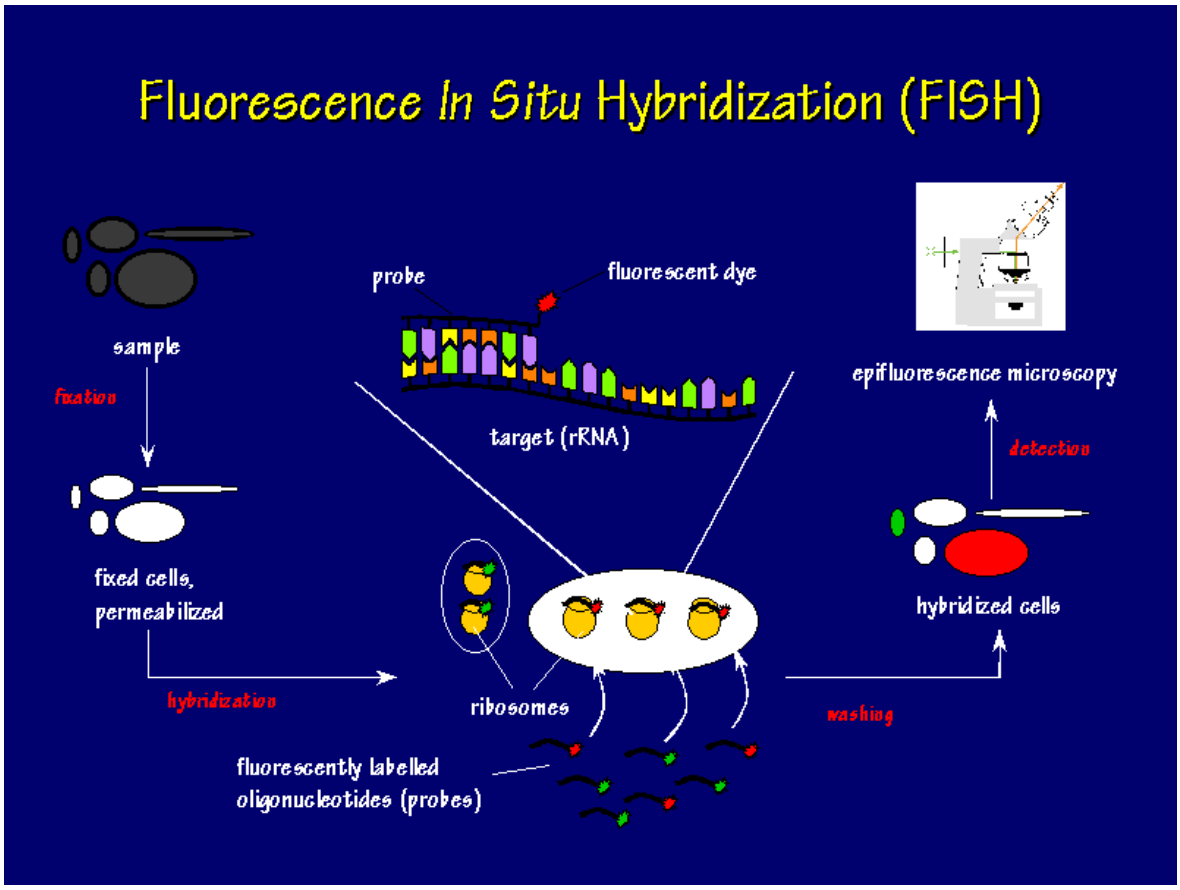


Figure 2.11. Fluorescent in situ hybridization flow chart

In addition, the microbial community dynamics could be analyzed by FISH (Fernandez et al., 1999). By chancing the environmental factors, dominant members of the community could be monitored by FISH. Harmsen et al. (1996) applied FISH to identify

syntrophic propionate-oxidizing bacteria, and this study revealed the distribution of bacteria and methanogens in anaerobic granular sludge systems resolved the phylogenetic affiliation and localization of important microbial populations in a full-scale UASB reactor treating brewery wastewater.

The main advantage of FISH that it does not need any DNA or RNA amplification and allows microscopic inspection of intact cells in the samples. The other important advantages of FISH technique are listed as follows:

- it is an easy and fast technique;
- it allows direct visualization of organisms without cultivation;
- it is generally quantative;
- it also allows quantification of specific microbial groups, in contrast to traditional methods and other molecular methods;
- it is possible to detect active microorganisms in the sample (Sanz, et al. 2006).

Despite the advantages above, FISH technique has its limitations and disadvantages like any other technique. One of the most important limitations of FISH is that not all bacterial and archaeal cells can be permeated by oligonucleotide probes using standart fixation protocols (Aman et al., 1995). Besides, the use of rRNA targeted oligonucleotide probes, which are covalently mono-labelled with fluorescent dye molecules, limits the sensitivity of the method and aggravates the use of FISH for identification of prokaryotes with low ribosome content per cell. The other disadvantages of FISH are as follows

- a priori knowledge of the studied ecosystem and the microorganisms to be detected is necessary, meaning combining with other techniques is obligate;
- if a particular microorganism is detected and quantified, the rRNA sequence of the microorganim must be known (in case corresponding probe has not yet been published);
- it is not always possible to design a specific probe for a certain group of microorganism, especially if metabolic criteria are applied;
- the design and assessing optimum conditions for hybridization for a new probe is a difficult dedication;
- quantification of microorganisms can be tedious and subjective (manual counting) or complex (image analysis).

2.5. Anaerobic Reactor Configurations

Anaerobic treatment systems are mostly preferred in terms of several advantageous such as high efficiency, lower excess sludge and biogas production. Reactor configurations and unites should be designed to achieve the best treatment. Reactor types are very important for cost efficiency, start-up, maintenance, management, and effluent quality. Many reactor configurations are used for treatment of munipical and industrial wastewaters or sludges.

Anaerobic Sequencing Batch Reactor : An anaerobic sequencing batch reactor (ASBR) process is one of the novel and promising high-rate anaerobic processes and has been used for treating organic wastewaters due to some advantages over the continuous processes, including better solids retention, efficient operating control, absence of primary or secondary settling, high organic matter removal efficiency and simple operation. (Ratusnezei et al, 2000, Lin, 2003) A typical cycle in an ASBR is composed of four steps: feed, reaction, settling and liquid withdrawal (Dague et al., 1992). Mixing is required during the reaction step in order to improve the mass transfer rates, increasing the overall organic matter uptake rates. For high strength wastewaters, mixing can be entirely supplied by recirculating the biogas produced during the anaerobic digestion (Sung et al, 1995). However, for low strength wastewaters the biogas production is insufficient to provide suitable mixing in the reactor and an alternative way of agitation may be necessary, such as liquid recycle or mechanical agitation (Brito et al., 1997; Pol et al., 1998; Ndon et al, 1997).

Completely Mixed Digester : The conventional anaerobic digester is the simplest anaerobic reactor design application of the conventional flow-through tank without any biomass recycle. These systems are suitable for wastewaters containing high concentrations of particulates or extremely high concentrations of soluble biodegradable organic materials. The average retention time of anaerobic microorganisms in the reactor (SRT) is almost equal to hydraulic retention time (HRT) with the chracateristics of the design. Process stability can be limited by the short SRTs and large reactor volumes are required to maintain necessary SRTs, due to slow growth of methanogens 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.

Anaerobic Contact Processes : The anaerobic contact process that overcomes the disadvantages of the conventional digester process by separating and recycling biomass back to the anaerobic reactor is consisted of a completely stirred digester, a settling tank and a sludge-recycling unit. The system SRT can be controlled independently from the HRT with the 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 kgCOD/m^3.day can be applied to the reactor with HRTs of range between 0.5 and 5 days.

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 carbon dioxide 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.

Anaerobic Expanded/Fluidized Bed Processes : 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. The expanded-bed anaerobic reactor is a variation of the fluidized-bed reactor and contains similar support media. The difference

is that fluid's upward flow velocity is not maintained as high as in the fludized-bed; thus, full bed fluidization does not result.

Upflow Anaerobic Sludge Blanket (UASB) Reactor : Lettinga, vam Velsen, de Zeeuw, and Hobma (1979) developed an important new reactor, UASB reactor, which has found wide application for the treatment of industrial and municipal wastewaters. In the upflow anaerobic sludge blanket reactor, the wastewater passing through an expanded bed (blanket) of highly active biomass which is kept in suspension is degraded. The biomass present in the reactors are dense granules or flocs of 1-5 mm with highly settleable. Microorganisms dominating the granule formation are found to be acetate-utilizing methanogens, especially *Methanothrix* and *Methanosarcina*. The upper part of the reactor is designed for the purpose of gas-solids separation, thus allowing biogas collection and internal sludge recycling. Rising gas during the degradation process provides the mixing force to maintain contact between the biomass and the wastewater. Therefore, the reactor can be operated without any internal mixing device. High treatment efficiency (65-95% COD removal) can be obtained with short hydraulic retention times and energy demand is low in the process.

Expanded Granular Sludge Bed (EGSB) Reactor : An EGSB reactor is a variant of the UASB reactor (Kato et al., 1994). The distinguishing feature is the faster rate of upward-flow velocity allowing 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.

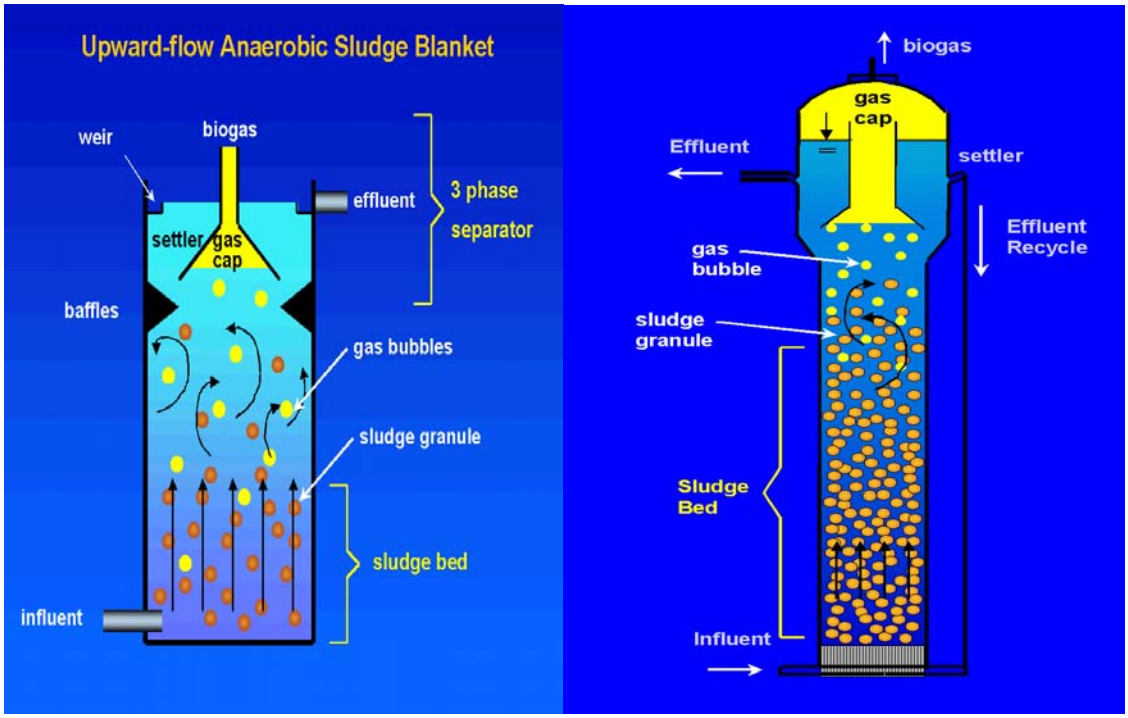


Figure 2.12. UASB and EGSB reactor configurations.

The biomass is present in a granular form. The granule size and inner structure seem to play a more relevant role in fully expanded EGSB reactors (Seghezzo, 1997). Accumulation of flocculent excess sludge between the sludge granules is also prevented (van der Last and Lettinga, 1992). Soluble pollutants are efficiently treated in EGSB reactors but suspended solids are not substantially removed from the wastewater stream due to the high upflow velocities applied. Recirculation of the effluent dilutes the influent concentration, but it was extensively proven that low strength wastewater can efficiently be treated in EGSB reactors (Kato et al., 1994; Kato, 1994).

Hybrid Systems : Anaerobic hybrid systems have simple design and require no special gas or sludge separation device. 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. So in a hybrid system as a combination of a UASB and an anaerobic filter in the top part of the UASB reactor can overcome the disadvantages of both of the configurations. There are numerous examples of such hybrid systems applied in the industry which make use of both of the systems hybridized.

Two-Phase Systems : Different groups of bacteria which can show variations with respect to physiology, nutritional requirements, growth, metabolic characteristics and sensitivity to environmental conditions play role in the anaerobic biodegradation of organic matter (Ghosh et al, 1975; Ince Kasapgil et al., 1995; Ince et al., 1995; Ince Kasapgil et al., 1997). Environmental conditions can be optimized for the acid and methane-forming bacteria by using two completely mixed biochemical reactors in series in two-phase systems. Although there are numerous chemical and physical separation techniques, it is generally accepted that the most appropriate method to achieve this is by means of kinetic control which provides the required growth rates of each in separate reactors (Pohland and Ghosh, 1971; Ghosh et al, 1975; Kasapgil et al., 1995). Although 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.

2.6. Environmental Factors Affecting Anaerobic Treatment Processes

2.6.1. 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 processes have been shown to be strongly affected by the temperature variations. Especially methane conversion of acetate to CH_4 is known as more sensitive to temperature than the acetate forming process (Stover et al., 1994). Methane production has been documented under a wide range of temperatures. In municipal wastewater treatment plants, 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. Thermophilic digestion operates at temperature ranges of 50-65°C. It allows higher loading rates and is also conductive to greater destruction of pathogens. One drawback of thermophilic digestion is its higher sensitivity to toxicants. 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.

2.6.2. Hydraulic 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. The retention times of mesophilic and thermophilic digesters range between 25 - 35 days, since anaerobic treatment based on attached growth have a lower HRT (1-10 days).

2.6.3. pH

pH is an important parameter which affects the solubility of substances and the reaction behavior of microorganisms thereby influencing performance of anaerobic digestion. Most methanogenic bacteria function in a pH range between 6.1 and 7.5. Optimum pH values for some methanogens are given in Table2.6. Deviations from this optimum may result in excess production and accumulation of acidic or basic conversion products such as organic fatty acids or ammonia respectively. It is reported that pH below 6.0 are inhibitory to methanogenic bacteria 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). Acidogenic bacteria produce organic acids, which tend to lower the pH of the bioreactor (Malina and Pohland, 1992). Under normal conditions, this pH reduction by the acidogenic bacteria is buffered by the bicarbonate which is produced by methanogens. Under adverse environmental conditions, the buffering capacity of the system can be upset, eventually stopping the production of methane. Acidity is inhibitory to methanogens than of acidogenic bacteria. 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 2.6. Optimum pH for some methanogenic *Archaea* (Gerardi, 2003)

Genus	**Optimum pH Range**
Methanosphaera	6.8
Methanothermus	6.5
Methanomicrobiales	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

2.6.4. Mixing

Mixing ensures the absolute contact between the reactor contents and the biomass. It 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.

2.6.5. 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..6.6. Inhibitors

Inhibitory materials are ususally the leading cause of anaerobic reactor failures since they are present in substantial consantrations in wastewaters and sludges (Chen Ye, 2007). A wide range of inhibitors is responsible for the occasional failure of anaerobic digesters. A substance may be called inhibitory when it causes an adverse shift in the microbial community or inhibiton of bacterial growth. Inhibition is usually indicated by a decrease of the steady-state rate of methane gas production and accumulation of organic acids (Kroeker et al., 1979).

Literature on anaerobic digestion shows serious variation in the inhibition levels reported for most substances. The major reason for these variations is the complexity of the anaerobic digestion process where mechanisms such as antagonism, synergism, acclimation and complexing could affect the phenomenon of inhibition. (Chen Ye, 2007)

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).

Ammonia 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 range of 1500 mg/L and 3000 mg/L may have inhibitory effect on methanogens at higher pH values.

The methanogens are the least tolerant to ammonia inhibition among the four types of anaerobic microorganisms (Kayhanian, 1994). As ammonia concentrations were increased

in the range of 4000-5700 mg/L, acidogenic populations in the granular sludge were hardly affected while the methanogenic population lost 56.5% of its activity (Koster and Lettinga, 1988). There are two different aspects in the literature about the sensitivity of aceticlastic and hydrogenotrophic methanogens to ammonia. Some research based on the comparison of methane production and growth rate indicated that the inhibitory effect was in general stronger for the aceticlastic than for the hydrogenotrophic methanogens (Koster and Lettinga, 1984; Zeeman et al., 1985; Sprott and Patel, 1986; Bhattacharya and Parkin, 1989; Robbins et al., 1989; Angelidaki and Ahring, 1993; Borja et al., 1996), while others observed the relatively high resistance of acetate consuming methanogens to high total ammonia nitrogen levels as compared to hydrogen utilizing methanogens (Zeeman et al., 1985; Wiegant and Zeeman, 1986).

Sulfide Inhibition : Sulfate is a common constituent of many industrial wastewaters (O'Flaherty et al., 1998). In anaerobic reactors, sulfate is reduced to sulfide by the sulfate reducing bacteria (SRB) (Koster et al., 1986; Hilton and Oleszkiewicz, 1988). Introduction of the waste streams and/or the biological production in the anaerobic digestion may cause the sulfides via reduction of sulfates or other sulphure-containing 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 mg/L 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 acclimation. Stronache et al. (1986) stated that sulphate concentrations in excess of 200 mg/L had a direct toxic effect on anaerobic systems.

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; 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 anaerobic processes is the accumulation of VFA produced by acidogenic bacteria. 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 including 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 inhibition of microbial growth was observed at 35 mg/L acetic acid and excess of 3000 mg/L propionic acid concentrations (Ionnati and Fisher, 1983). 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. Krocker (1979) reported that reactor failure can be generally expected at the concentrations above 10 mg/L of unionized acids.

Light Metal Ions Inhibition : Sodium, potassium magnesium and calcium are the important light metal ions in anaerobic systems. They are required for microbial growth and affect specific growth rate like any other nutrient (Chen et al., 2008). Moderate concentrations of these ions situmulate microbial growth in anaerobic systems. On the other hand, excessive amounts slow down the growth, and even higher concentrations can cause severe inhibition or toxicity (Soto et al., 1993b).

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. The heavy metals which have a particular concern include chromium, iron, cobalt, copper, zinc, cadmium, and nickel (Jin et al., 1998). Heavy metals are not biodegradable and can accumulate to potentially toxic concentrations (Sterritt and Lester, 1980).

Organic Chemicals : Many organic chemicals that are a source of food for anaerobic microorganisms at low concentrations can show inhibitory effects at higher concentrations.

A wide range of organic compounds can inhibit anaerobic degradation. 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 accumulation of apolar pollutants in bacterial membranes causes the membrane to swell and leak, disrupting ion gradients and eventually causing cell lysis (Heipieper et al., 1994; Sikkema et al., 1994). 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. Acetate-utilizing methanogenic cultures were used in the study for the analyses and found concentrations that resulted in a 50% reduction in gas production. Their results for selected compounds are summarized in Table 2.7. The study indicates concentrations that could cause problems in anaerobic treatment systems.

Table 2.7. Concentrations of organic compounds that reduce gas production by 50% (IC_{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

2.6.7. Methanol Inhibition in Anaerobic Processes

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.

Table 2.8. Basic properties of methanol

Molecular formula	CH_3OH
Molar mass	32.04 g/mol
Appearance	colorless liquid
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
Acidity (pK_a)	~ 15.5
Viscosity	0.59 mPa.s at 20 °C
Dipole moment	1.69 D (gas)

Methanol is a main pollutant in some specific wastewaters like the evaporator condensate of pulp and paper industries, pharmaceutical industry, coal-gasification plants, potato-starch producing gactories, and landfill leachates. Such wastewaters can be treated anaerobically (Berube and Hall 2000, Minami et al., 1991, Paulo et al., 2001, Yamaguchi et al., 2001).

Under anaerobic conditions methanol can be directly metabolised by methanogens and homoacetogens. It can also be oxidised to H_2 and CO_2, provided a low hydrogen partial pressure is sustained by hydrogenotrophic methanogens. 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., 1979, 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 13C-labelled substrates and specific inhibitors it was shown that in an anaerobic methanol-fed thermophilic bioreactor, about 50% of the 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).

Methanol is degraded by methanogens, sulfate reducing bacteria and homoacetogens. In mixed cultures methanol potentially supports a complex food chain as shown in Figure 2.14. The important groups that plays key a role in degradation of methanol in anaerobic reactors are as follows:

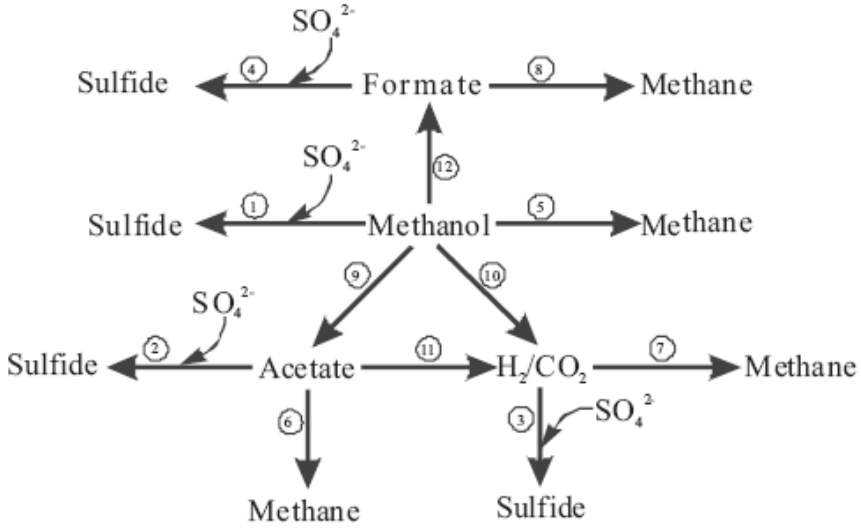


Figure 2.13. Anaerobic methanol mineralization (Weijma et al., 2000).

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 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 (Weijma et al., 2001).

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.

Table 2.9. 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.

Mixed cultures : 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.

Competition for methanol : Florencio (1994) studied the competition between methanogens and homoacetogens for methanol in mesophilic UASB reactors in detail. The Ks 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.

Anaerobic treatment of pharmaceutical wastewater containing organic solvents such as methanol, isopropanol, ethyl acetate and methylene chloride etc. has been investigated by using upflow anaerobic filter (UAF) continous stirred tank reactor (CSTR)

and hybrid reactor configurations (Ince et al., 2002, Oz et al., 2004; Oktem et al., 2008) It has been reported that the hybrid UASB and UAF reactors may become a preferred option for this kind of wastewaters containing organic solvents. However, the CSTR did not performed well and was severely affected by the nature of the raw pharmaceutical wastewater. These distinct differences in the results of similar studies in terms of feeding strategy and wastewater type could be attributed to different reactor configurations.

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 cause 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. 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. IC_{50} concentrations have been found to be 950 mM for a granular sludge treating citric for methanol acid, 1350 mM for a sludge treating alcohol distillery wastewater and 400 mM for a non-granular sludge from a cattle manure.

3. AIM OF THE STUDY

Anaerobic treatment is a favorable option for a wide range industrial wastewaters such as pharmaceutical, pulp and paper, food and beverage, petrochemical , alcohol dairy, distilleries, textile, leachates. It is also used for municipal solid wastes, wastewaters, agricultural wastes and manures. However, inhibitory wastewaters containing organic solvents may affect activity and composition of methanogens in anaerobic bioreactors. Treatment of the inhibitory waste streams by anaerobic processes is still being investigated. Defining effect of organic solvents on both microbial community structure and activity changes can lead to improvements in the understanding of bioreactors treating wastewaters containing organic solvents, thereby obtaining better reactor performance in terms of higher degradation capacity with higher biogas production. For a better understanding of the processes, FISH and SMA tests were used as key procedures to evaluate effects of organic solvents on microbial community and methanogenic activity. Therefore, the study, aims to investigate the effects of methanol on methanogenic activity and quantative distribution of methanogenic *Archaea*.

4. MATERIALS AND METHODS

4.1. Operation of Lab-Scale Anaerobic Batch Reactor

In this study, a laboratory-scale glass reactor with an active volume of 1.6 L was used as an anaerobic batch reactor. During the study, the reactor was kept in a water bath to control the temparature stabile at 35±2 °C. Mixing was provided by magnetic stirrers, which run at a speed of 90 rpm. The anaerobic batch reactor was operated for 47 days. The pH of the reactor was maintained between 6.8-7.2. Influent COD of the anaerobic reactor was increased gradually from 2500 mg/L to 9000 mg/L, resulting in an increase of substrate to biomass ratio (S_o/X_o) from 0.25 to 0.9. Total solid (TS) and total volatile solid (TVS) concentration of the lab-scale anaerobic batch reactor sludge was 13500 mg/L and 10000 mg/L respectively.

4.2. Wastewater Characteristics

A synthetic wastewater made up of glucose as main sole carbon and energy source was used for feed. 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; $CoCl_2.6H2O$, 2000 mg/L; $MnCl_2$, 318 mg/L; $CuCl_2$, 24 mg/L; ZnCl2, 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 liter synthetic wastewater.

4.3. Seed Sludge Characteristics

Inoculum sludge was taken from a full-scale EGSB reactor used during the anaerobic stage of a two stage anaerobic-aerobic biological treatment system at a brewery. Total wet volume and volumetric loading rate of full-scale are 2280 m^3 and 15 kg COD/

m^3, respectively. Reactor diameter is 14 m and total height is 15.5 m. 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 L^{-1} $CaCO_3$ respectively. Total solid (TS) and total volatile solid (TVS) concentration of the anaerobic granular sludge was 80 000 mg/L and 70 000 mg/L respectively.

4.4. Analytical Techniques

During this study pH, COD were monitored in representative intervals. Gas compositions for SMA tests were monitored via computerized unit. Gas compositions for SMA tests were determined using a Hewlet Packard 6850 gas chromatograph (GC) with a thermal conductivity detector (HP Plot Q column 30 m x 530 μm). Due to the granular characteristics of the reactor sludge, total solids and total volatile solids (TS/TVS) were measured. During the SMA tests, suspended solids and volatile suspended solids (SS/VSS) were measured. All analyses were carried out according to Standard Methods (APHA, 1997).

4.5. Specific Methanogenic Activity Tests

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 solenoid 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 send an electrical signal to a control interface that activated the three-way solenoid 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 (3s 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. A photographic view and experimental set-up of SMA test unit are shown in Figures 4.1 and 4.2, respectively. The device used for calibration of the eight digesters with their respective gas flow meters was carried out by using a very sensitive Health Care Pump.

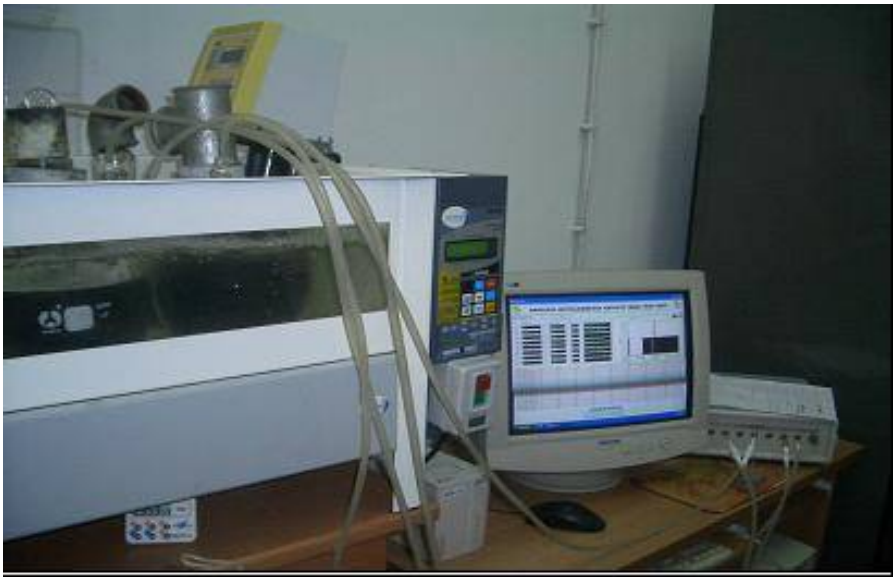


Figure 4.1. Photographic view of SMA test unit.

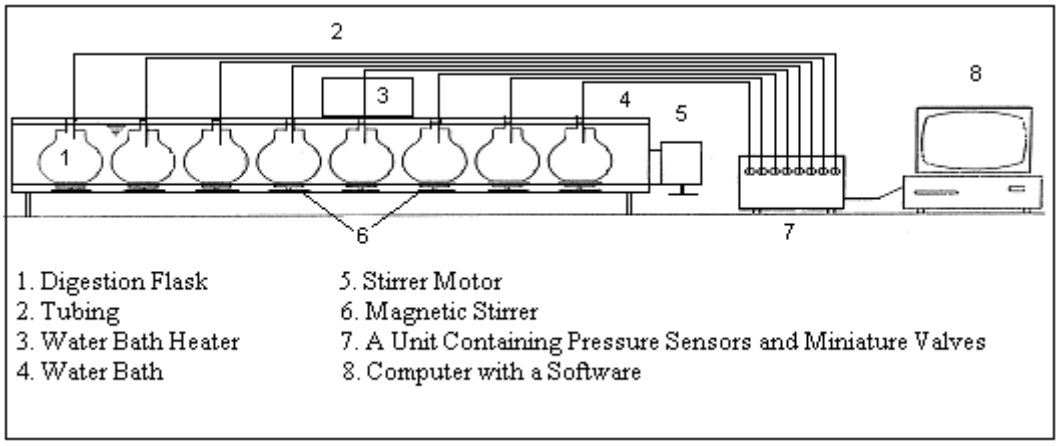


Figure 4.2. Experimental set-up for SMA test unit

4.5.1. Experimental Procedure for Specific Methanogenic Activity Test

The laboratory routine for SMA test is given as follows: The volatile suspended solid content (VSS) of the sludge sample to be analyzed was determined before the test was started (preferably 12 hour in advance). The concentration of volatile suspended solid (VSS) in the reactors was brought about 2000 mg/L by diluting sludge sample with a mineral stock solution given in Table 4.3. The pH of the reactors were adjusted to 7.0. Reactors were flushed with helium gas about a period of 10 minutes to maintain anaerobic conditions in the reactor. The taps of the reactors were closed immediately after flushing and all connections of the SMA test were greased in order to prevent air leakage. Temperature of the reactor content was kept at 35±0.5 °C by heating the water bath. The samples were acclimatized for 12-16 hours. The gas production during the acclimatization period was neglected. After the acclimatization period acetate was introduced to the SMA reactors as a substrate. Mixing system was opened and data collection system was reset. Biogas production was saved automatically for every hour. Methane concentration was determined at regular intervals by taking 1 mL gas sample. The volume of methane produced per unit of time is calculated using Equation 4.1.

Table 4.1. Mineral stock solution for methanogenic activity tests
(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

4.5.2. Feed and Seed for Methanogenic Activity Tests

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. Approximately 72% of the methane formed during anaerobic digestion of complex substrate results from acetic acid (McCarty, 1964). Acetate concentrations in range of 1000-4000 mg/L were initially tested in order to find maximum methanogenic activity during the SMA tests. 2000 mg/L acetate concentration was found to be optimum. Finally, a VFA mixture of 2000 mg/L acetate, 500 mg/L propionate, 500 mg/L butyrate was used to measure overall methanogenic activity (Soto et al., 1993).

SMA reactors were seeded with two different sludges, namely seed sludge and lab-scale anaerobic batch reactor. The seed sludge from the EGSB reactor was inoculated into the lab-scale anaerobic batch reactor. After 47 days of operation period, sludge samples were taken for SMA tests. All samples were diluted to 2000 mg VSS/L for SMA tests as it was described in the laboratory routine.

4.5.3. Calculation of Specific Methanogenic Activity

The gas produced in the reactor was sent to a gas-washing flask. The methane content of the gas was measured by gas chromatograph. The potential methane production was calculated by the formula expressed below:

Specific Methanogenic activity was calculated as:

$$\text{SMA (mLCH4/gVSS.d)} = (A \times B \times C \text{ x } 24) / (D \times E) \quad (4.1)$$

A: Biogas production per hour (mL/h)
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)

4.6. Fluorescent In Situ Hybridization (FISH)

4.6.1. Sampling and Short Term Fixation

Three different types of sludge samples were collected for FISH studies: (1) from the bottom level of the full scale EGSB reactor, (2) from lab-scale anaerobic batch reactor and (3) from methanol added SMA reactors. Then, the samples were transferred into sterile containers with the addition of absolute ethanol (1:1, v/v) on-site immediately. The samples were stored at -20°C and standart paraformaldehyde fixation carried out within 3 days.

4.6.2. 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.

4.6.3. Hybridization

16S rRNA-targeted oligonucleotide probes used in this study and their target microbial groups nucleotide sequences are listed in Table 4.4 and Figure 4.3. Optimal hybridization conditions for each probe are also given in Table 4.5. All probes were obtained commercially (Qiagen Corp.).

Table 4.2. 16S rRNA-targeted oligonucleotide probes used in this study

Probe	**Target Group**	**Probe sequence (5'-3')**	**Labelling (5')**	**Reference**
MC1109	*Methanococcales*	GCAACATAGGGCACGGGTCT	CY3	Raskin et al., 1994a
MB310	*Methanobacteriales*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin et al., 1994a
MG1200	*Methanomicrobiales* relatives	CGGATAATTCGGGGCATGCTG	CY3	Raskin et al., 1994a
MSMX860	*Methanosarcinaceae*	GGCTCGCTTCACGGCTTCCCT	CY3	Raskin et al., 1994a
MS1414	*Methanosarcina* + relatives	CTCACCCATACCTCACTCGGG	CY3	Raskin et al., 1994a
MS821	*Methanosarcina*	CGCCATGCCTGACACCTAGGCCAGC	CY3	Raskin et al., 1994a
MX825	*Methanosaeta*	TCGCACCGTGGCCGACACCTAGC	TAMRA	Raskin et al., 1994a
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	CY3	Stahl et al., 1988
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	Fluorescein	Amman et al. 1990a
UNIV1392	Virtualy all known organisms	ACGGGCGGTGTGTAC	TAMRA	Pace et al., 1986
NON338	Non sense probe	ACTCCTACGGCAGGCAGC	TAMRA	Manz et al., 1992

For each sample hybridization, two negative controls were prepared; one of these controls was used to assess non-specific binding (with Non338 probe), 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 three EGSB sludge samples were also stained using DAPI staining to visualize intact cells in the samples.

Table 4.3. Optimum hybridization conditions for oligonucleotide probes (Kolukirik, 2004)

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

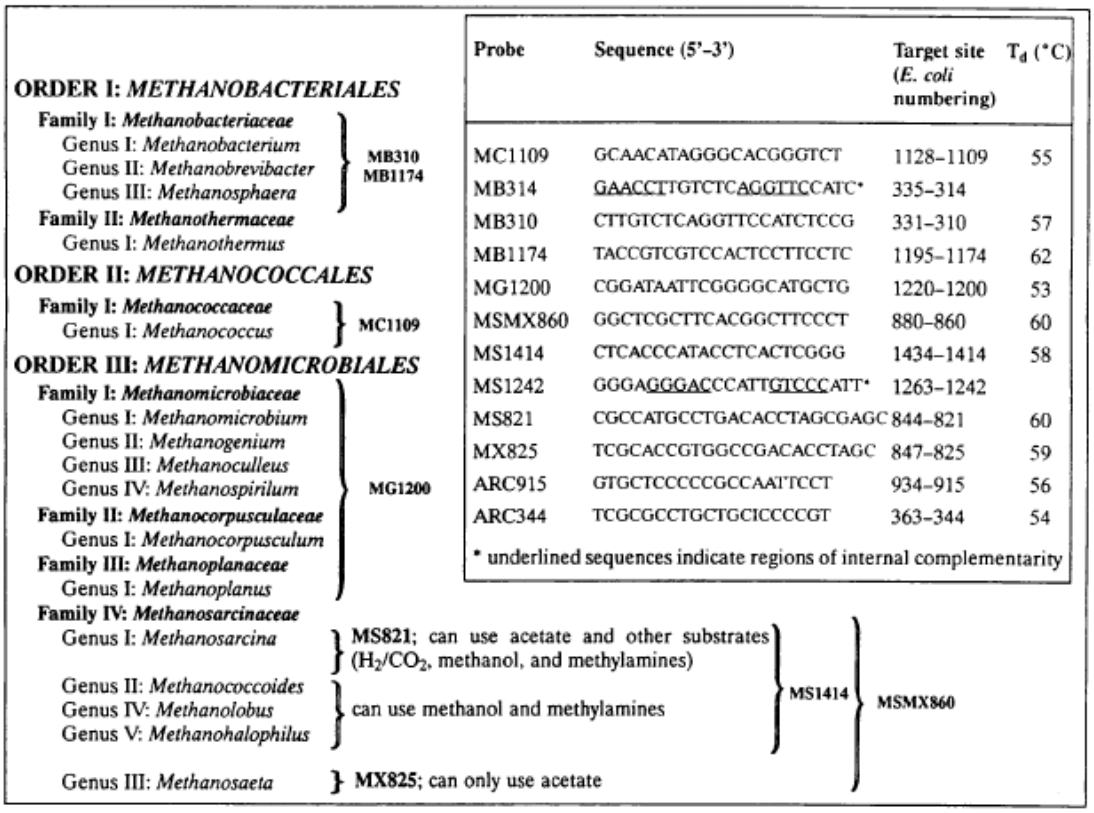


Figure 4.3. Classification of methanogens in relationship to the oligonucleotide probes characterized (Raskin et al., 1994)

200µL of the fixed samples were washed twice with PBS and once with MilliQ water. Then the fixed samples dehydrated at room temperature in increasing concentrations of ethanol (50, 80, and 100%). Dehydrated samples were resuspended in 40µL of hybridization buffer (0.9M NaCl, 2mg/mL Ficoll, 2mg/mL Bovine Serum Albumen, 2mg/mL polyvinyl pyrrolidone, 5mM EDTA, pH 8.0, 25 mM NaH_2PO_4, pH 7.0, 0.1% SDS, 5-35% deionised formamide) and prehybridized at the intended hybridization temperature for 20 minutes. After prehybridization, 2µL of probe (50 ng/µL) was added and incubated at the optimal hybridization 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 (1985) for 15 min at the optimal washing temperature before a final wash in MilliQ water. Optimum hybridization conditions for each probe used in this study are given in Table 4.5. The cells were resuspended in 200µL of MilliQ water, and a 10 µL aliquot was placed on a gelatin-coated slide and air dried. 10 µL of DABCO (1,4-diazabicyclo[2.2.2]octane) [Sigma D-2522]: 0.233g DABCO800 µL ddH_2O 200 µL TRIS-HCl (pH=7.2) was added to the cells, and a coverslip was applied to the preparation and sealed with nail polish before epifluorescence microscopy.

4.6.4. DAPI Staining

The total cells present in the samples were previously determined by counting 4, 6-diamin phenylindol (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 MilliQ 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 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 MilliQ water for 1 minute

in each of them. Slides were dried in incubator and covered with lamel by enamel.

4.6.5. Visualization

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 6.3 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 4.5.

Table 4.4. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used

Fluorochrome	**Color of Fluorescence**	**Maximum excitation wavelength (nm)**	**Maximum emission wavelength (nm)**	**Filter cube used**
FLUOS	Green	494	518	U-MWIB
TAMRA	Orange	555	580	U-MWG
CY3	Red	552	565	U-MWG
DAPI	Blue	365	397	U-MWG

4.6.6. Quantification

Quantification of microoorganisms in the sludge samples collected was conducted using Image-Pro Plus 6.3 image analysis software (Figure 3.4). Quantification involves counts of total microorganisms with DAPI staining and counts of specific methanogenic groups with other oligonucleotide probes using FISH.

For each sample, firstly DAPI Stain was used to determine the average number of total microorganisms. For all times, counts for 10 random fields of view were obtained for eah sample, and the average cell count was calculated. Average of the counts gave the representative number of total microorganisms in each sample.

Quantification of methanogens involves application of FISH with oligonucleotide probes specific for different methanogenic groups given in Table 4.4. For all times, counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated. Hence, a representative number of microorganism in each group were found.

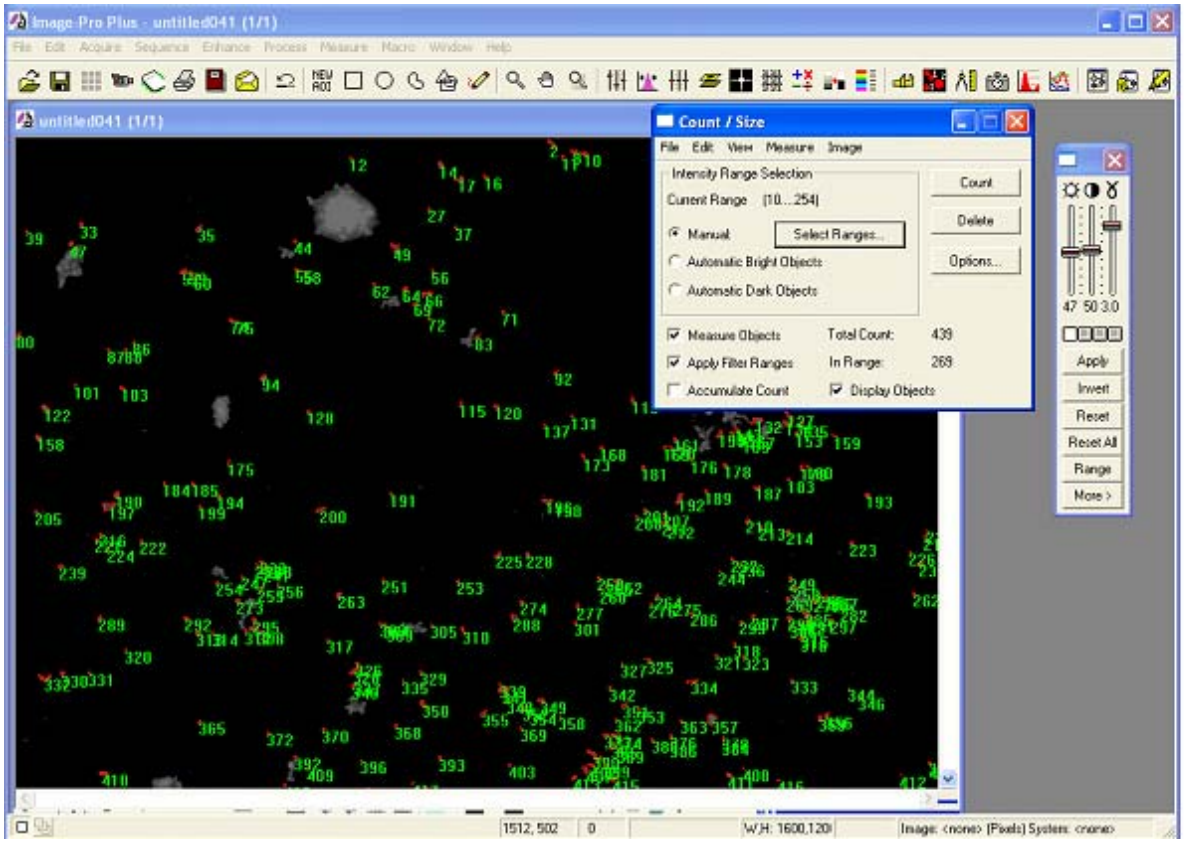


Figure 4.4. Capture image of Image Pro Plus 6.3

5. RESULTS AND DISCUSSION

5.1.Performance of the 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 methanol effect on methanogenic activity and microbial community.

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 with a subsrtate to biomass ratio of 0.9. 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 5.1. shows the performance of the lab-scale anaerobic batch reactor.

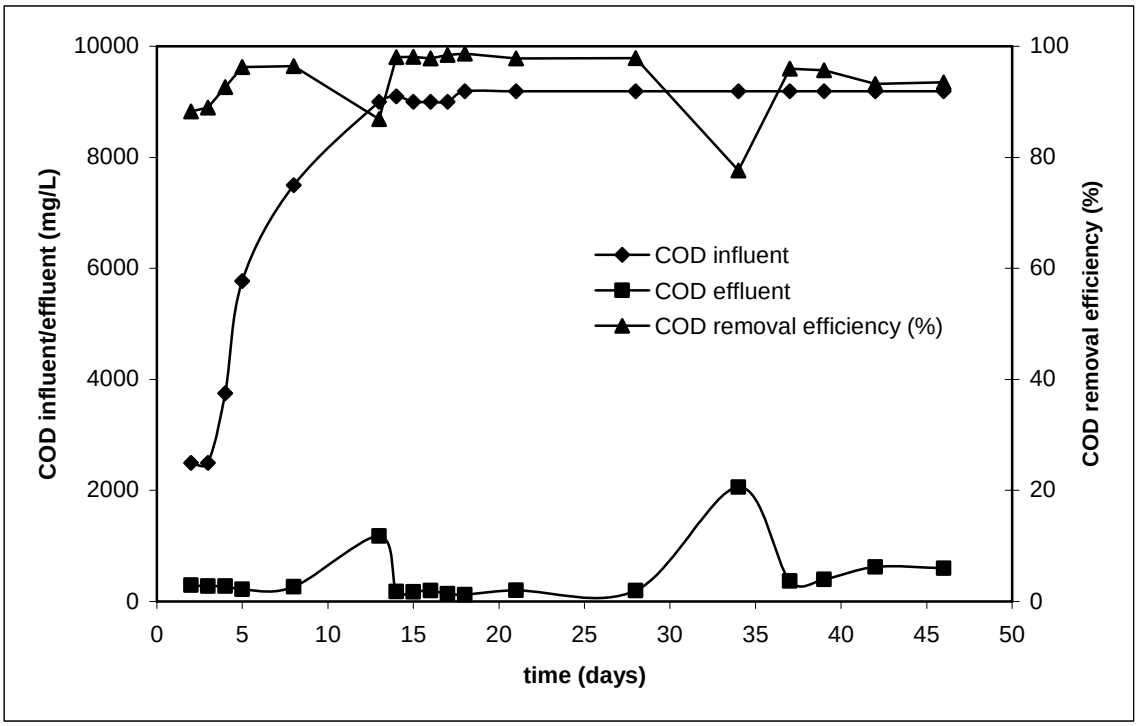


Figure 5.1. Performance of lab-scale anaerobic batch reactor

5.2. Assessing Methanol IC_{50} for SMA Tests and FISH Studies

SMA tests were carried out with the seed sludge from the EGSB reactor to determine methanol concentration that resulted in 50% inhibition in SMA (IC_{50}), which was calculated from the linear regression of SMA as a function of methanol concentration. Methanol concentrations ranging from 0.1 M to 1.5 M were used and 0.4 M was found to be the IC_{50} that resulted in 50% inhibition. 2000 mg/L acetate was used as substrate in the SMA reactors and a control reactor without any solvent was used for each test unit. The results are given in Figures 5.2 and 5.3. 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 methanol concentration that is found to be IC_{50} value, was used in the SMA tests to determine the effects of methanol on methanogenic activity. Then, methanol-induced inhibition of acetoclastic and hydrogenotrophic methanogenic populations were determined for each sludge to evaluate the methanol effect on these microbial populations by FISH studies.

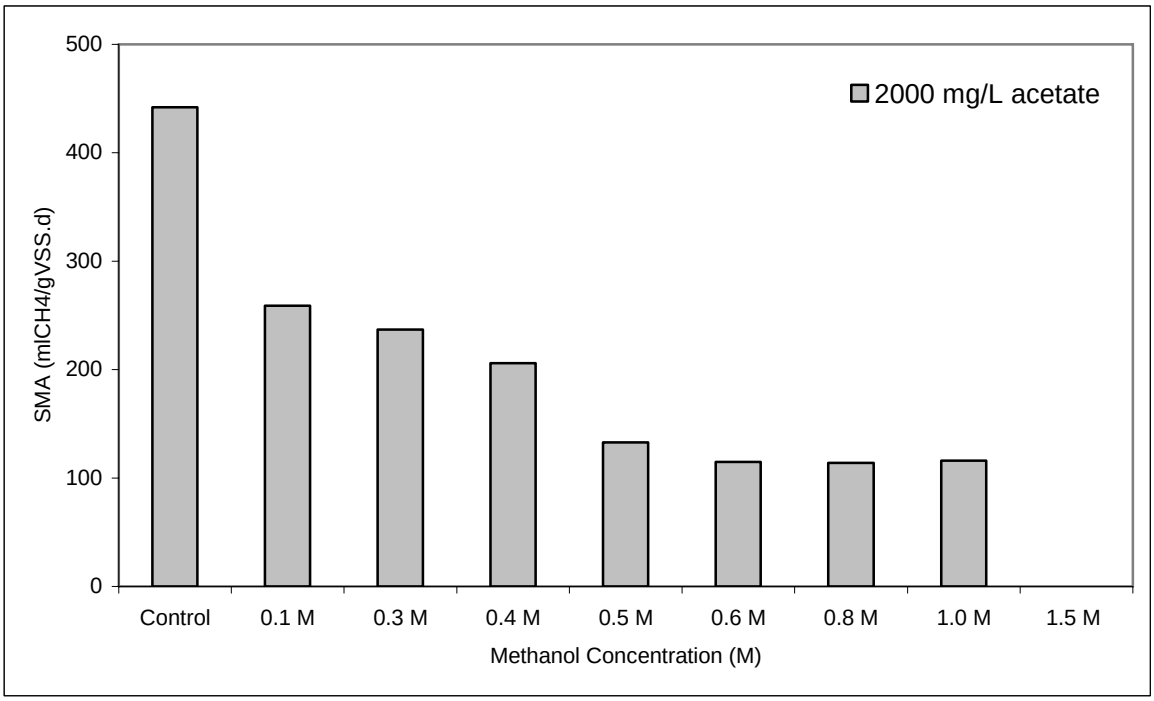


Figure 5.2. Specific methanogenic activity of seed sludge at various methanol concentrations

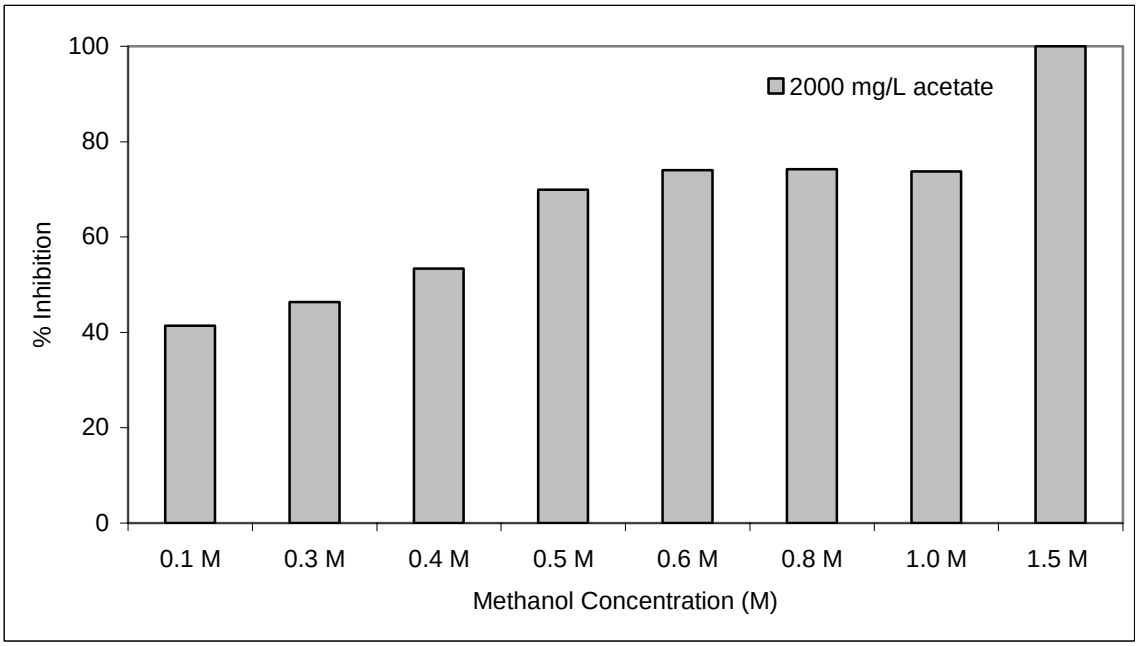


Figure 5.3. Percent inhibition on SMA at various methanol concentrations

5.3. Methanogenic Activity Test Results

Methanogenic activity tests had initially been carried out to determine maximum methanogenic activity of the seed sludge, then lab-scale anaerobic batch reactor sludge and finally methanol added anaerobic sludge from the anaerobic batch reactor.

Two different substrates were used to determine specific methanogenic activity. 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. Firstly, acetate was used as substrate in order to measure the potential acetoclastic methanogenic activity. Then, 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., 1993a).

In a previous study, 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)

5.3.1. Methanogenic Activity of Seed Sludge

SMA tests were applied to the seed sludge that was taken from the EGSB reactor. According to the results, maximum acetoclastic activity of the seed sludge was found to be 453 mLCH4/gVSS.d at 2000 mg/L acetate (Figure 5.4). Compared to anaerobic sludge activities reported in the litrerature (>300 mLCH4/gVSS.d), 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 mLCH4/gVSS.d (Figure 5.5).

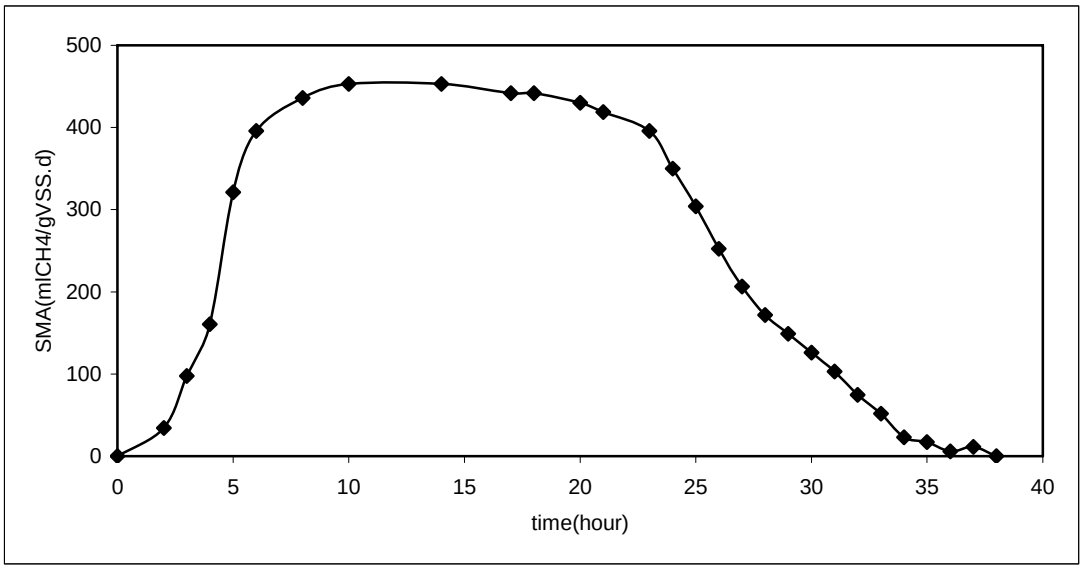


Figure 5.4. Acetoclastic methanogenic activity of the seed sludge (2000 mg/L acetate concentration)

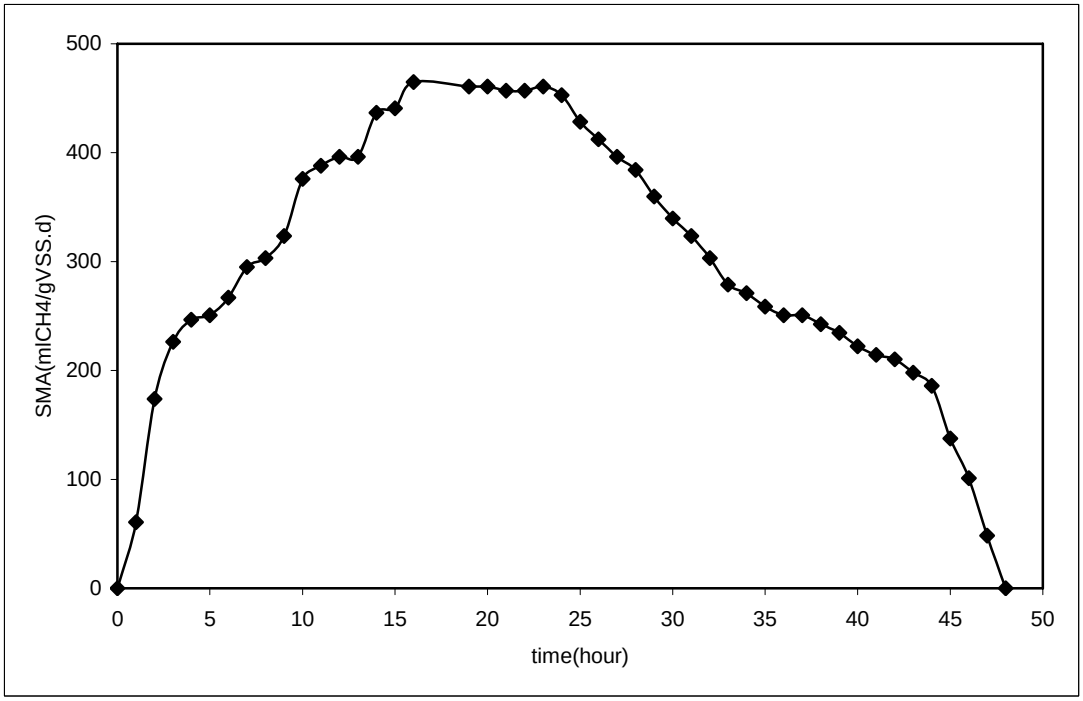


Figure 5.5. Overall methanogenic activity of the seed sludge (VFA mixture:2000 mg/L acetate, 300 mg/L butyrate, 500 mg/L propionate)

5.3.2. Methanogenic Activity of 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 mLCH4/gVSS.d at 2000 mg/L acetate and 490 mLCH4/gVSS.d, respectively as illustrated in Figures 5.6 and 5.7.

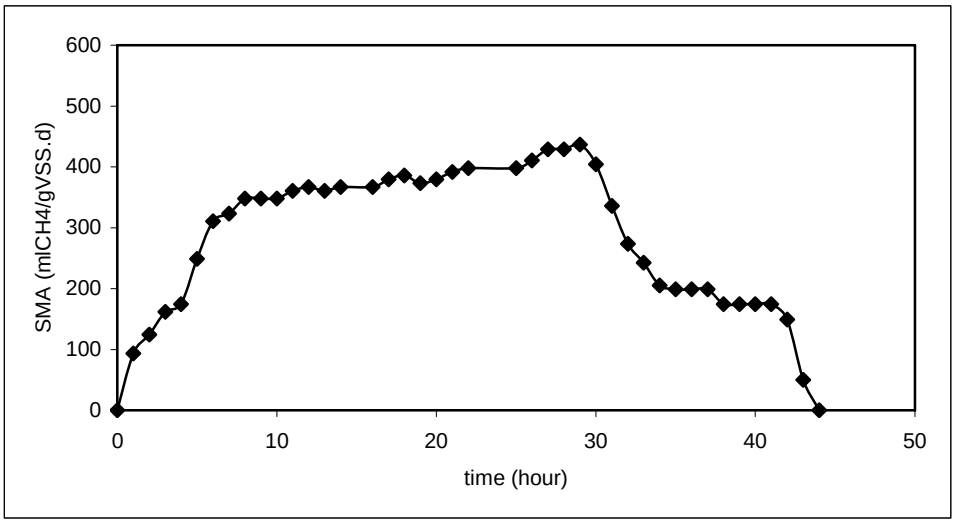


Figure 5.6. Acetoclastic methanogenic activity of the lab-scale anaerobic batch reactor

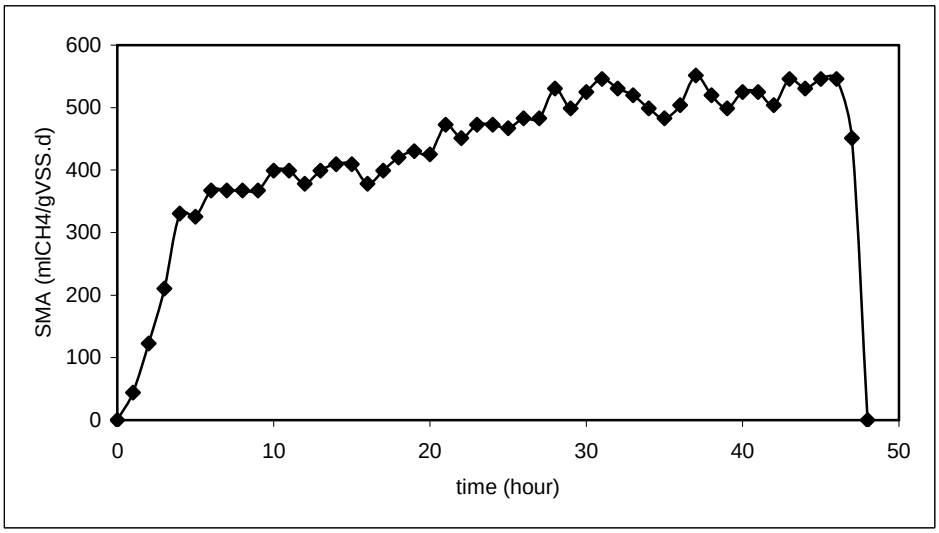


Figure 5.7. Overall methanogenic activity of the lab-scale anaerobic batch reactor

5.3.3. Effect of Methanol on Methanogenic Activity of Anaerobic Sludge

Methanogenic activity tests had been carried out to determine the effect of methanol on anaerobic sludge which was taken from the lab-scale anaerobic batch reactor. 0.4 M methanol concentration was applied to the SMA reactors, as it was previously found that 0.4 M methanol concentration inhibited 50% of the methane production in the SMA test reactors.

According to SMA test results, acetoclastic methanogenic activity of the 0.4 M methanol added anaerobic sludge was found to be 252 mLCH4/gVSS.d (Figure 5.8). When the results of SMA test carried out with anaerobic reactor sludge were compared with those, a 43 % decrease in acetoclastic activity was observed (Figure 5.10).

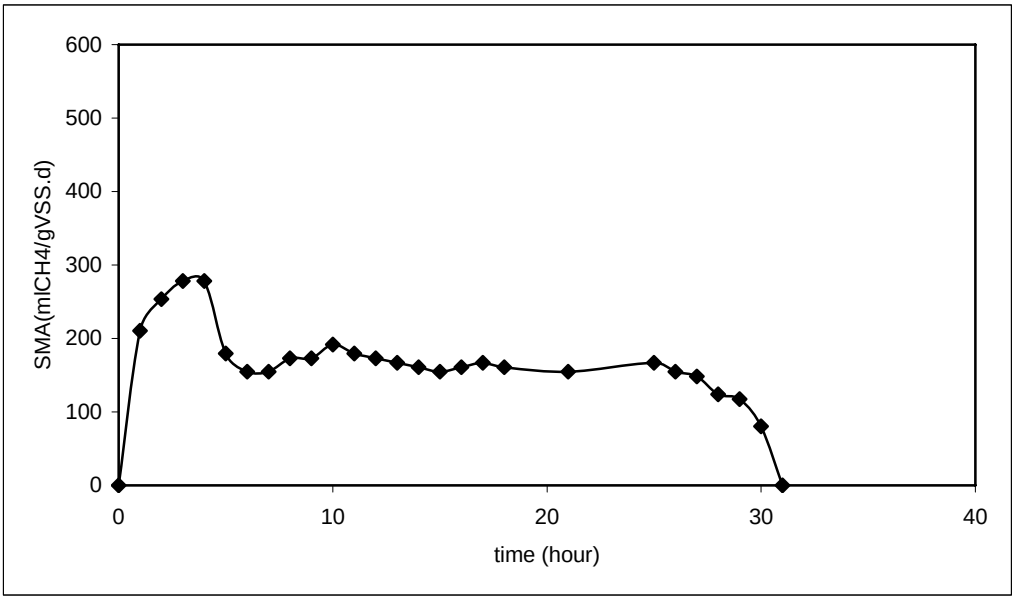


Figure 5.8. Acetoclastic methanogenic activity of SMA-Methanol-Acetate

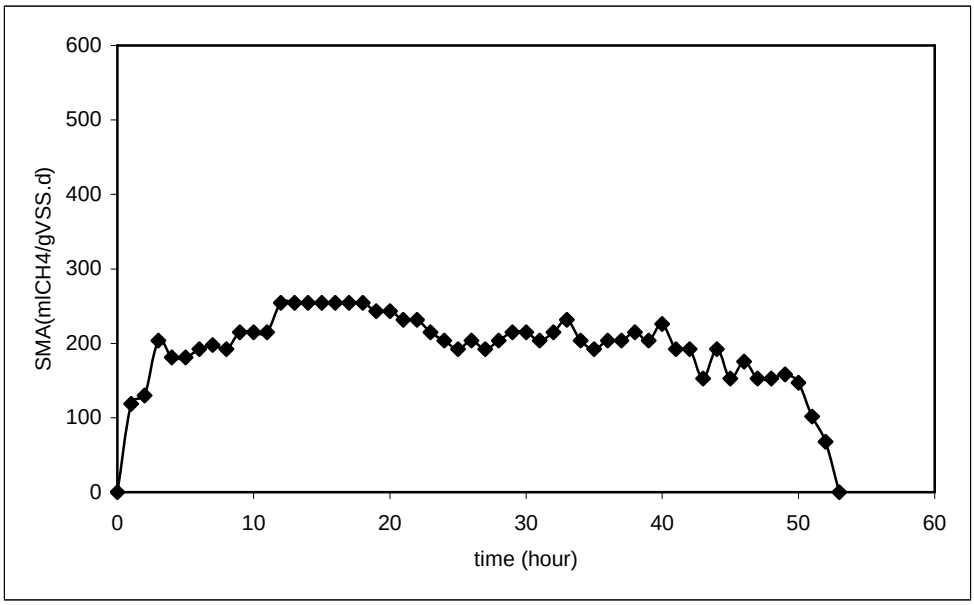


Figure 5.9. Overall methanogenic activity of SMA-Methanol-VFAmix

The overall methanogenic activity of the 0.4 M methanol added anaerobic sludge was found to be 221 mLCH4/gVSS.d (Figure 5.9). Similarly, a dramatic decline was observed in the overall methanogenic activity when compared with the anaerobic batch reactor sludge. Overall methanogenic activity decreased from 490 mLCH4/gVSS.d to 221 mLCH4/gVSS.d, indicating a 55% decrease (Figure 5.11).

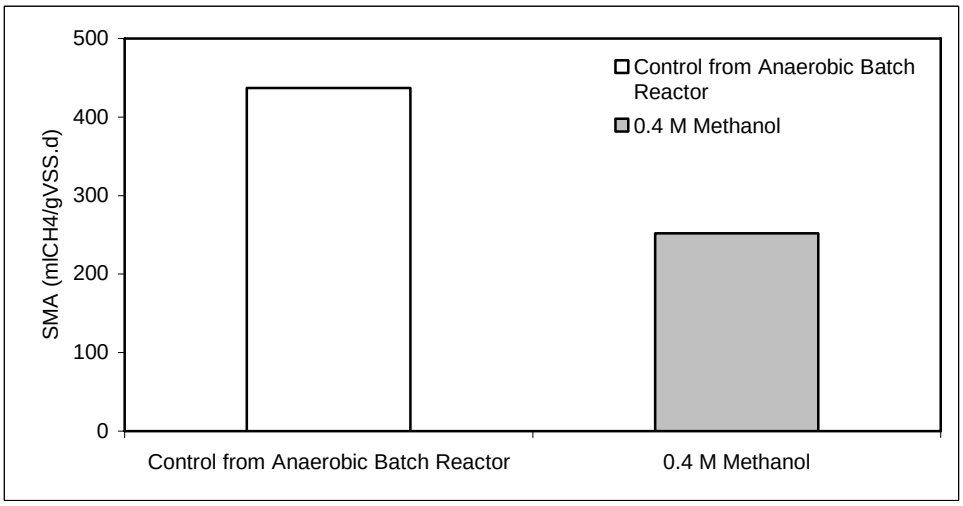


Figure 5.10. Methanol effect on acetolastic methanogenic activity of lab-scale anaerobic batch reactor sludge (2000 mg/L acetate concentration)

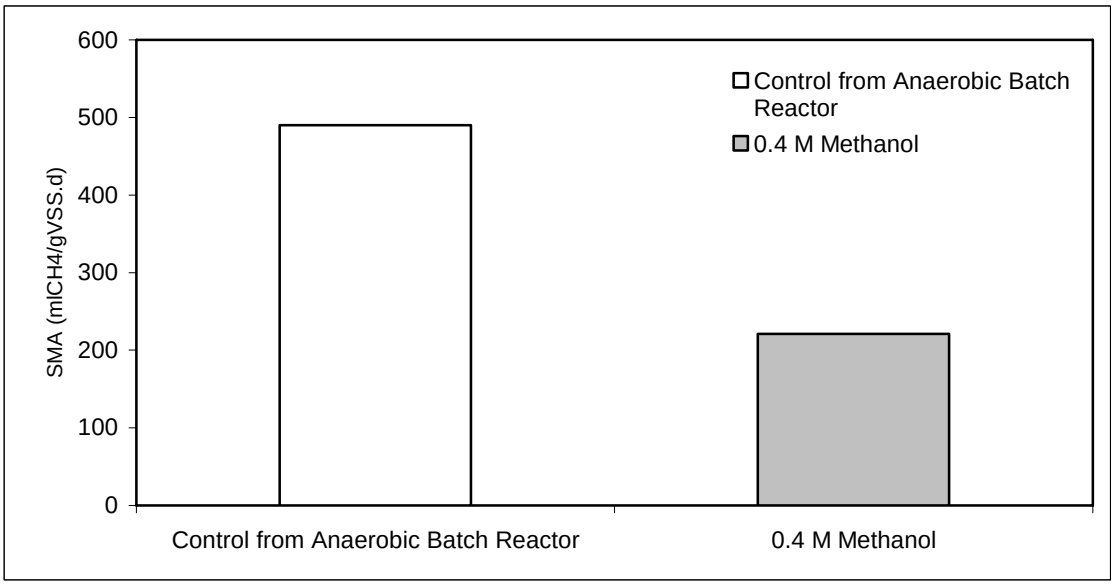


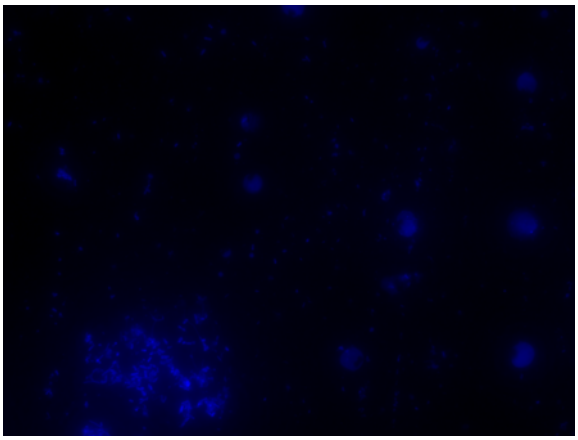
Figure 5.11. Methanol effect on overall methanogenic activity of lab-scale anaerobic batch reactor sludge (VFA mixture)

5.4. FISH Results

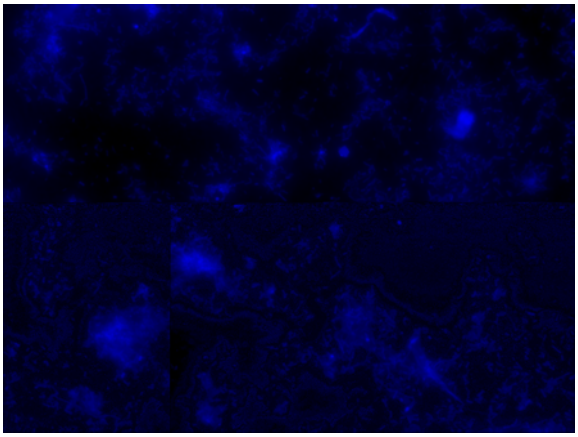
The microbial community structure of the seed sludge taken from the EGSB reactor, from lab-scale anaerobic batch reactor sludge and methanol-added SMA reactor sludges were characterized using fluorescent rRNA targeted oligonucleotide probes specific for Bacteria, *Archaea* and phylogenetically defined groups of Methanogens. 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.

5.4.1. FISH Results of Seed Sludge from the Full-Scale EGSB Reactor

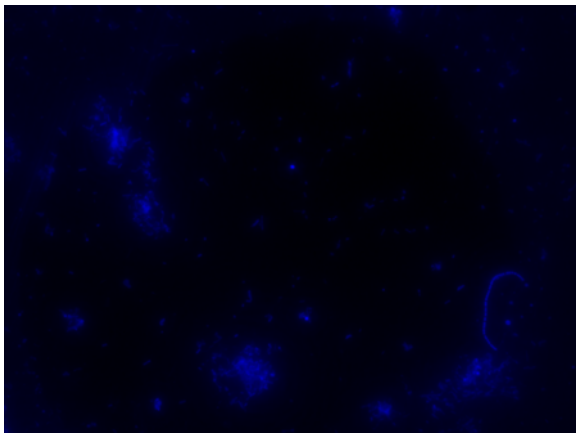
As mentioned above, before hybridization, DAPI staining was applied to the sludge samples to indicate intact cell concentration. 1/40 dilution factor and 5 µL sample volume were decided in accordance with the DAPI staining applied to the seed sludge from EGSB reactor. These dilution factors were decided to be optimum and applied to the FISH analyses that are done during the study. DAPI results of the seed sludge are shown in Figure 5.12.



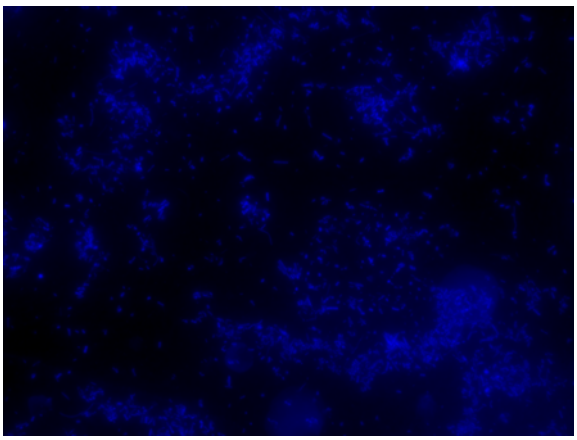
1/20 dilution factor, 5 µL sample



1/20 dilution factor, 10 µL sample



1/40 dilution factor, 5 µL sample



1/40 dilution factor, 15 µL sample

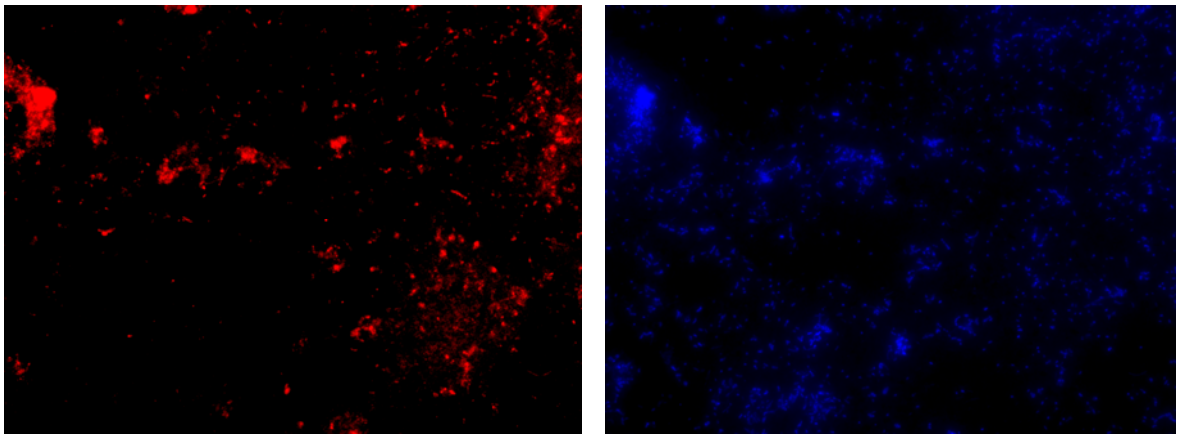
Figure 5.12. DAPI results of seed sludge

As can be seen from Table 5.2., total active microorganisms of the seed sludge was found to be 80.1% ± 4.0 % (mean ± standard deviation) (with Univ1392 probe). 42.2% ± 2.0% of the seed sludge consisted of Eubacteria (with Eubmix probe), since 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), 18.4% ± 1.6% *Methanomicrobiales* (with Mg1200 probe). Epifluorescence micrographs of the seed sludge are shown in Figure 5.13.

The Archaeal subpopulation mostly were found to be *Methanosaeta* spp.. The high percentage of the acetoclastic methanogens also corresponded with the activity test results. Two acetate-utilizing methanogenic genera, *Methanosarcina* and *Methanosaeta*, have been identified as important methanogens in granular sludge from anaerobic reactors (de Zeeuw, 1984; Grotenhuis, 1988; Hulshoff, 1989; Schmidth, 1996). 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). *Methanosaeta* spp. are known to grow only on acetate (Jetten et al., 1992). 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 5 to 10 times higher (Jetten et al., 1992; Zinder, 1990). It is generally assumed that *Methanosaeta* spp.. improves granulation and result in more stable reactor performance; consequently, *Methanosaeta* spp. should be favored over *Methanosarcina* spp.. *Methanosaeta* spp.. was more numerous than *Methanosarcina* spp.. in the seed sludge that is used in this study as shown in Table 5.2, indicating the seed sludge used in this study is in good quality in terms of granulation and stabilization (Schmidt et al., 1999)

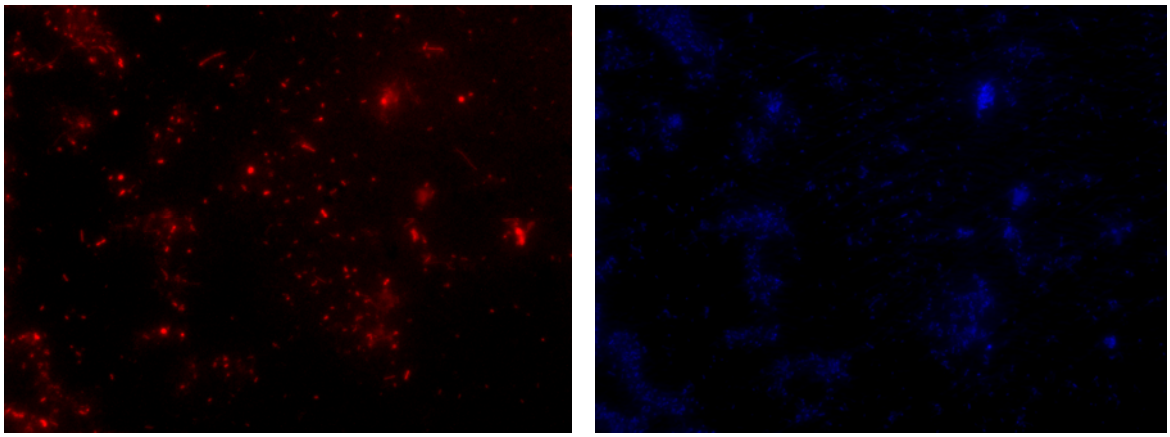
In the seed sludge, the percentage of *Methanomicrobiales* relatives (8.4% of the active microbial community) was higher than *Methanobacteriales* and *Methanococcales* relatives. In some studies in the literature *Methanobacteriales* relatives reported as dominant in hydrogenotrophic methanogens, while in some other studies *Methanomicrobiales* were reported as dominant. The predominance of

Methanomicrobiales and less abundance of *Methanobacteriales* and *Methanococcales* in a subpopulation of hydrogenotrophic methanogens are difficult to explain, since the competition for common substrates among different hydrogenotophic methanogens has been studied less extensively than the competition for acetate among acetoclastic methanogens (Raskin et al., 1996).



(a) (b)

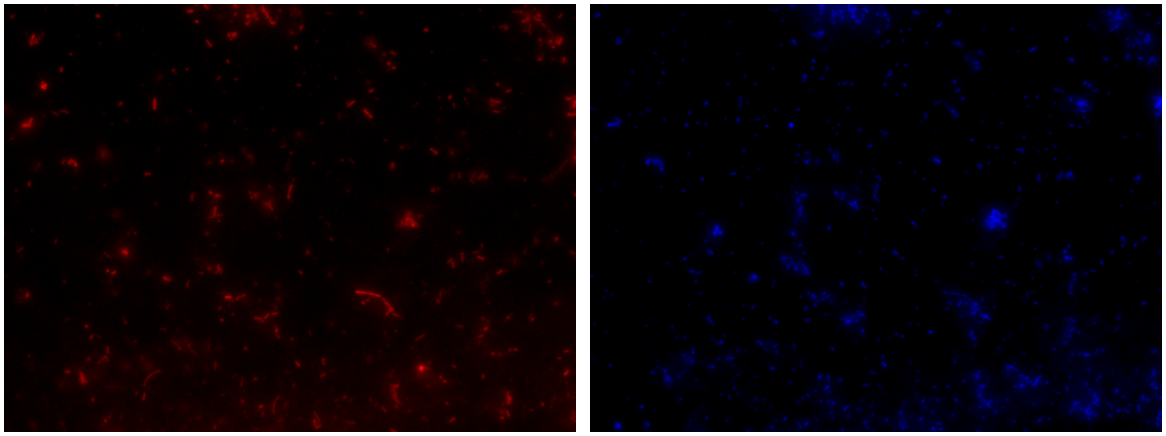
Active cells hybridized with UNIV1392 probe



(a) (b)

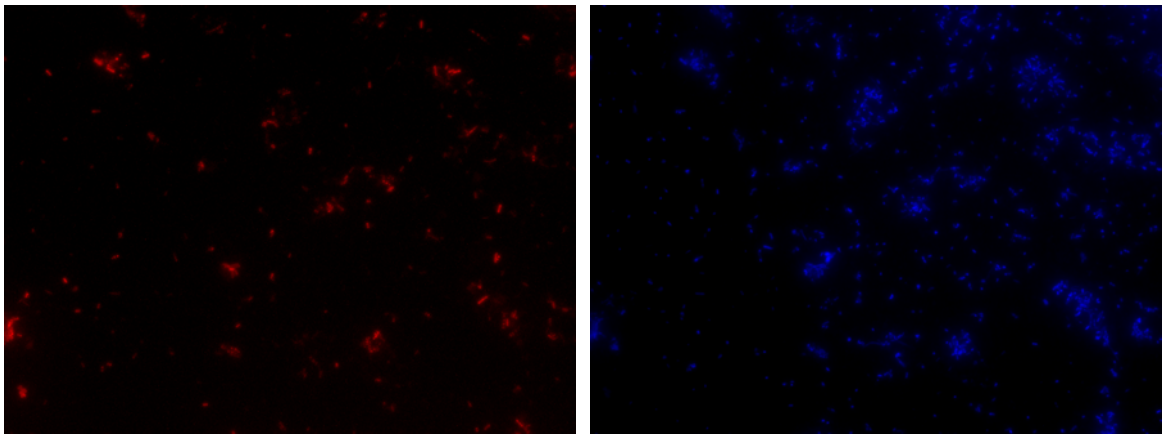
Eubacteria hybridized with EUBMIX probe

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



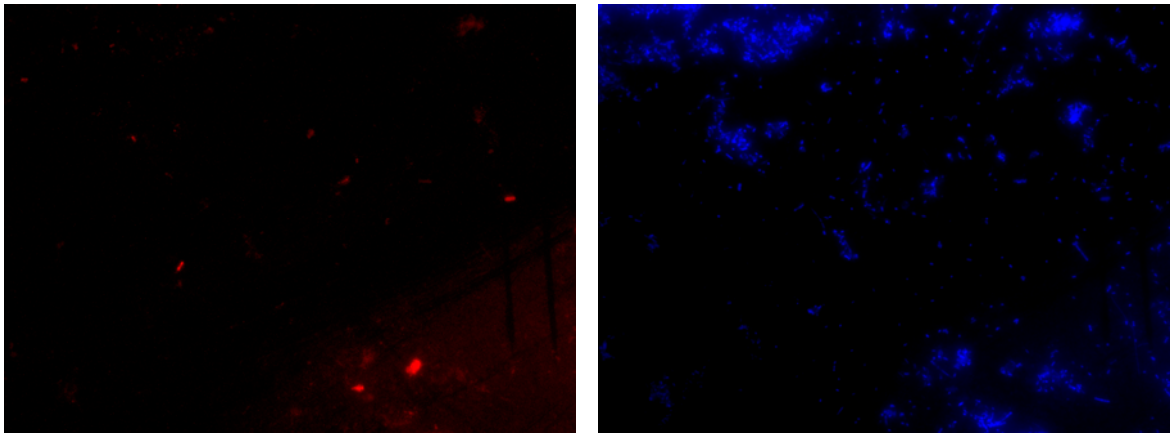
(a) (b)

Archaea hybridized with ARC195 probe



((a) (b)

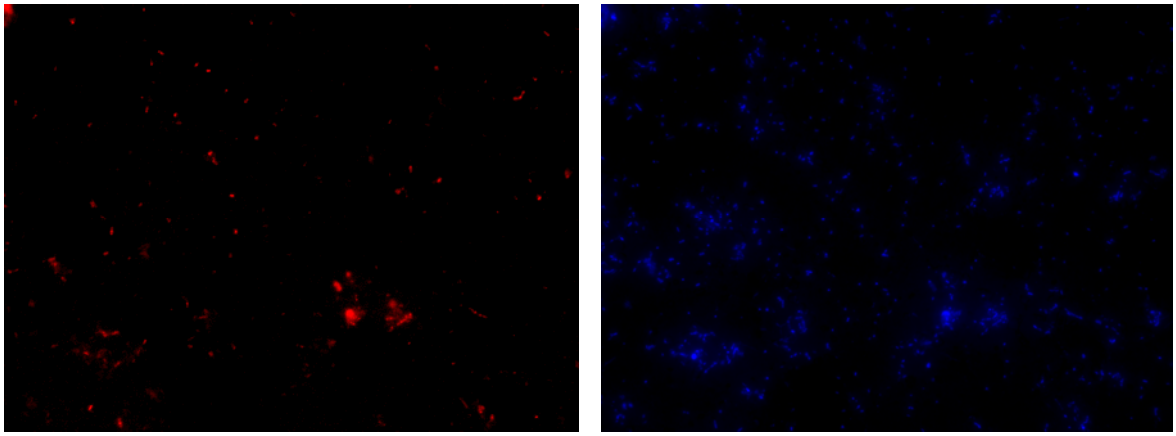
Methanosaeta hybridized with MX825 probe



(a) (b)

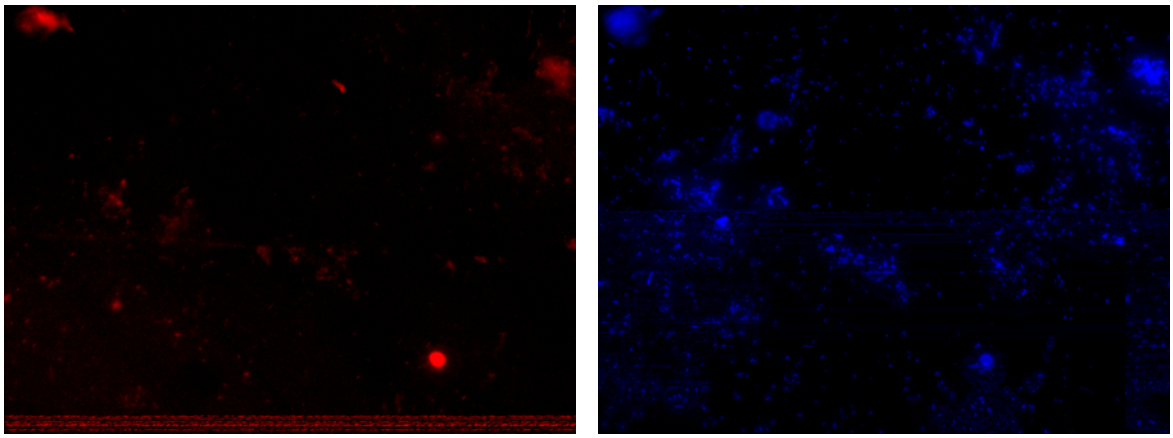
Methanosarcina hybridized with MS821 probe

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



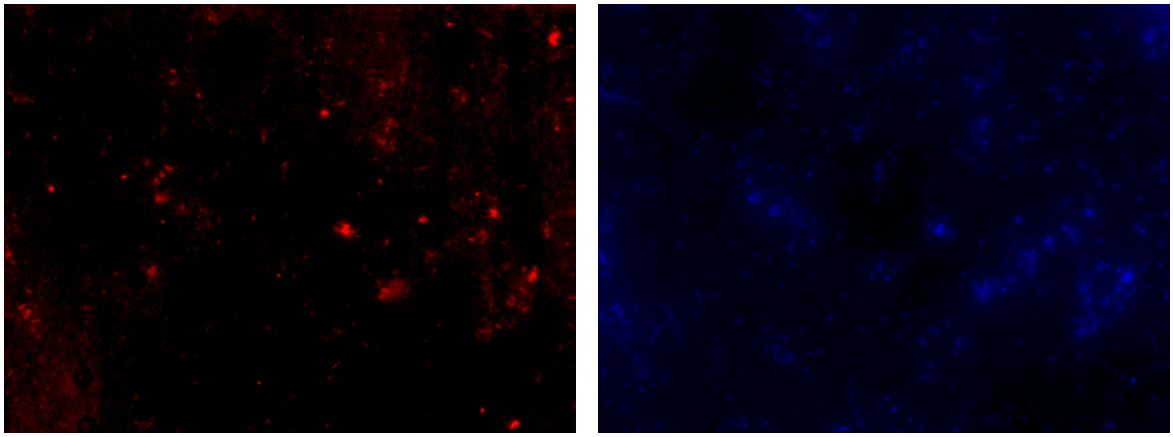
(a) (b)

Methanobacteriales hybridized with MB310 probe



(a) (b)

Methanococcales hybridized with MC1109 probe



(a) (b)

Methanomicrobiales hybridized with MG1200 probe

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

5.4.2. FISH Results of Sludge From the Anaerobic Batch Reactor

73.2% ± 1.2% of the cells in the lab-scale anaerobic batch reactor sludge gave positive signal with UNIV1392 probe, that is, 73.2% of the microorganisms were metobolically active. Bacterial and Archaeal population were detected 49.6 % ± 1.1 and 57.1 % ± 0.8 respectively. The Archaeal subpopulation 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). Epifluorescence micrographs of the anaerobic sludge from the anaerobic batch reactor are shown in Figure 5.17.

Table 5.1. FISH results of seed sludge and anaerobic batch reactor sludge

	Seed Sludge	Anaerobic Batch Reactor
Active Cells	80.1 ± 4.0	73.2 ± 1.2
Eubacteria	42.2 ± 2.0	49.6 ± 1.1
Achaea*	62.3 ± 1.2	57.1 ± 0.8
*Methanosaeta***	32.4 ± 0.8	54.2 ± 0.8
*Methanosarcina***	8.2 ± 1.5	30.1 ± 1.4
*Methanobacteriales***	17.1 ± 1.3	14.2 ± 2.2
*Methanococcales***	12.5 ± 1.1	8.6 ± 0.3
*Methanomicrobiales***	18.4 ± 1.6	8.2 ± 1.7

*within active population; **within *Archaeal* subpopulation

Compared to the seed sludge there is a slight increase in the percentage of Eubacteria and a slight decrease in Archaeal population in the anaerobic reactor sludge which was fed with glucose during the operation period. Also, there was an increase in the ratio of acetoclastic methanogens. *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). The elevated amount of *Methanosaeta* spp. 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* spp. improves granulation and result in more stable reactor performance.

Table 5.2. Standardized FISH results of seed sludge and anaerobic batch reactor sludge

	Seed Sludge	Anerobic Batch Reactor
Methanosaeta	15.9	22.6
Methanosarcina	4.0	12.6
Methanobacteriales	7.0	5.9
Methanococcales	5.9	3.6
Methanomicrobiales	8.4	3.4

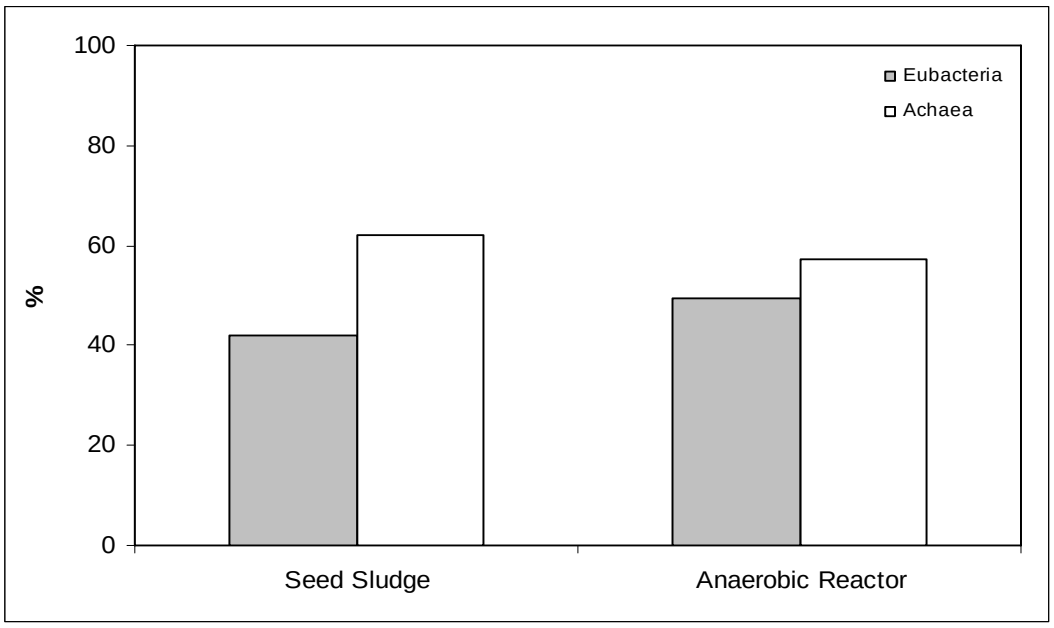


Figure 5.14. Distribution of microbial composition (%) in seed and anaerobic batch reactor sludges

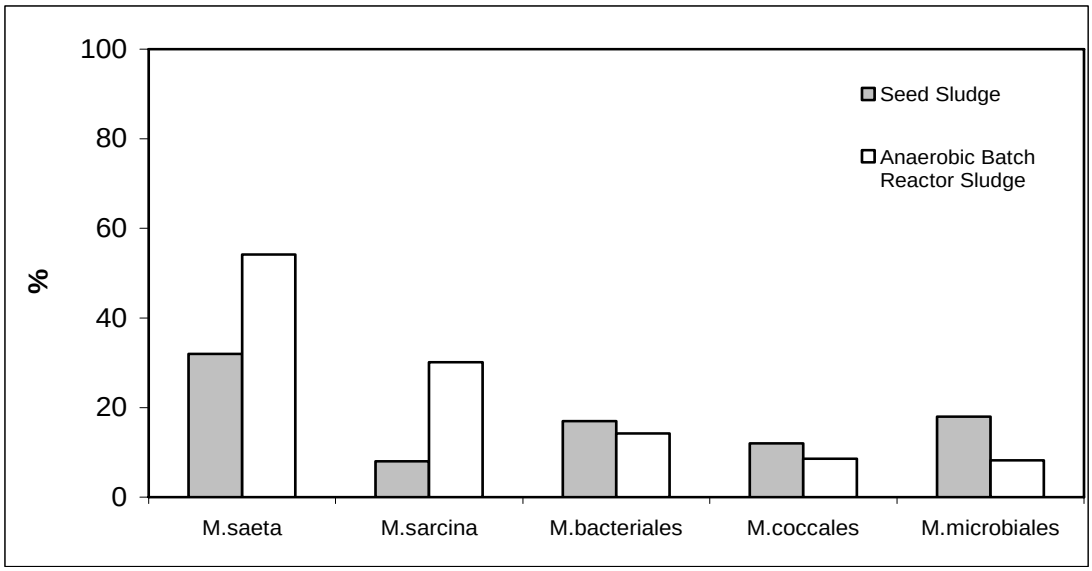


Figure 5.15. Distribution of *Archaeal* subpopulation (%) in seed and anaerobic batch reactor sludges

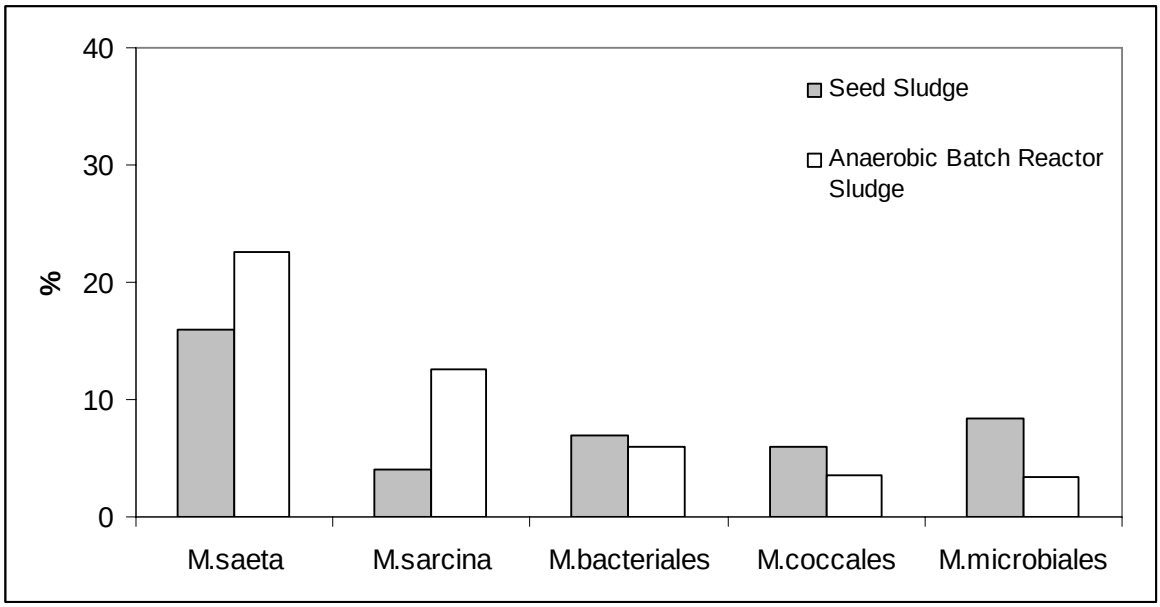
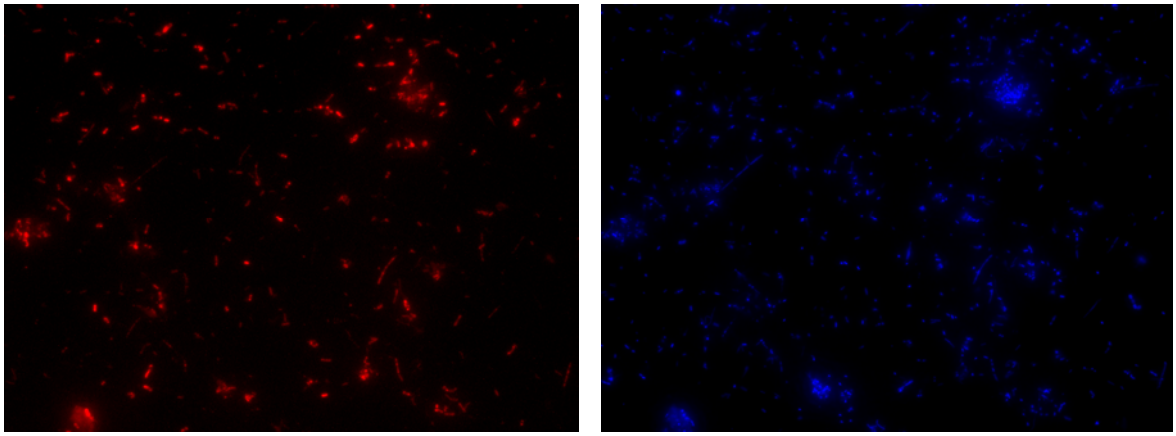
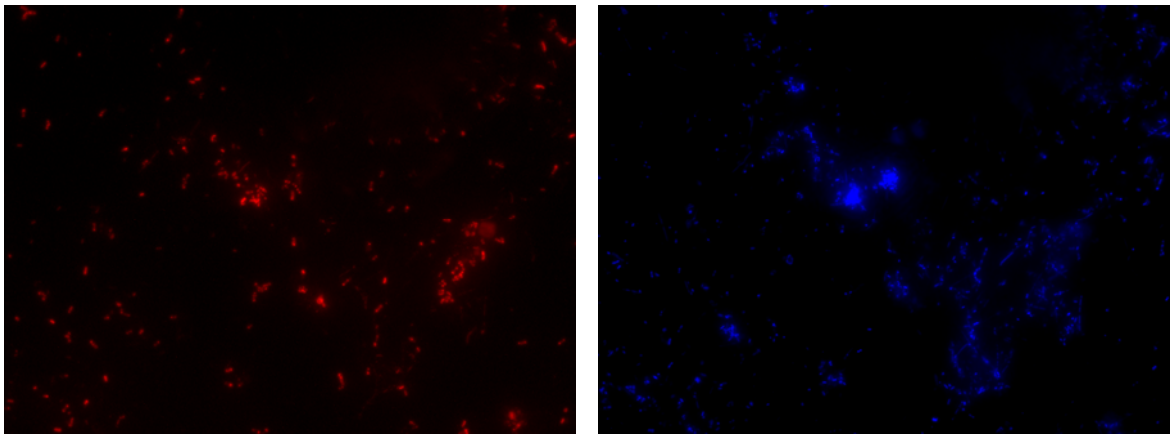


Figure 5.16. Standardized FISH results of seed and anaerobic batch reactor sludges



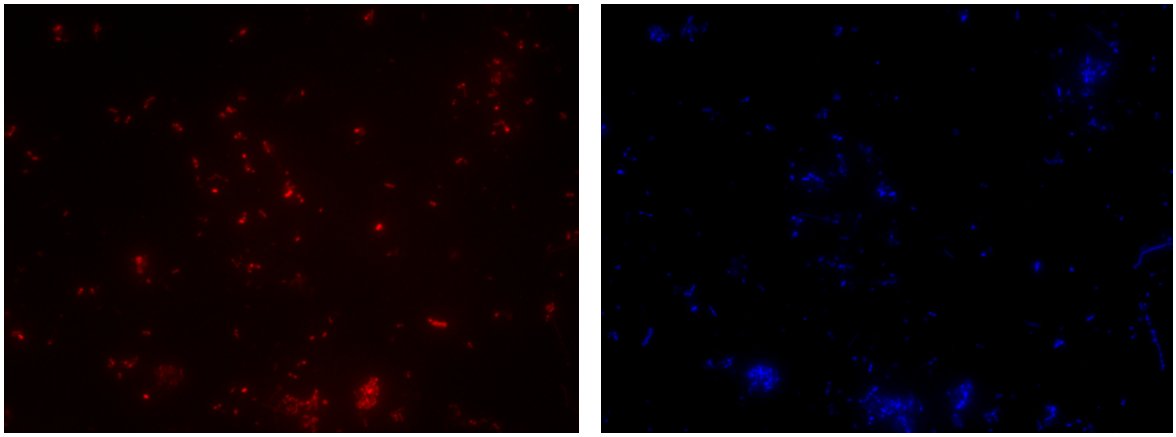
(a) (b)

Active cells hybridized with UNIV1392 probe



(a) (b)

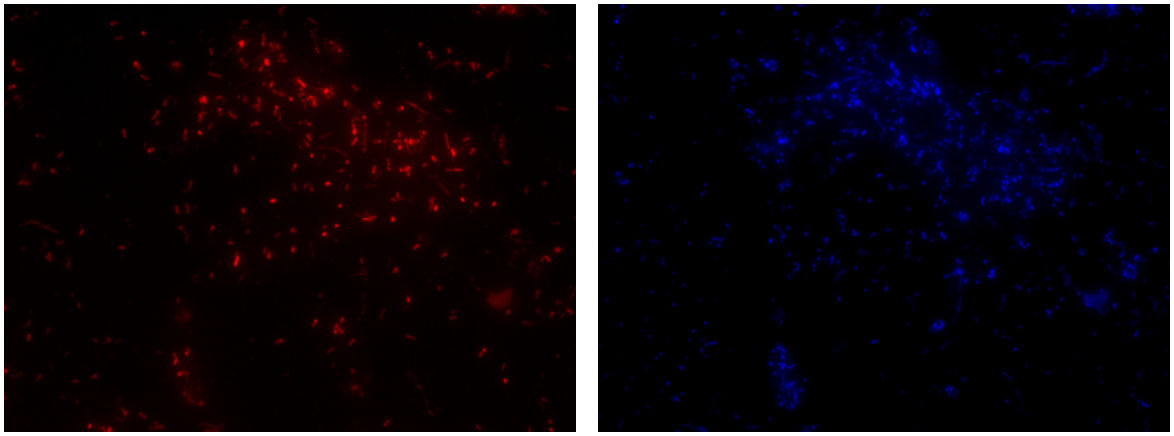
Eubacteria hybridized with EUBMIX probe



(a) (b)

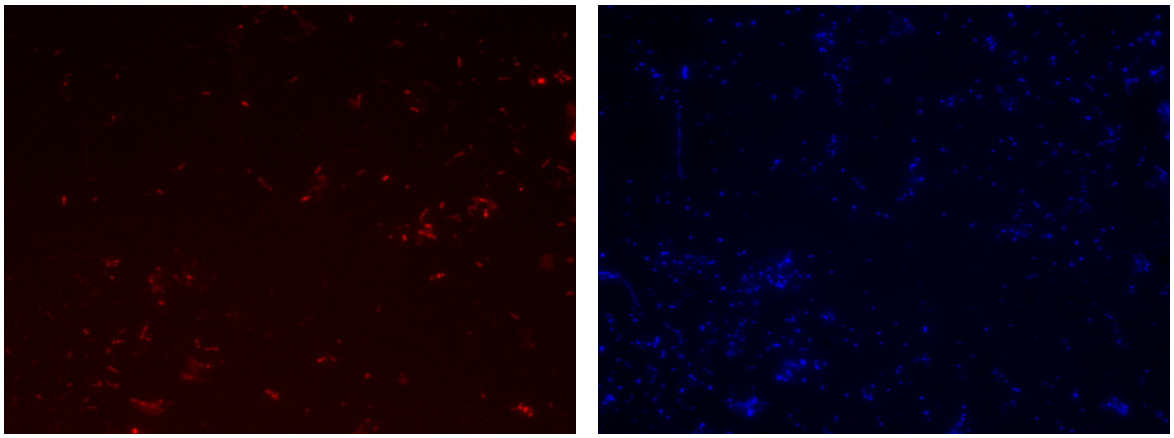
Archaea hybridized with ARC195 probe

Figure 5.17. Epifluorescence micrographs of the hybridized anaerobic batch reactor sludge samples. (a) Fluorescent and (b) DAPI images are in the same field



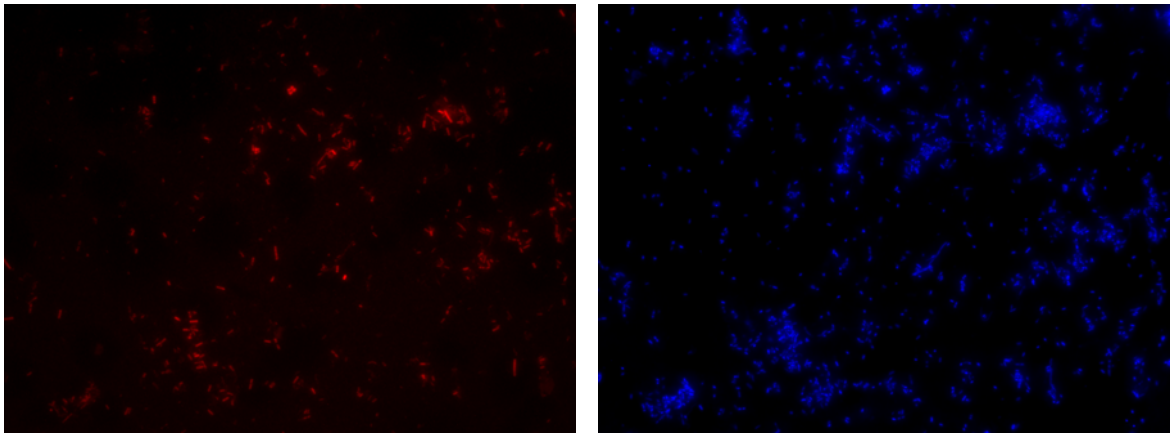
(a) (b)

Methanosaeta hybridized with MX825 probe



(a) (b)

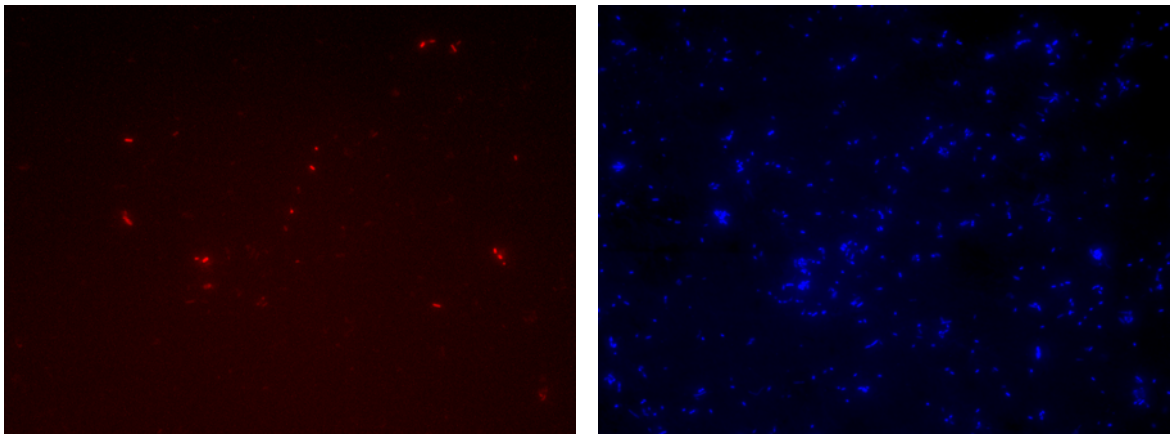
Methanosarcina hybridized with MS821 probe



(a) (b)

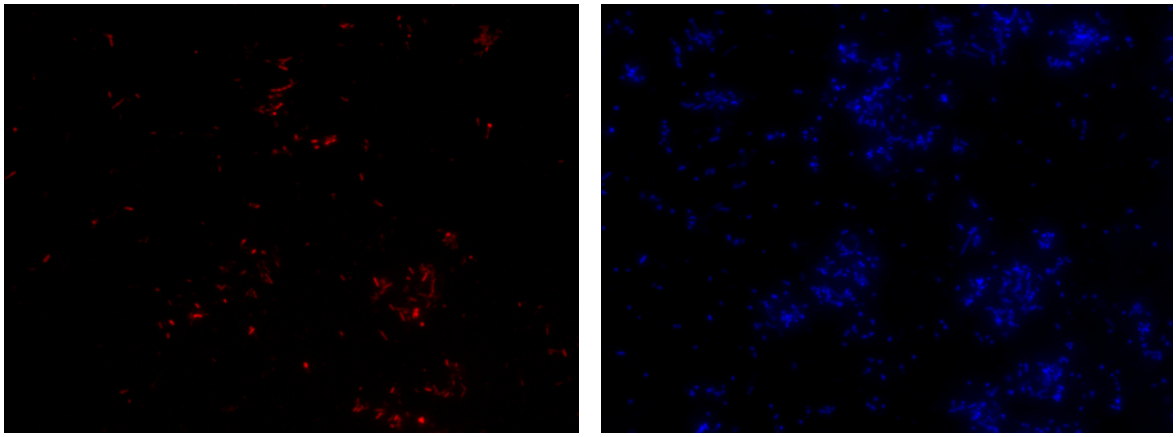
Methanobacteriales hybridized with MB310 probe

Figure 5.17. (continued) Epifluorescence micrographs of the hybridized anaerobic batch reactor sludge samples. (a) Fluorescent and (b) DAPI images are in the same field



(a) (b)

Methanococcales hybridized with MC1109 probe



(a) (b)

Methanomicrobiales hybridized with MG1200 probe

Figure 5.17. (continued) Epifluorescence micrographs of the hybridized anaerobic batch reactor sludge samples. (a) Fluorescent and (b) DAPI images are in the same field

5.4.3. FISH Results of Methanol-added SMA Reactors

FISH was applied to the sludge samples taken from the methanol-added SMA test reactors in order to indicate the microbial community change in the sludge samples. The microbial community structure of the SMA samples were characterized using FISH technique in order to emphasize the effect of methanol on microbial community of anaerobic sludge. In order to differentiate two different SMA measurements based on different substrates, SMA samples were named as SMA-Methanol-Acetate (2000mg/L

acetate, 0.4 M Methanol) and SMA-Methanol-VFAmix (VFA Mix, 0.4 M Methanol) regarding to the substrates which were used in the SMA test reactors.

In SMA-Methanol-Acetate, active cells, Eubacteria and Archaeal population were detected as 64.8% ± 2.8%, 51.1% ± 2.5%, 48.0% ± 1.9% respectively. FISH results shows that the percentage of the genuses of *Methanosaeta* and *Methanosarcina* were found as 40.5% ± 1.4% and 19.1% ± 1.4%, respectively. In addition, archaeal subpopulation composed of 34.0% ± 2.3% *Methanobacteriales*, 9.4% ± 1.6% *Methanococcales,* , 14.3% ± 1.1% *Methanomicrobiales* were found in SMA-Methanol-Acetate. Epifluorescence micrographs of SMA-Methanol-Acetate are shown in Figure 5.21.

In SMA-Methanol-VFAmix which was fed with a VFA Mixture, 59.1% ± 2.4% of the cells were metabolically active. 48.3 ± 1.6 and 47.5 ± 2.1 of these active cells were belonged to domain Eubacteria and Archaea respectively. Archaeal subpopulation were consisted of 38.4 ± 1.8, 17.4 ± 1.9, 36.2 ± 2.1, 8.2 ± 1.2 and 16.1 ± 1,8 of the genuses of *Methanosaeta*, *Methanosarcina*, *Methanobacteriales*, *Methanococcales* and *Methanomicrobiales* respectively.

Table 5.3. FISH results of anaerobic batch reactor sludge, SMA-Methanol-Acetate and SMA-Methanol-VFAmix

	Control: Anaerobic Batch Reactor Sludge	SMA-Methanol-Acetate	SMA-Methanol-VFAmix
Active Cells	73.2 ± 1.2	64.8 ± 2.8	59.1 ± 2.4
Eubacteria	49.6 ± 1.1	51.1 ± 2.5	48.3 ± 1.6
Achaea*	57.1 ± 0.8	48.0 ± 1.9	47.5 ± 2.1
*Methanosaeta***	54.2 ± 0.8	40.5 ± 1.4	38.4 ± 1.8
*Methanosarcina***	30.1 ± 1.4	19.1 ± 1.4	17.4 ± 1.9
*Methanobacteriales***	14.2 ± 2.2	34.0 ± 2.3	36.2 ± 2.1
*Methanococcales***	8.6 ± 0.3	9.4 ± 1.6	8.2 ± 1.2
*Methanomicrobiales***	8.2 ± 1.7	14.3 ± 1.1	16.1 ± 1.8

*within active population; **within *Archaeal* subpopulation

Table 5.4. Standardized FISH results of FISH results of anaerobic batch reactor sludge, SMA-Methanol-Acetate and SMA-Methanol-VFAmix

	Control: Anaerobic Batch Reactor Sludge	SMA-Methanol-Acetate	SMA-Methanol-VFAmix
Methanosaeta	22.6	12.6	10.8
Methanosarcina	12.6	5.9	4.9
Methanobacteriales	5.9	10.6	10.2
Methanococcales	3.6	2.9	2.3
Methanomicrobiales	3.4	4.4	4.5

Table 5.5. Relative percent change in the microbial community of methanol-added SMA samples compared to control reactor (Anaerobic batch reactor)

	SMA-Methanol-Acetate	SMA-Methanol-VFAmix
Methanosaeta	- 44.2	- 52.2
Methanosarcina	- 53.2	- 61.1
Methanobacteriales	+ 79.7	+ 72.9
Methanococcales	- 19.4	- 36.1
Methanomicrobiales	+ 29.4	+ 32.3

* Microbial community of control reactor was assumed to be 100%.

When anaerobic reactor sludge was compared to SMA test samples, a dramatic decrease was seen in the ratio of acetoclastic methanogens. *Methanosaeta* spp.. showed a 44.2% decrease in the SMA-Methanol-Acetate. Similarly, *Methanosaeta* spp.. decreased from 22.6% to 10.8, indicating a 52.2% decrease in the SMA-Methanol-VFAmix. In addition, *Methanosarcina* spp.. showed a 53.2% decrease in the SMA-Methanol-Acetate and 61.1% decrease in the SMA-Methanol-VFAmix. These results corresponded with the activity test results indicating adverse effect of methanol on anaerobic sludge; a 43% decrease in the acetoclastic methanogenic activity. On the other hand, *Methanobacteriales* spp.. increased from 5.9% to 10.6% and 10.2% in the SMA-Methanol-Acetate and SMA-

Methanol-VFAmix respectively. These results may point out that *Methanobacteriales* spp.. showed better resistance to stress conditions like inhibitory effect of methanol.

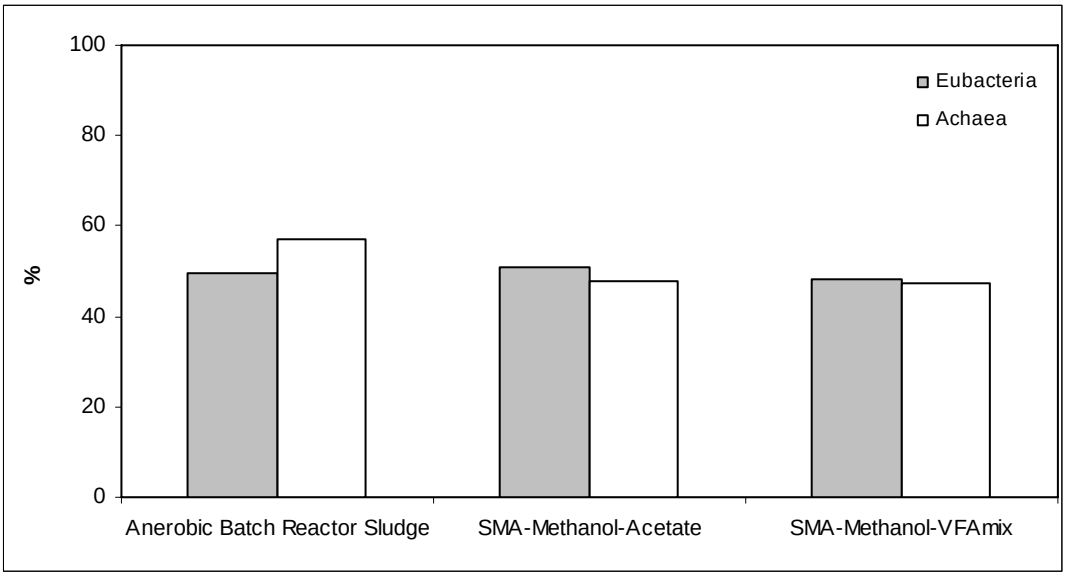


Figure 5.18. Distribution of microbial composition (%) in anaerobic batch reactor, SMA-Methanol-Acetate and SMA-Methanol-VFAmix

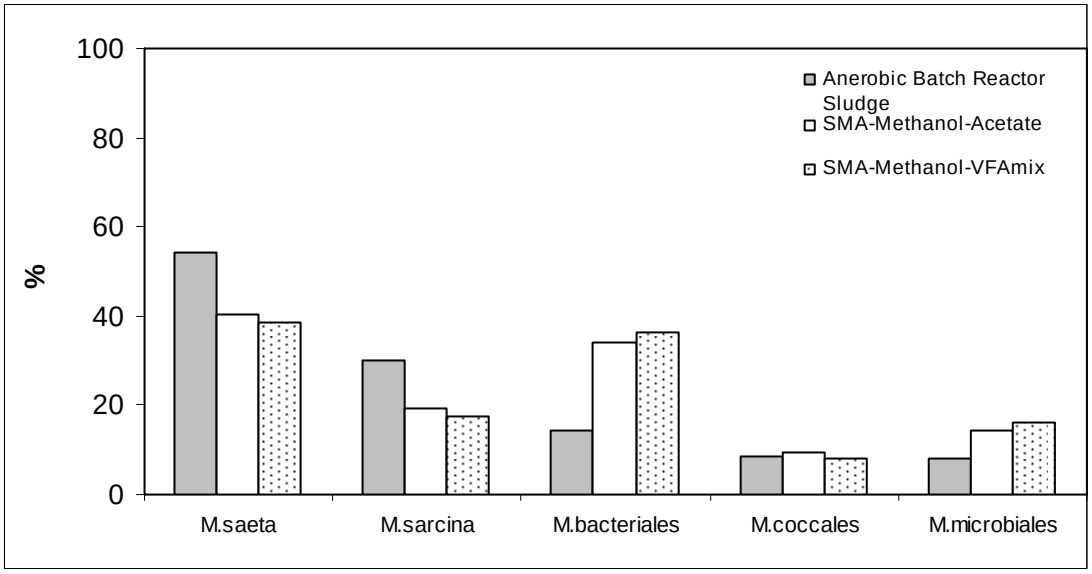


Figure 5.19. Distribution of Archaeal subpopulation (%) in anaerobic batch reactor, SMA-Methanol-Acetate and SMA-Methanol-VFAmix

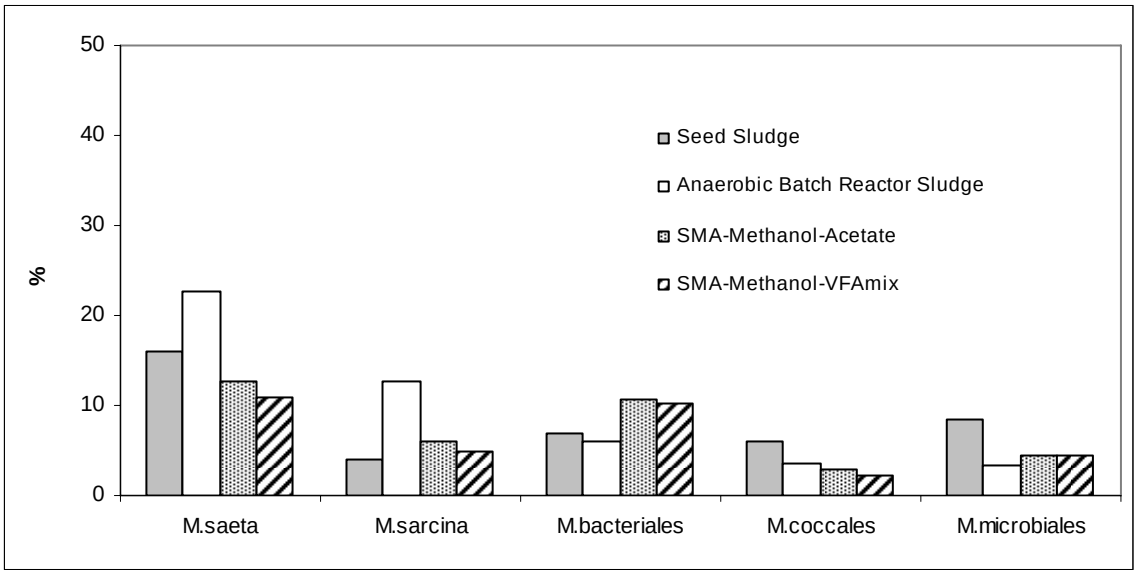
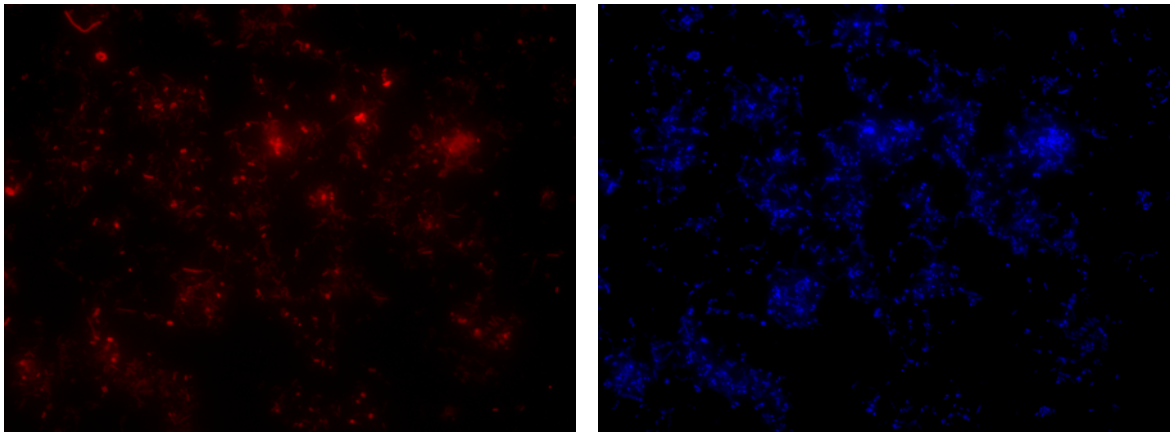
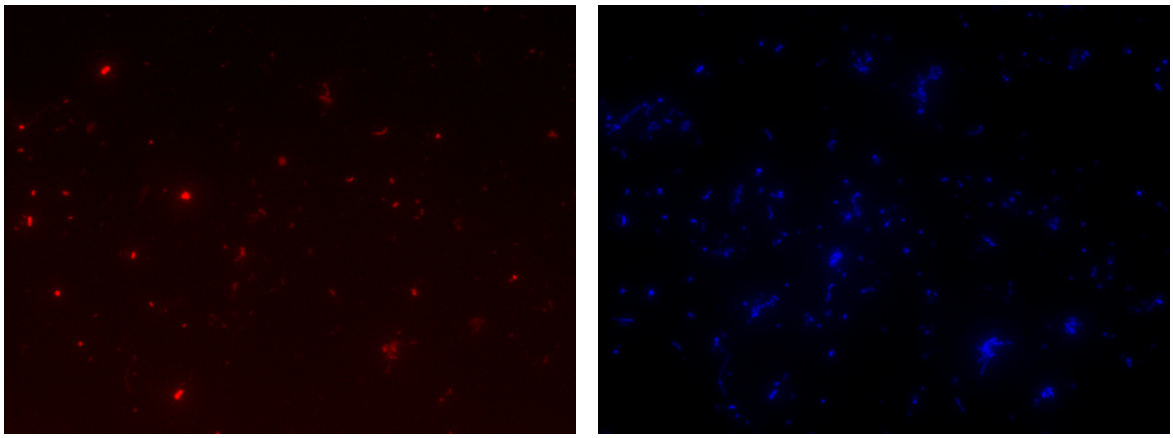


Figure 5.20. Standardized FISH results of seed sludge, anaerobic batch reactor sludge, SMA-Methanol-Acetate and SMA-Methanol-VFAmix



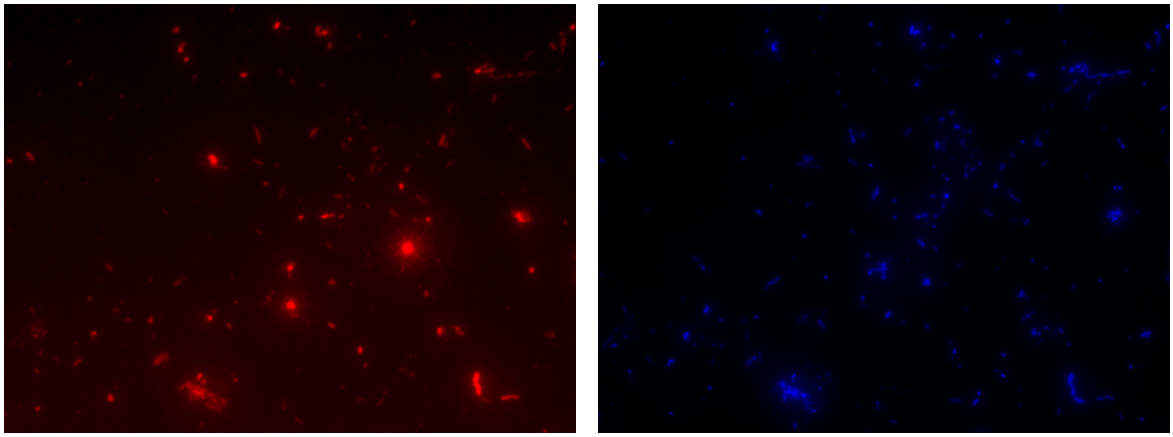
(a) (b)

Active cells hybridized with UNIV1392 probe



(a) (b)

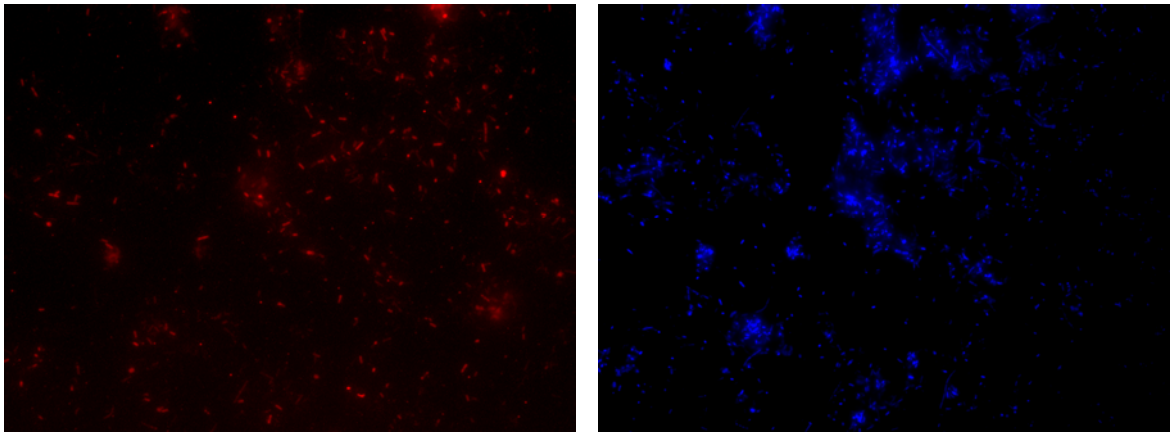
Eubacteria hybridized with EUBMIX probe



(a) (b)

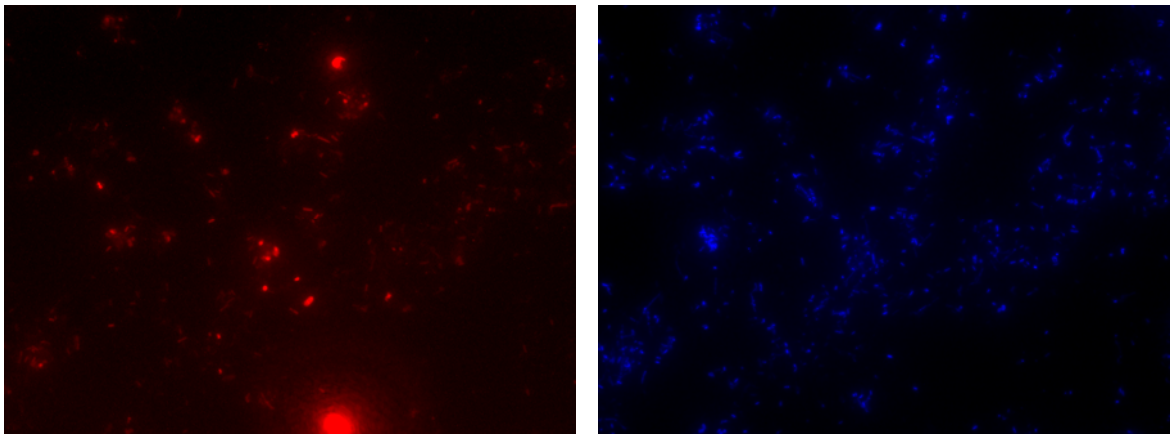
Archaea hybridized with ARC195 probe

Figure 5.21. Epifluorescence micrographs of the SMA-Methanol-Acetate. (a) Fluorescent and (b) DAPI images are in the same field



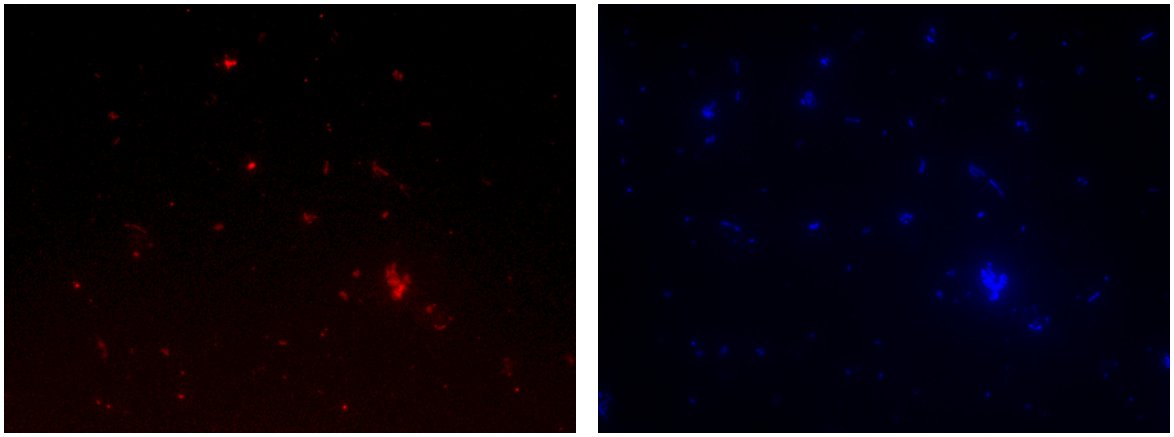
(a) (b)

Methanosaeta hybridized with MX825 probe



(a) (b)

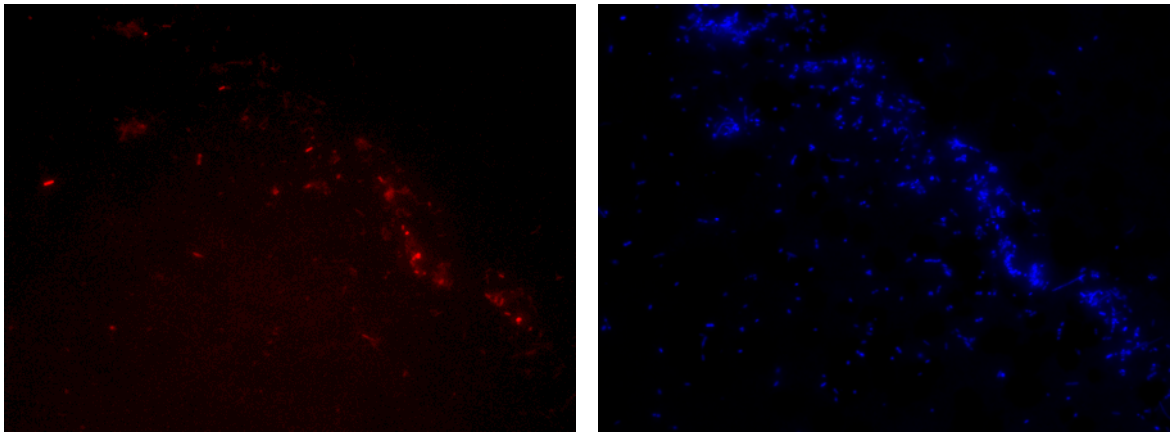
Methanosarcina hybridized with MS821 probe



(a) (b)

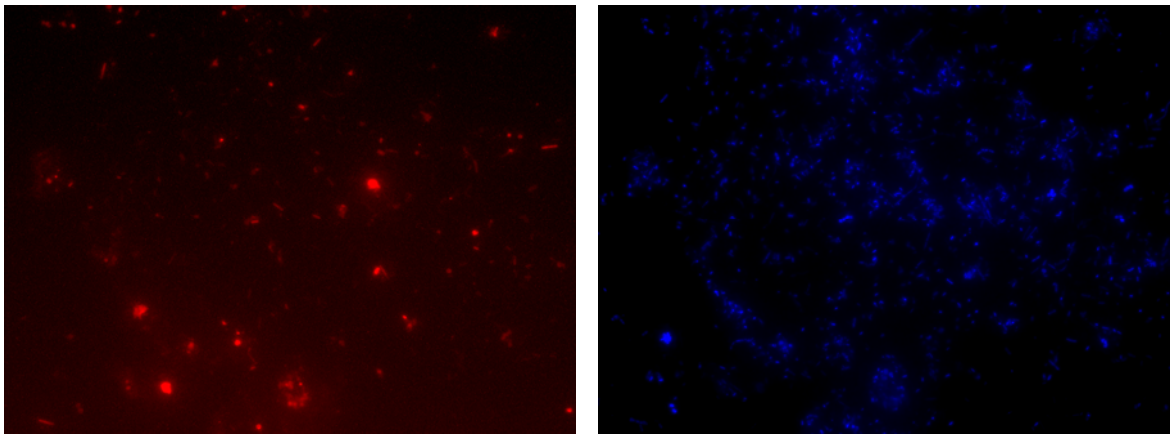
Methanobacteriales hybridized with MB310 probe

Figure 5.21. (continued) Epifluorescence micrographs of the SMA-Methanol-Acetate. (a) Fluorescent and (b) DAPI images are in the same field



(a) (b)

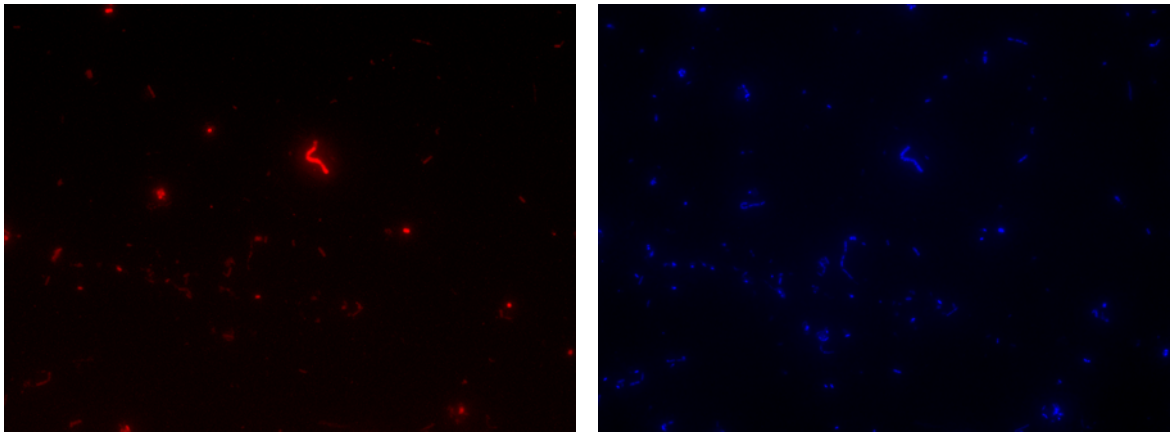
Methanococcales hybridized with MC1109 probe



(a) (b)

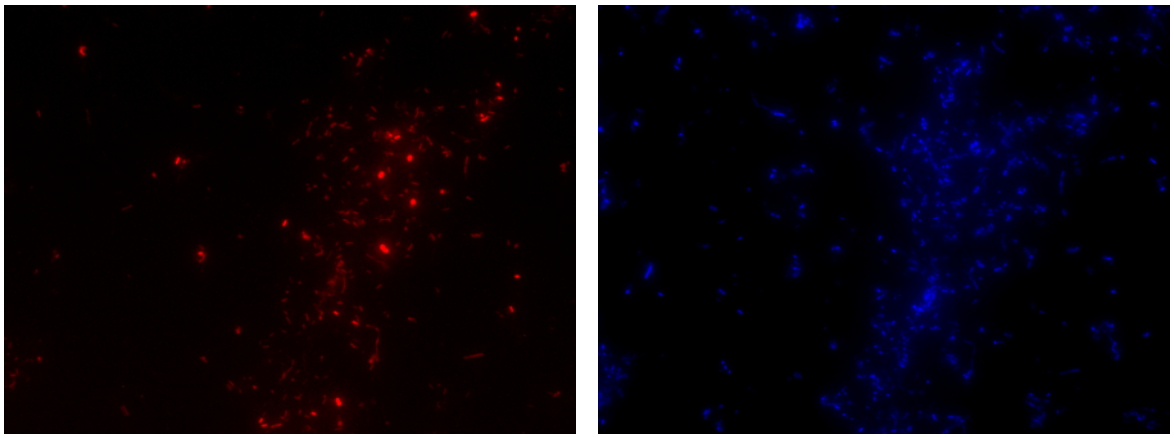
Methanomicrobiales hybridized with MG1200 probe

Figure 5.21. (continued) Epifluorescence micrographs of the SMA-Methanol-Acetate. (a) Fluorescent and (b) DAPI images are in the same field



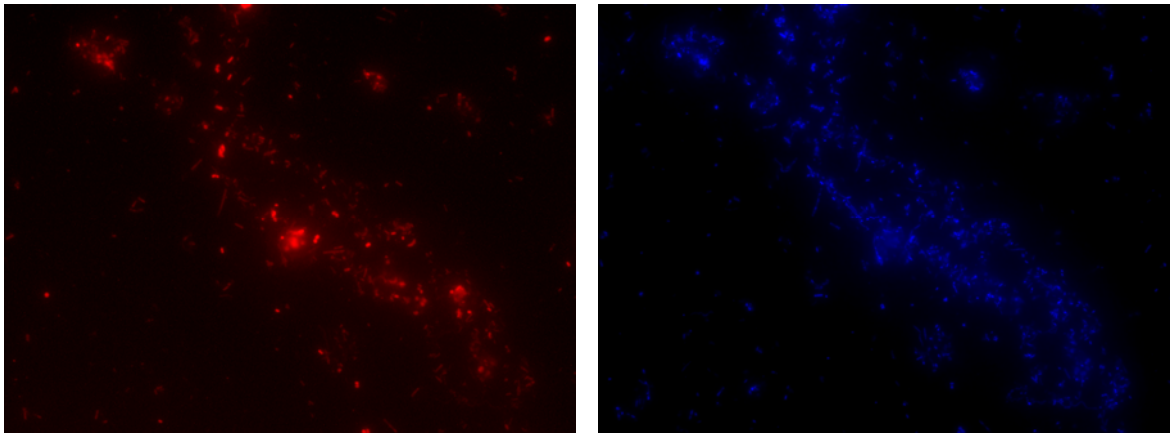
(a) (b)

Active cells hybridized with UNIV1392 probe



(a) (b)

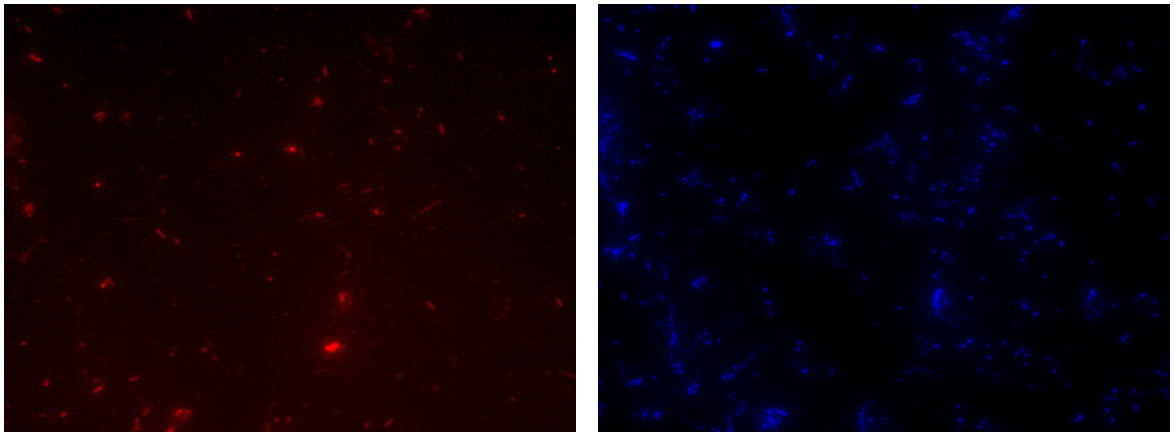
Eubacteria hybridized with EUBMIX probe



(a) (b)

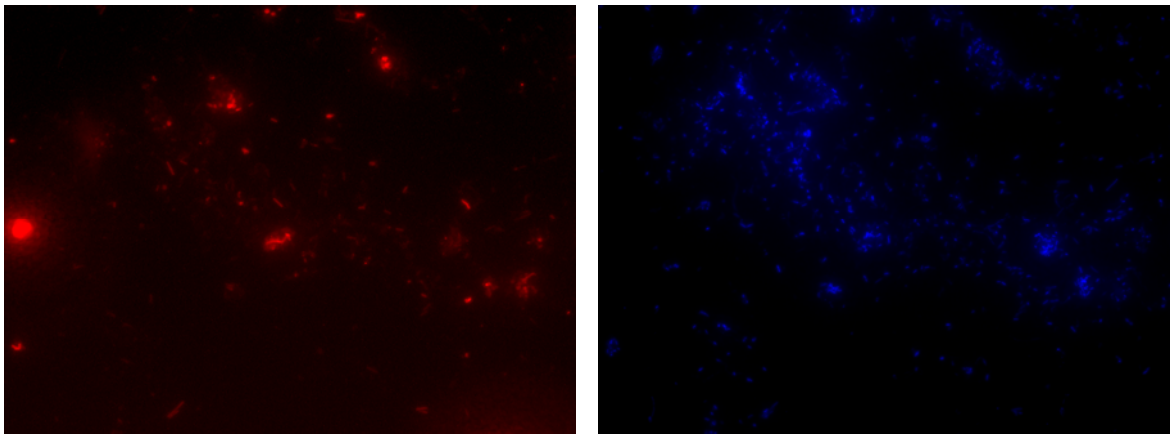
Archaea hybridized with ARC195 probe

Figure 5.22. Epifluorescence micrographs of SMA-Methanol-VFAmix. (a) Fluorescent and (b) DAPI images are in the same field



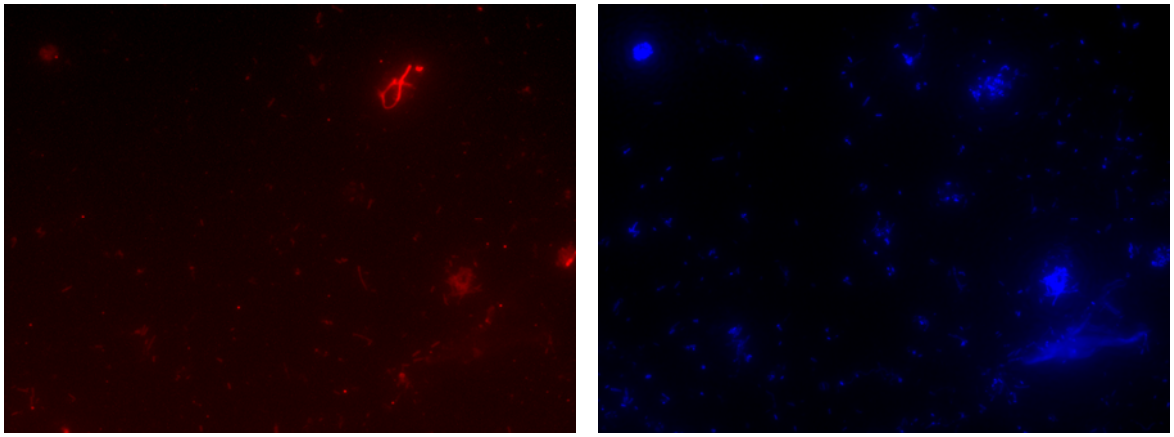
(a) (b)

Methanosaeta hybridized with MX825 probe



(a) (b)

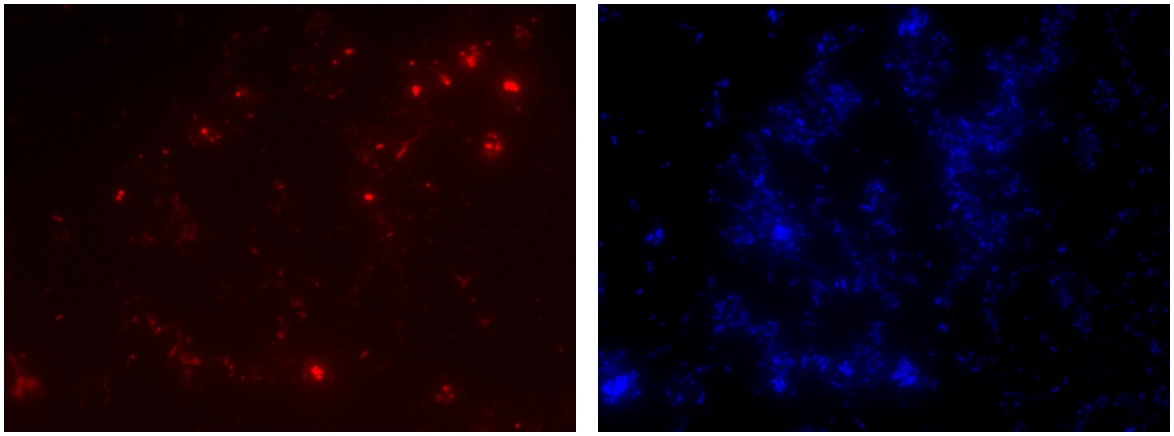
Methanosarcina hybridized with MS821 probe



(a) (b)

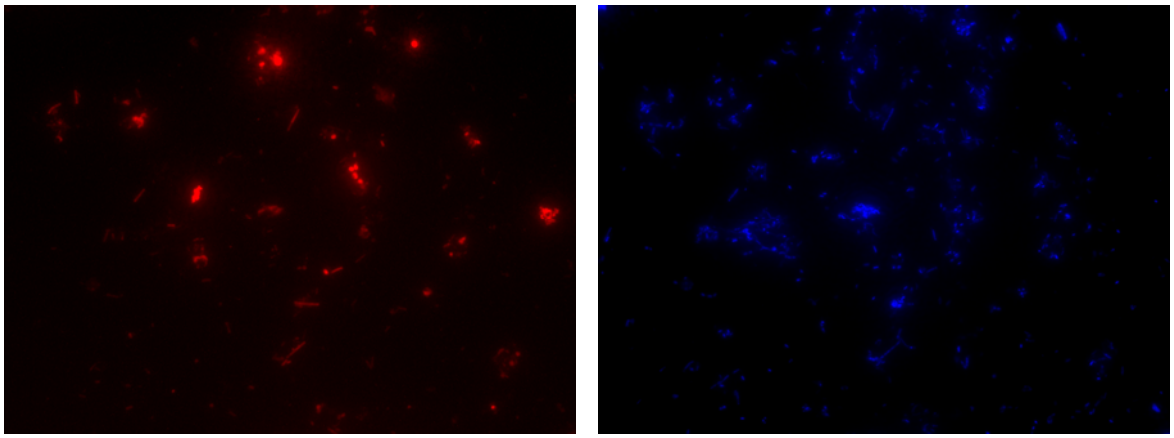
Methanobacteriales hybridized with MB310 probe

Figure 5.22. (continued) Epifluorescence micrographs of SMA-Methanol-VFAmix. (a) Fluorescent and (b) DAPI images are in the same field



(a) (b)

Methanococcales hybridized with MC1109 probe



(a) (b)

Methanomicrobiales hybridized with MG1200 probe

Figure 5.22. (continued) Epifluorescence micrographs of SMA-Methanol-VFAmix.

(a) Fluorescent and (b) DAPI images are in the same field

The anaerobic seed sludge used in this study has a high acetoclastic and total methanogenic capacity of 453 mLCH4/gVSS.d and 461 mLCH4/gVSS.d, respectively. Predominance of acetoclastic methanogens to hydrogenotrophic methanogens were observed.*Methanosaeta* spp. (32.4%) was the predominant methanogen in the seed sludge among *Archaea*.

The seed sludge taken from anaerobic EGSB reactor was inoculated into a lab-scale anaerobic reactor treating synthetic wastewater. The reactor was operated for 47 days. At

the end of the operation period, anaerobic sludge was taken for SMA tests and FISH studies. 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 437 mLCH4/gVSS.d, and an overall methanogenic activity of 490 mLCH4/gVSS.d. 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.

SMA tests were carried out with lab-scale anaerobic reactor sludge with the addition of methanol and two different substrates: acetate and a VFA mixture to to emphasize the effect of methanol on methanogenic activity. The results showed that acetoclastic methanogenic activity show a 43% decrease when compared to lab-scale anaerobic reactor. Similarly, overall methanogenic activity decreased from 490 mLCH4/gVSS.d to 221 mLCH4/gVSS.d. A distinct shift in archaeal community structure was observed in methanol added SMA reactors, especially with a profilation of *Methanobacteriales* spp. and a parallel decrease in *Methaanosaeta* and *Methanosarcina* spp.. The reason for this shift in population structure from acetate-utilizing methanogens to hydrogen-utilizing methanogens is unclear. However, it can be suggested that hydrogenotrophs, especially *Methanobacteriales* spp. are more resistant to stress conditions, i.e methanol inhibition.

CONCLUSIONS

Initial seed sludge quality in terms of high methanogenic activity and a well-balanced microbial diversity is of great importance in anaerobic reactors. Therefore, seed sludge taken from a full-scale anaerobic EGSB reactor treating a brewery wastewater which has a high methanogenic activity and active methanogenic composition has been chosen in this study for further inhibition studies;

Activity test results showed that the anaerobic seed sludge has a high acetoclastic and total methanogenic capacity of 453 mLCH4/gVSS.d and 461 mLCH4/gVSS.d, respectively. According to FISH results, *Methanosaeta* spp. (32.4%) were the predominant methanogen in the seed sludge among archaea followed by *Methanomicrobiales* (18.4%), *Methanobacteriales* (17.1%), *Methanococcales* (12.5%) and *Methanosarcina* (8.2%).

Activity test results of lab-scale anaerobic batch reactor (control) showed that the methanogenic activity has not shown a significant decrease compared to seed sludge with a acetoclastic methanogenic activity of 437 mLCH4/gVSS.d, and an overall methanogenic activity of 490 mLCH4/gVSS.d. Besides, FISH results sustain this statement with an increase in relative abundance of *Methanosaeta* spp. An increase in the relative abundance of acetoclastic methanogens was accompanied by a corresponding decrease in the relative abundance of hydrogenotrophic methanogens compared to seed sludge.

The inhibition concentration ranges vary widely for specific toxicants. These ranges also vary with the characteristics of biological sludge, meaning IC_{50} for a specific inhibitory substance may show differences with biological sludge that is studied. IC_{50} for methanol was found to be 0.4 M for the seed sludge and this concentration was used in the SMA tests to evaluate the effects of methanol on methanogenic activity and microbial community.

SMA test results showed that acetoclastic methanogenic activity show a 43% decrease when compared to control reactor. Similarly, overall methanogenic activity show

a 55% decrease in the SMA reactor sludge. Acetoclastic methanogens, *Methanosaeta* spp. and *Methanosarcina* spp.. showed a dramatic decrease while hydrogenotrophic methanogens, especially *Methanobacteriales* spp. showed an increase in SMA reactors. The results showed that there was a distinct shift from acetoclastic methanogens to hydrogenotrophic methanogens in response to stress conditions. Overall results showed that *Methanosaeta* population is of great importance in terms of reactor stability and performance. However, *Methanosaeta* spp. are not tolerant to stress conditions such as inbitory substances. In direct contradiction, *Methanobacteriales* spp. showed higher resistance to strees conditions.

RECOMMENDATIONS

This study is a part of a completed project (06Y102D, 'Determining effects of organic solvents/organic solvent mixtures on methanogenic and non-methanogenic activity', Bogazici University Research Foundation). In this project, effect of methanol and other chosen solvents on different sludges sources taken from one-phase anaerobic and two-phase anaerobic reactors-including acid and methane reactors were investigated. In this study, the effect of methanol (IC_{50} for the selected sludge) on methanogenic activity and microbial community of the sludge taken from one phase anaerobic reactor was evaluated by SMA tests and FISH. However, it should be mentioned that the study has been carried out with non-acclimated sludges in order to determine the acute effect of methanol on microbial diversity. For further study, the study should be carried out by acclimated sludge to methanol.

Proteobacterial population should be identified and quantified to clearly understand non-methanogenic steps together with methanogenic archaeal composition. Moreover, sulphate reducing bacteria which are mostly encountered in anaerobic reactors should be identified to observe syntrophic relationships.

In this study, the results of FISH were correlated with SMA and gave information on quantity of active archaeal methanogenic community. Verification of the microbial composition by other molecular methods such as DGGE, cloning, real time PCR will also help to achieve a better understanding in the whole microbial composition in anaerobic sludge samples.

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APPENDIX A: RAW SMA DATA

Table A.1. SMA test results of seed sludge from full-scale anaerobic EGSB reactor

EGSB.Seed Sludge (2000 mg/L acetate)			EGSB.Seed Sludge (VFA Mixture)		
time(hour)	pulse(1/hour)	SMA (ml CH4 /gVSS/day)	time(hour)	pulse(1/hour)	SMA (ml CH4 /gVSS/day)
0	0	0	0	0	0
1	4	23	1	15	61
2	6	34	2	43	174
3	17	97	3	56	226
4	28	161	4	61	247
5	56	321	5	62	251
6	69	396	6	66	267
7	71	407	7	73	295
8	76	436	8	75	303
10	79	453	9	80	323
11	77	442	10	93	376
12	75	430	11	96	388
13	77	442	12	98	396
14	79	453	13	98	396
15	76	436	14	108	437
16	76	436	15	109	441
17	77	442	16	115	465
18	77	442	17	114	461
19	72	413	18	116	469
20	75	430	19	114	461
21	73	419	20	114	461
22	75	430	21	113	457
23	69	396	22	113	457
24	61	350	23	114	461
25	53	304	24	112	453
26	44	252	25	106	429
27	36	206	26	102	412
28	30	172	27	98	396
29	26	149	28	95	384
30	22	126	29	89	360
31	18	103	30	84	340
32	13	75	31	80	323
33	9	52	32	75	303
34	4	23	33	69	279
35	3	17	34	67	271
36	1	6	35	64	259
37	2	11	36	62	251
38	0	0	37	62	251
			38	60	243
			39	58	234
			40	55	222
			41	53	214
			42	52	210
			43	49	198
			44	46	186
			45	34	137
			46	25	101
			47	12	49
			48	0	0

Table A.2. SMA test results of lab-scale anaerobic batch reactor

Anaerobic Batch Reactor (2000 mg/L acetate)			Anaerobic Batch Reactor (VFA Mixture)		
time(hour)	pulse(1/hour)	SMA (ml CH4 /gVSS/day)	time(hour)	pulse(1/hour)	SMA (ml CH4 /gVSS/day)
0	0	0	0	0	0
1	15	93	1	9	44
2	20	124	2	25	123
3	26	162	3	43	211
4	28	174	4	63	331
5	40	249	5	62	326
6	50	311	6	70	368
7	52	323	7	70	368
8	56	348	8	70	368
9	56	348	9	70	368
10	56	348	10	76	399
11	58	361	11	76	399
12	59	367	12	72	378
13	58	361	13	76	399
14	59	367	14	78	410
15	53	330	15	78	410
16	59	367	16	72	378
17	61	379	17	76	399
18	62	386	18	80	420
19	60	373	19	82	431
20	61	379	20	81	425
21	63	392	21	90	473
22	64	398	22	86	452
23	57	354	23	90	473
24	59	367	24	90	473
25	64	398	25	89	467
26	64	398	26	92	483
27	69	429	27	92	483
28	69	429	28	101	530
29	70	437	29	95	499
30	65	404	30	100	525
31	54	336	31	104	546
32	44	274	32	101	530
33	39	243	33	99	520
34	33	205	34	95	499
35	32	199	35	92	483
36	32	199	36	96	504
37	32	199	37	105	551
38	28	174	38	99	520
39	28	174	39	95	499
40	28	174	40	100	525
41	28	174	41	100	525
42	24	149	42	96	504
43	8	50	43	104	546
44	0	0	44	101	530
			45	104	546
			46	104	546
			47	86	452
			48	0	0

Table A.3. SMA tests results of methanol added reactor sludges

SMA-Methanol-Acetate			SMA-Methanol-VFAmix		
time(hour)	pulse(1/hour)	SMA (ml CH4 /gVSS/day)	time(hour)	pulse(1/hour)	SMA (ml CH4 /gVSS/day)
0	0	0	0	0	0
1	34	210	1	21	119
2	41	254	2	23	130
3	45	278	3	36	204
4	45	278	4	32	181
5	29	179	5	32	181
6	25	155	6	34	192
7	25	155	7	35	198
8	28	173	8	34	192
9	28	173	9	38	215
10	31	192	10	38	215
11	29	179	11	38	215
12	28	173	12	45	254
13	27	167	13	45	254
14	26	161	14	45	254
15	25	155	15	45	254
16	26	161	16	45	254
17	27	167	17	45	254
18	26	161	18	45	254
19	12	74	19	43	243
20	13	80	20	43	243
21	25	155	21	41	232
22	19	118	22	41	232
23	17	105	23	38	215
24	8	49	24	36	204
25	27	167	25	34	192
26	25	155	26	36	204
27	24	148	27	34	192
28	20	124	28	36	204
29	19	118	29	38	215
30	13	80	30	38	215
31	0	0	31	36	204
			32	38	215
			33	41	232
			34	36	204
			35	34	192
			36	36	204
			37	36	204
			38	38	215
			39	36	204
			40	40	226
			41	34	192
			42	34	192
			43	27	153
			44	34	192
			45	27	153
			46	31	175
			47	27	153
			48	27	153
			49	28	158
			50	26	147
			51	18	102
			52	12	68
			53	0	0