

INVESTIGATING THE METHANE PRODUCTION EFFICIENCY OF SINGLE-
AND TWO-PHASE ANAEROBIC DIGESTION AND POPULATION
DYNAMICS OF A SECOND-PHASE ANAEROBIC DIGESTION

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POPULATION DYNAMICS OF A SECOND-PHASE ANAEROBIC
DIGESTION**

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ABSTRACT

INVESTIGATING THE METHANE PRODUCTION EFFICIENCY OF SINGLE- AND TWO-PHASE ANAEROBIC DIGESTION AND POPULATION DYNAMICS OF A SECOND-PHASE ANAEROBIC DIGESTION

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The aim of this thesis study was to investigate the methane production efficiency of single-phase and two-phase anaerobic digestion (AD) in batch reactors and anaerobic sequencing batch reactor (ASBR). It was also aimed to monitor the population dynamics and change in the quantity of acetoclastic methane-producers of the second-phase of a two-phase ASBR.

Two sets of batch reactors were conducted, namely, Batch Reactor Set-1 and Batch Reactor Set-2. The aim of Batch Reactor Set-1 was to investigate the effect of initial substrate concentration to initial microorganism concentration ratio (S/X_0) on methane yield. To this purpose, batch reactors were conducted at different initial S/X_0 ratios of 0.5, 1, 2, 3 and 4 g sCOD/g VSS. Results revealed that the highest methane yield (267 ± 5 mL CH_4 /g COD_{added}) and anaerobic treatability were observed at S/X_0 ratio of 1 g COD/g VSS. The results also indicated the applicability of the anaerobic seed sludge and 1 g COD/g VSS as the optimum S/X_0 ratio for the following experiments.

Batch Reactor Set-2 was conducted to investigate and compare the methane production efficiency of a single-phase AD and the second phase of a two-phase AD system. To this purpose, sucrose and effluent of a dark fermentative sequencing batch reactor (DF-SBR) operated with sucrose (Tunçay, 2015) were used as substrates. The highest

methane yield and content in biogas were observed as 344 ± 20 mL CH₄/g COD_{added} and 83%, respectively, in the second phase of the two-phase AD. Two-phase AD resulted in 39% increase in methane yield compared to its single-phase counterpart.

ASBR study was conducted to investigate the effect of hydraulic retention time (HRT) and solid retention time (SRT) on methane production, and archaeal and bacterial population dynamics, the latter performed with denaturing gradient gel electrophoresis (DGGE). The change in the quantity of acetoclastic methane-producers, namely, *Methanosaeta* and *Methanosarcina* sp. with respect to the changing SRT and HRT conditions was also investigated with quantitative polymerase chain reaction (qPCR) analysis. Thus, an ASBR fed with the effluent of the DF-SBR was operated. Results showed that the highest average methane yield was achieved at 20 days of SRT and 6 days of HRT as 343 ± 17 mL CH₄/g COD_{added}. The highest average methane productivity was observed as 1794 ± 279 mL CH₄/L/day at SRT and HRT values of 10 and 0.7 days, respectively. *Methanosaeta* sp. was found to be the dominant specie among the archaeal group during all HRT and SRT combinations (10-20 days of SRT and 0.7 to 6 days of HRT). Although *Methanosarcina* species was not found in sequence analysis, qPCR results revealed the existence of *Methanosarcina* species through the whole operation period. Yet, *Methanosaeta* dominated the ASBR for all HRT and SRT combinations studied. Gradual decrease in HRT from 6 days to 0.7 days resulted in three fold decrease in *Methanosaeta* (9.5×10^{14} to 3.1×10^{14} gene copy/g VSS) and nearly five fold increase in *Methanosarcina* concentration (9.4×10^{12} to 5.1×10^{13} gene copy/g VSS) at 20 days of SRT. On the other hand, decrease in SRT from 20 days to 10 days did not lead any significant change in the concentration of *Methanosaeta* and *Methanosarcina* species in the ASBR.

Keywords: Archaea, bacteria, DGGE, hydraulic retention time, methane yield, molecular analysis, population dynamics, productivity, solid retention time, qPCR

ÖZ

TEK VE İKİ-AŞAMALI ANAEROBİK ÇÜRÜTMEDE METAN ÜRETİM VERİMİNİN VE İKİNCİ AŞAMA ANAEROBİK ÇÜRÜTMEDE POPÜLASYON DİNAMİĞİNİN ARAŞTIRILMASI

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Bu yüksek lisans tezinin amacı kesikli ve anaerobik ardışık kesikli reaktörlerde (AAKR) tek aşamalı ve iki-aşamalı anaerobik çürütmenin (AD) metan üretimi verimi üzerine etkisini araştırmaktır. Ayrıca, mikrobiyal popülasyon dinamiği ve asetiklastik metan üreticilerin nicel değişiminin iki aşamalı anaerobik sistemin ikinci aşaması olan AAKR’de araştırılması da amaçlanmıştır.

Kesikli Reaktör Seti-1 ve Kesikli Reaktör Seti-2 olmak üzere iki farklı kesikli reaktör deney seti kurulmuştur. Kesikli Reaktör Seti-1’in amacı, başlangıç sübstrat derişimin başlangıç mikroorganizma oranının (S/X_0) metan üretim verimine etkisini araştırmaktır. Bu amaçla, başlangıç S/X_0 oranı 0,5, 1, 2, 3, ve 4 g KOİ/g UAKM olan kesikli reaktörler kurulmuştur. Sonuçlar, en yüksek metan üretim veriminin (267 ± 5 mL CH_4 /g KOİ_{eklenen}) ve anaerobik artırılabilirliğin 1 g KOİ/g UAKM S/X_0 oranında elde edildiğini göstermiştir. Bu sonuç, kullanılan anaerobik aşı çamurunun ve 1 g KOİ/g UAKM S/X_0 oranının takip eden çalışmalar için de uygulanabilir olduğunu göstermiştir.

Kesikli Reaktör Seti-2, tek-aşamalı AD ve iki-aşamalı AD’nin metan üretim verimlerinin karşılaştırılması amacıyla gerçekleştirilmiştir. Bu amaçla, sukroz ve

süstrat olarak sukrozun kullanıldığı bir karanlık fermentatif ardışık kesikli reaktörün (KF-AKR) çıkış suyu (Tunçay, 2015) bu çalışmanın bir diğer süstratı olarak kullanılmıştır. En yüksek metan üretim verimi ve metan yüzdesi, sırasıyla, 344±20 mL CH₄/g KO₂eklenen ve %83 olarak iki-aşamalı AD'nin ikinci aşamasında elde edilmiştir. İki-aşamalı AD, tek-aşamalı eşleniğine kıyasla %39 daha fazla metan üretim verimi sağlamıştır.

AAKR çalışması, hidrolik bekletme süresi (HBS) ve katı bekletme süresi (KBS)'nin metan üretimi, ve archaeal ve bakteriyel popülasyon dinamiğine etkisini araştırmak amacıyla kurulmuştur. Popülasyon dinamiği, denaturant gradyan jel elektroforezi (DGGE) ile araştırılmıştır. Asetiklastik metan-üreticiler olan *Methanosaeta* ve *Methanosarcina* türlerinin değişen KBS ve HBS değerlerine göre nicel değişimleri de, eş zamanlı polimeraz zincir reaksiyonu (qPCR) analizleri ile araştırılmıştır. Bu amaçla, bir AAKR, KF-AKR çıkış suyu ile işletilmiştir. Sonuçlar, en yüksek ortalama metan üretim veriminin 343±17 mL CH₄/g çKO₂eklenen ile 20 gün KBS ve 6 gün HBS değerlerinde elde edildiğini göstermiştir. En yüksek ortalama metan üretim hızı ise 2362 mL CH₄/L/gün ile, sırasıyla, 10 gün KBS ve 0,7 gün HBS'de gözlenmiştir. *Methanosaeta concilii* türü, archaea türleri içinde, çalışılan tüm HBS ve KBS kombinasyonları süresince (10-20 gün KBS ve 0,7-6 gün HBS) baskın tür olarak belirlenmiştir. Sekans analizlerinde gözlemlenmemiş olmasına rağmen, qPCR sonuçları tüm işletim süresince *Methanosarcina* türünün var olduğunu göstermiştir. Ancak, *Methanosaeta*, çalışılan tüm HBS ve KBS kombinasyonları için AAKR'yi domine etmiştir. KBS 20 gün iken, HBS'nin kademeli olarak 6 günden 0,7 güne düşürülmesi, *Methanosaeta* ($9,5 \times 10^{14}$ 'den $3,1 \times 10^{14}$ gen kopyası/g UAKM'ye) türünün derişiminin üç katı azalmasına, *Methanosarcina* türünün derişiminin ise beş kat artmasına ($9,4 \times 10^{12}$ 'den $5,1 \times 10^{13}$ gen kopyası/g UAKM'na) sebep olmuştur. Öte yandan, KBS'nin 20 günden 10 güne düşürülmesi, *Methanosaeta* and *Methanosarcina* türlerinin derişimlerinde herhangi önemli bir değişime yol açmamıştır.

Anahtar Kelimeler: Archaea, bakteri, DGGE, hidrolik bekletme süresi, metan üretim verimi, moleküler analiz, popülasyon dinamiği, katı bekletme süresi, qPCR

To my family with endless love...

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ABBREVIATIONS

ASBR	Anaerobic sequencing batch reactor
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
FBR	Fluidized bed reactor
EGSB	Expanded granular sludge bed reactor
HAc	Acetic acid
HRT	Hydraulic retention time
MBR	Membrane bio-reactor
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
OLR	Organic loading rate
PBR	Packed bed reactor
S	Substrate
SBR	Sequencing batch reactor
sCOD	Soluble chemical oxygen demand
SRT	Solid retention time
TAN	Total ammonifiable nitrogen
TSS	Total suspended solids
TVS	Total volatile solids
UASB	Upflow anaerobic sludge blanket
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
X ₀	Initial volatile suspended solids

CHAPTER 1

INTRODUCTION

Anaerobic digestion (AD) process has long been applied to the treatment of domestic and industrial wastes/wastewaters (Park et al., 2010). Nowadays, this process has been frequently employed to produce renewable bioenergy like hydrogen and methane as well as to treat the wastes/wastewaters/industrial by-products. Especially, due to current imperative global issues such as petroleum depletion and global warming, world wide attention has been paid to the AD process for bioenergy production. Thus, AD processes have been developing to improve the renewable energy production. In this way, changes in reactor configurations, operational parameters and environmental conditions, microbial consortium analysis and phase separation studies have been acting in concert (Weiland, 2010).

With the study of Ghosh and Poland (1976), it was understood that digestion phase separation has a direct positive effect on bioenergy production with AD process. After this point, two-phase AD process has been focused on rather than single-phase AD process in the literature studies (Demirer and Othman 2008). The main difference between single and two-phase AD is to use two anaerobic reactors consecutively in two-phase AD and one single anaerobic reactor in single-phase AD process. Two consequent reactors of two-phase AD can be named as acidogenesis and methanogenesis reactors. By this way, more suitable environmental conditions can be provided to each microbial consortium (acidogens and methanogens) of an AD process (Demirer and Othman, 2008). Hereby, two-phase AD system has several advantages over single-phase systems, such as ease of the selection and enrichment of different bacteria and archaea species in each reactor, increased process stability, and enhanced buffering of the methanogenesis phase pH by the prior acid phase (Speece, 1996).

In addition to phase separation, the other approach used in developing the process stability and energy recovery for AD processes is the reactor type selection. The anaerobic sequencing batch reactor (ASBR), developed by Dague et al. (1992), has not been used as widely as other processes in wastewater treatment industry. However, SBR technology does hold some advantages over other systems with its flexible operation regarding the fact that it uses the same vessel for both reacting and settling the wastewater (Nyon and Dague, 1994). In addition to reactor type, hydraulic and solid retention times (HRT and SRT) are the two of the operational parameters which affect the energy yield and treatment efficiency (Speece, 1996).

The improvements in understanding of both the microbial communities and processes in anaerobic reactors are essential to design and control anaerobic systems effectively (Zinder, 1993). Application of molecular methods such as denaturing gradient gel electrophoresis (DGGE) has led to new insights into microbial processes and species in biological reactors (De Bok et al., 2006). In addition, by using quantitative polymerase chain reaction (qPCR), quantitative analyses can be made for specific microbial species responsible for a specific degradative function (Muyzer et al., 1993; 1998). These molecular applications may make it possible to design better anaerobic treatment processes and higher methane production (Conklin et al., 2006). During AD, 70% of the methane is produced by acetoclastic methane producers (Speece, 1996). Currently, it is known that there are two acetoclastic methanogenic genera (subfamily in the classification of microorganisms consisting of more than one specie), namely, *Methanosarcina* and *Methanosaeta* (De Bok et al., 2006; Conklin et al., 2006; Hackstein, 2010). The research studies investigating the effects of HRT and SRT on archaeal and bacterial population dynamics and the change in the quantity of acetoclastic methane-producers (*Methanosaeta sp.* and *Methanosarcina sp.*) are limited (Lee et al., 2011; Ma et al., 2013).

Considering the above mentioned points, the aim of this thesis was set as to investigate the methane production efficiency of single-phase and two-phase AD in batch reactors and ASBR, respectively. For anaerobic batch reactor operations S/X_0 ratio (g COD/g VSS) is an important parameter to reveal the methane production potential of

anaerobic sludge from different wastewaters (Neves et al., 2004). Thus, it was initially aimed to research the methane production potential of the seed to be used in all experiments. It was also aimed to monitor the changes in methane production efficiency, microbial population dynamics and quantity of methane producers of a second-phase of the two-phase ASBR operated at different operational conditions which are HRT and SRT. To this purpose, three different reactor experiments were conducted. Two of them were experienced with batch reactors, namely, Batch Reactor Set-1 and Batch Reactor Set-2 and the last one was an ASBR. By this way scope of the thesis defined as follows;

- To determine the optimum initial substrate to biomass (S/X_0) ratio leading to the highest methane production yield (via Batch Reactor Set-1)
- To investigate the methane production efficiency difference between single-phase and two-phase AD (via Batch Reactor Set-2)
- To investigate the effects of HRT and SRT on the second-phase of a two-phase ASBR system (i.e. fed with the effluent of a preliminary dark fermentative SBR) (via ASBR)
- To investigate the effects of HRT and SRT on archaeal and bacterial population dynamics in the second phase of the two-phase ASBR system via DGGE (via ASBR)
- To investigate the change in the quantity of methane-producers (*Methanosaeta sp.* and *Methanosarcina sp.*) with respect to the changes in the operational parameters via qPCR (via ASBR)

CHAPTER 2

LITERATURE REVIEW

2.1 Anaerobic Digestion (AD)

Rapid industrialization, increasing urbanization and uncontrolled population growth lead to an increase in energy demand. Most of the energy demand in the world is met by fossil fuels; thus, consumption of fossil fuels will continue in a continuous and unsustainable manner (Park et al., 2010). In addition, the use of fossil fuels leads to dangerous greenhouse gas emissions and results in climate change, global warming and rather environmental problems such as air pollution (Xie et al., 2008). Thus, resolution to the environmental problems and sustainable development objectives has increased the demand for renewable energy resources. The search for alternative energy and fuels has motivated researchers to focus on renewable and sustainable means of getting them instead of relying on the conventional way of energy and fuel production.

Organic wastes are valuable energy resources that can be used for renewable energy production. When they are disposed to the environment without treatment, they result in problems such as air and soil pollution, surface and groundwater degradation, and emissions of greenhouse gases. Biochemical systems, in which organic wastes are converted to biohydrogen or methane, have important role on the basis of environmental protection via waste treatment reduction of the greenhouse gas emissions, and in response to the energy demand by production of renewable/sustainable energy (Giordano et al., 2010). On that basis, AD is one of the important biochemical systems. At the beginning, this technology had been used for sludge degradation; however, the application of it has been expanded for handling of different kind of domestic processes' wastes and also industrial wastes, wastewaters and by-

products. Nowadays, AD has been considered to produce renewable bioenergy like hydrogen and methane by not only treating the wastes/wastewaters, but also using industrial and agricultural by-products (Park et al., 2010). Especially, due to current imperative global issues such as petroleum depletion and global warming, world-wide attention has been paid to the AD process for bioenergy production. As a result a huge amount of study researched the improvement of energy efficiency of application and benefit of the AD process for hydrogen and/or methane production (Park et al., 2010).

Comparing with the other biological treatment processes, AD has some critical advantages based on economically feasible and environmentally safe perspectives such as;

- ✓ Production of usable energy in the form of methane, hydrogen,
- ✓ Less sludge production,
- ✓ Lower installation space requirement and operational cost,
- ✓ No need for aeration and associated energy costs,
- ✓ Low production of stabilized sludge,
- ✓ Low nutrient requirements,
- ✓ Little if any energy requirement,
- ✓ Reduction of greenhouse gas emissions,
- ✓ Suitable for high organic loading rates,
- ✓ Plain technology (relatively simple in operation and maintenance),
- ✓ Biodegradation of aerobically non-biodegradables such chlorinated organics,
- ✓ Anaerobic sludge can be stored unfed (provision of seasonal treatment which is important especially for campaign industries) (Weiland, 1993; Speece, 1996; Weiland, 2010).

On the other hand, AD processes have some disadvantages to maintain the system stability compared to aerobic systems such as;

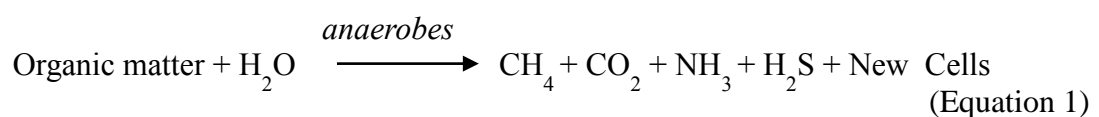
- ✓ Neutral pH and mesophilic or thermophilic temperature conditions must be strictly maintained.

- ✓ Anaerobic digestion generally achieves organic pollution reduction in the region of 85-90%. A second (usually aerobic) step is therefore often needed to attain satisfactory removal of COD.
- ✓ AD systems are more sensitive for COD load fluctuations than aerobic systems to maintain the treatment efficiency.
- ✓ Bacteria require minimum amounts of salts for optimum growth. However if salts are allowed to accumulate beyond the requirements, digestion is inhibited.
- ✓ Hydrogen sulphide, which is one of the output of AD and component of the biogas, is extremely corrosive and its presence requires the purchase of more robust and therefore expensive generators.
- ✓ The high initial costs required to develop an anaerobic digester are often the biggest obstacle to their implementation
- ✓ Cost of employing people with the necessary skills adds to both the initial capital and running costs.
- ✓ Acids are produced as intermediates during the digestion process. If sufficient alkalinity is not present, this will cause an increase in pH and inhibit the digestion (Fullhage et al., 1993; Speece, 1996; Weiland, 2000).

All of these disadvantages contribute to the fact that AD requires more stringent process controls than the more robust aerobic treatment. Thus, many developments have been going on such as reactor configuration changes, deeply understanding on biochemical process necessities and microbial community analysis in anaerobic digesters to eliminate the disadvantages of AD systems (Weiland, 2010).

2.1.1. Process Description

AD is a biochemical process where microorganisms break down complex organic materials in the absence of oxygen as shown below briefly in Equation 1 (Speece, 1996).



During AD, the major gas product is methane, which is a valuable and renewable energy source for heat and electricity generation (McCarty, 2001). In addition H₂, ethanol and volatile fatty acids (VFAs) productions are the important by-products of AD in terms of energy generation and input of various chemical synthesis process for commercial industries (Azbar and Speece, 2001; Krajnc et al., 2007).

In AD process, the four fundamental biochemical processes occur simultaneously (Gerardi, 2003). These are called as hydrolysis, acidogenesis, acetogenesis and methanogenesis particularly. In the hydrolysis process, macro-molecules like proteins, polysaccharides and fats are converted into molecules with a smaller atomic mass that are soluble in water such as peptides, saccharides and fatty acids, respectively. This biochemical flow scheme is shown in Figure 2.1.

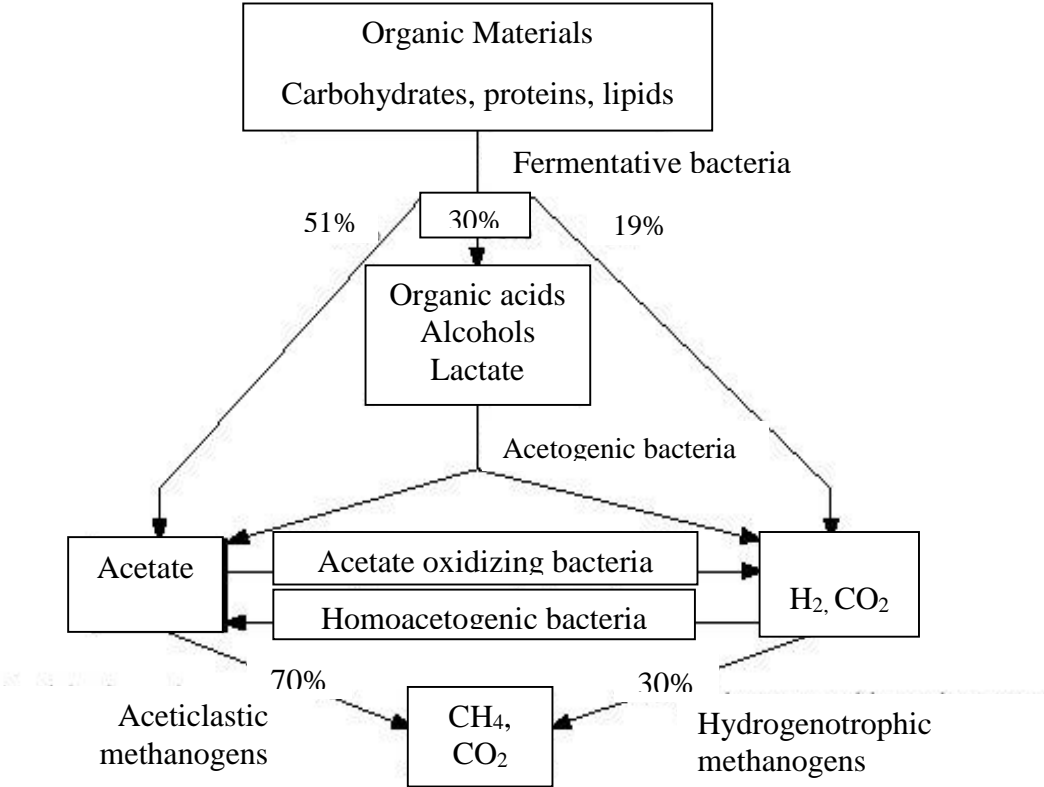


Figure 2-1 Biochemical process flow scheme of anaerobic digestion (Gerardi, 2003)

The hydrolysis or solubilisation process is carried out by exo-enzymes excreted by fermentative bacteria. Hydrolysis is a relatively slow process and generally it limits the rate of the overall anaerobic digestion process (Park et al., 2005).

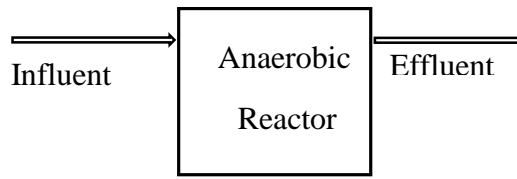
The second step of the AD process is acidogenesis. This process results in the conversion of the hydrolysed products into simple molecules with a low molecular weight, like volatile fatty acids (acetic acid, propionic acid, butyric acid, etc), alcohols, aldehydes and gases like CO₂, H₂ and NH₃. Acidogenesis is performed by a very diverse group of bacteria, the majority of which are strictly anaerobic (Weiland, 2010). Moreover, except the anaerobes, there are always bacteria present that will use oxygen whenever it is available. The presence of these bacteria is important to remove all oxygen that might be introduced into the system. Thus, anaerobic conditions could be also controlled by the process itself. In addition, the acidogenic bacteria are able to metabolise organic material down to a very low pH of around 4 (Weiland, 2010).

In the third step, acetogenesis, the products of the acidification are converted into acetic acids, H₂, and CO₂ by acetogenic bacteria. The first three steps of anaerobic digestion are often grouped together as acid fermentation. It is important to note that in the acid fermentation organic matter is transformed into a form suitable as substrate for the subsequent process of methanogenesis (Parawira, 2004).

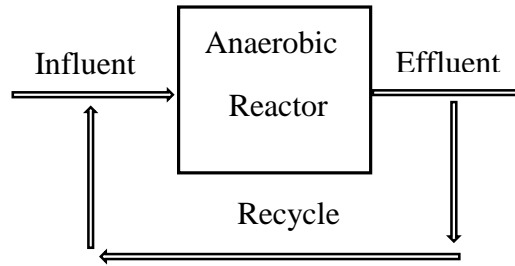
In the final step of the anaerobic digestion process, the products of the acid fermentation (mainly acetic acid) are converted into CH₄ and CO₂ (Speece, 1996). Then, organic materials are removed as the produced CO₂ and CH₄. In each of the four fundamental sequential steps, the catabolic reactions described above develop together with anabolic activity. The free energy released in the reactions is partially used for synthesis of the anaerobic bacterial populations. As the energy release from fermentative catabolism is relatively small, the yield coefficient is much lower than in aerobic processes. Therefore, a large fraction of the digested organic matter is converted into biogas (85 to 95 percent) and less sludge production occurs (Weiland, 2010). In order to maintain an anaerobic sludge with a high metabolic activity, it is necessary to apply favourable environmental conditions. Among these factors the most

important ones are temperature, pH, the absence of toxic materials and the availability of nutrients. The methanogens are very sensitive to adverse environmental conditions and for this reason it is always attempted to maintain optimal conditions for them.

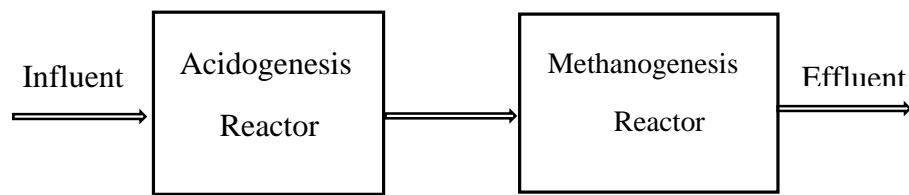
In literature, some fundamental AD process configurations are present such as single-stage, single-phase, two-stage, two-phase AD which affect not only physical scheme but also biochemical processes in the system. The words phase and stage are used interchangeably in the literature. Yet, studies also defined the words in a different way. The main difference between the single & two-stages AD and single & two-phase AD is the recycle of the effluent in the “stage” called AD processes (Azbar and Speece, 2001). For example two-stage AD configuration refers to two consecutive reactors in which a common microbial consortium is recycled between the second-stage methanogenesis reactor and the first-stage acidogenesis reactor. Thus, same microorganisms are exposed to different environmental conditions as well as diverse substrate and metabolic intermediate concentrations in the acidogenesis reactor of a two-stage AD system. Staging can be accomplished in both suspended growth (two consecutive continuously stirred tank reactors or CSTR) and attached growth systems (packing of dense granules or biofilms) (Azbar and Speece, 2001). However, in two-phase AD systems, there is no recycle component of the influent of acidogenesis reactor. Therefore, each microbial species (acidogens and methanogens mainly) activates in their specific environmental conditions. Figure 2.2 represents the configuration differences between these mentioned AD systems briefly.



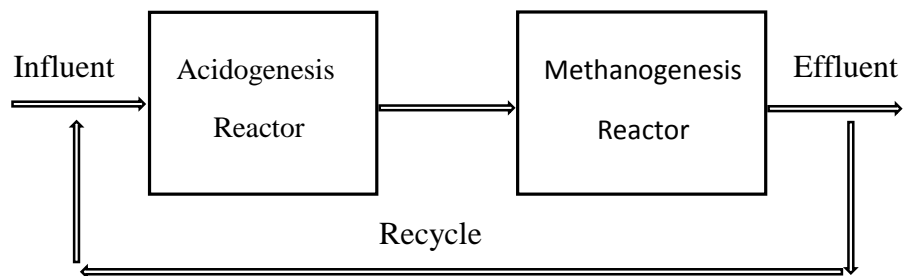
a) Single-phase AD scheme



b) Single-stage AD scheme



c) Two-phase AD scheme



d) Two-stage AD process scheme

Figure 2-2 a) Single-phase AD, b) Single-stage AD, c) Two-phase AD, d) Two-stage AD schemes (Azbar and Speece, 2001)

2.1.2. Two-Phase AD

Two-phase AD process was developed by Ghosh and Poland (1971) to increase the both treatment and methane production efficiency. The basic idea of two-phase AD is to provide optimal environmental and operational conditions for two different dominant groups of syntrophic microorganisms which are acidogens and methanogens (Ghosh and Poland, 1971). Pohland and Ghosh (1971) successfully employed two sequential reactors to separate the acid-forming phase from the methane-forming phase with improved performance and monitored 17% more methane production rate in two-phase AD. Table 2.1 indicates the results of some studies where single-phase is compared to two-phase AD in terms of methane yield and content. As seen in Table 2.1, independent of the reactor type and the carbon source used, both methane yield and methane content of the biogas increase.

In two-phase systems, as mentioned previously the first phase and second phase are called as acidogenesis phase and methanogenesis phase, respectively (Figure 2.1c). In the first-phase, acidogenic microorganisms become dominant by applying various pre-treatment methods on seed sludge (inoculum) and operational conditions which favor acidogenic microbial growth, while preventing methanogenic activity. In the second-phase, however, methanogens become dominant by the time with respect to supportive environmental conditions. The major practical parameters for selectively enrichment of acidogens and methanogens in the corresponding phase are pH (environmental condition) and solid retention time (SRT) (operational condition). SRT is strongly related with growth kinetics. Enrichment of acidogenic microorganisms for the first-phase could be set by applying a low SRT which is short enough to repress methanogenic activity (Hobson and Wheatley 1993; Guerrero et al., 1999). A short SRT in the first-phase washed out the slower-growing methanogenic microorganisms while the faster-growing acidogenic microorganisms remained (Massey and Pohland, 1978). For anaerobic digesters, SRTs of 2 hours to 2 days are reported to be suitable for the accomplishment of an efficient acidification process (Speece, 1996).

Maintaining different pH intervals for each phase increases the efficiency of both acidogenic and methanogenic microorganisms in their respective reactors. For

example, in single-phase AD process, VFAs tend to accumulate, which lowers the pH values to the levels of below 6.5, inappropriate for methanogenic activity. This situation affects the anaerobic reactor sustainability adversely on the basis of methane production and external pH control is needed (Hobson and Wheatley, 1993). However, in two-phase AD system biogas production rate and methane yield can be increased, in a system functioning without external pH control. Methane formation takes place within a relatively narrow pH interval, from about 6.5 to 8.5 with an optimum interval between 7.0 and 8.0. The process is severely inhibited if the pH decreases below 6.0 or rises above 8.5 (Hobson and Wheatley, 1993; Wang et al., 1999). On the other hand, optimum pH interval for the first-phase (acidogenesis) of the two-phase AD is generally reported as 4-5.5 in literature (Wang et al., 1999). Thus, in two-phase AD studies, the consequent lack of methane production was verified, and significant concentrations of VFA were noted in the acidification reactor. After the prescribed contact time in the acidification reactor, a sample of the contents was then transferred to a methanogenic reactor to observe gas production and COD reduction (Massey and Pohland, 1978; Ghosh and Klass, 1978; Cohen et al., 1980).

Two-phase anaerobic systems have been extensively studied and numerous advantages of phase separation over conventional anaerobic digestion (single-phase studies) have been demonstrated (Pohland and Ghosh, 1971; Massey and Pohland, 1978; Cohen et al., 1980; 1982; Demirer and Chen, 2004; Yilmaz and Demirer, 2008; Demirer and Othman, 2008). Some of these advantages include increased process stability and control, need of smaller reactor volumes and high tolerance to toxicity and shock loads. These advantages enable the two-phase AD systems be used to treat many kinds of wastes from following sources such as distillery, landfills, coffee making industry, cheese whey and dairy industry, starch, fruit and vegetable industry, pulp and paper industry, olive mill industry, dye industry, primary and activated sludge and solid wastes (Ke et al., 2005; Kyazze et al., 2007; Nasr et al., 2012).

Table 2-1 Comparison of two-phase and single-phase AD in terms of methane yield and methane content in biogas

Reference	Reactor Type	Carbon Source	Single-Phase		Two-Phase	
			CH ₄ yield (mL/g sCOD _{added})	CH ₄ (%)	CH ₄ yield (mL/g sCOD _{added})	CH ₄ (%)
Bull et al. (1983) ^a	FBR	Glucose	268	73	356	77
Weiland, (1993) ^a	CSTR	Sugar beet pulp	210	59	230	72
Nasr et al. (2012) ^b	Batch	Thin stillage	260	-	330	-
Yeoh (1997) ^b	CSTR	Cane molasses alcohol stillage	155	59	168	67
Wust (2003)	UASB	Cheese ww	-	55	-	66
Koç et al. (2014) ^a	Batch	Fermented molasses	311	68	461	85

a: Methane yield was calculated for 1 atm at 35°C

b: Methane yield was calculated for standard temperature and pressure (STP)

FBR: Fluidized bed reactor, CSTR: Completely stirred tank reactor

UASB: Up-flow anaerobic sludge blanket

ww: wastewater

2.2 Factors Affecting AD

2.2.1 Hydraulic Retention Time (HRT)

HRT is the average time that liquid (wastewaters) retained in the reactors (Speece, 1996). Short HRTs result in a smaller reactor size and thus provide more economical treatment options. Thus, AD systems might become more economically feasible at low HRTs. These developments have enabled a variety of dilute soluble and colloidal wastes, such as sewage, to be treated economically while keeping the HRT to a minimum (1.3–20 hours) (Speece, 1996). In this way, efficient organic (COD) destruction at short HRTs without the accumulation of excessive residual organic matter and intermediate products, such as VFAs and methane production can be investigated (Speece, 1996).

It should be noted that HRT also varies depending on the suspended and attached growth features of the AD systems. Ngon and Dague (1997) studied with ASBRs and achieved 80–90% sCOD removal efficiency at various dilute substrate concentrations and temperatures of 35, 25 and 20 °C at HRTs less than 24 hours. On the other hand, Rincon et al. (2008) reported that AD process, which used olive mill wastewater as substrate, failed when OLR was higher than 9.2 g COD/L day and HRT was lower than 17 days.

2.2.2 Substrate to biomass (S/X₀) ratio

S/X₀ ratio (g COD/g VSS) is an important parameter to test the anaerobic biodegradability and methane production potential of wastewaters in batch reactors (Neves et al., 2004). Theoretically S/X₀ ratio has an effect only on the kinetics and not on the ultimate methane yield which depends on the organic matter content (Raposo et al., 2006). It is reported that too high S/X₀ ratio may be toxic while too low S/X₀ ratio may prevent induction of the enzyme necessary for biodegradation (Prashanth et al., 2006). Each substrate has its optimum S/X₀ ratio, considering the potential amount of VFAs produced and its capacity to buffer the medium due to the ammonium produced by the hydrolysis of proteins (Lesteur et al., 2010). For these

reasons S/X_0 ratio should be recognized as one of the major parameters affecting the results of anaerobic assays. Previous work on the effect of S/X_0 ratio indicates different ratios. The ratio proposed by Owen (1979) was approximately 1, while Chynoweth (1993) suggested a ratio of 2. Neves (2004) used a range of S/X_0 ratios such as 2, 1, 0.74, and 0.43 for effective AD.

2.2.3 Food-to-microorganism (F/M) ratio

F/M ratio (g COD/g VSS/day) is a process control factor based upon maintaining a specified balance between available food materials (BOD or COD) and the microorganisms (VSS) concentration regarding with specific time intervals in biological reactors. According to Tanaka et al. (1997), F/M ratio should be in the range of 0.45-0.5 g COD/g VSS/day in order to solubilize cells efficiently. Pranshanth et al. (2006) found the best value of F/M ratio between 0.57- 0.68 g COD/g VSS/day for anaerobic digestion. In the study of Braguglia et al. (2006) they mentioned that the optimum range for F/M ratio was found between 0.5 and 2 g COD/g VSS/day for untreated excess sludge.

2.2.4 Solid retention time (SRT)

SRT is the average time that sludge retained in the biological reactor; in other words, bacteria and archaea species are retained in the digester (Speece, 1996). Depending on the required treatment, biogas production efficiencies and operational conditions applied (such as temperature, waste characteristics, mixing, etc.), different SRTs may suitable in a wide range. However, SRT must be kept long enough to avoid from wash-out of methanogens. Providing sufficient residence time to microorganisms, especially for slow-growing methanogens, results with sustainable and effective treatment and biogas production applications (Gerardi, 2003). Lawrence and McCarty (1969) found that methanogenesis failed when the SRT was 2.5–4 days due to washout of the methanogens. The limiting values of the minimum SRT for acetoclastic and hydrogenotrophic methanogens are reported to be 4 and 0.76 days, respectively (Rittmann and McCarty, 2001). In addition to the effect on wash-out limits, SRT value has an effect on enrichment of dominant acetoclastic methanogenic microorganism

species in anaerobic digesters. Lee et al. (2011) reported that 15 days and a lower value of SRT values resulted in dominance of *Methanosarcina* specie over *Methanosaeta* specie. On the other hand, Shigematsu et al. (2003) noted that SRT is not the only operational parameter which affects the relative dominance of any acetoclastic methanogens in AD system. For example, it was reported that *Methanosaeta* species dominate at low acetate concentrations (10^{-6} – 10^{-3} M acetate) while *Methanosarcina* species have a competitive advantage at higher acetate concentrations (Shigematsu et al., 2003; Zinder, 1993).

2.2.5 Organic loading rate (OLR)

OLR is the amount of substrate fed per unit volume of biological reactor in a unit period of time and is very crucial for digester performance (Rajeshwari et al., 2000). OLR is closely linked to loading of organics (COD) in the form of methane and the number of methanogens retained in the digester (SRT). In other words, high methanogenic activity by biomass immobilization ensures efficient removal of organics and enables high OLRs. Romano and Zhang (2008) claim that optimal OLRs are dependent on various operational parameters including the substrate, type of reactor, HRT, nutrients and alkalinity. In suspended and attached growth reactors, typical OLR values are reported as 0.25–3 and 10–100 g COD/L/day respectively (Rajeshwari et al., 2000).

2.2.6 Temperature

Anaerobic microorganisms, especially methanogens, are strongly influenced by temperature which makes digestion process preferable at mesophilic (30–35 °C) and thermophilic (50–60 °C) temperatures (Gerardi, 2003). On the other hand, the use of new or modified bioreactors also enables the use of psychrophilic (<20 °C) temperatures for anaerobic treatment of different effluents since they sustain required residence times for methane producers to grow (Connaughton et al., 2006).

2.2.7 pH

AD is a strongly pH dependent process. Although each of the microbial groups prefers specific pH ranges, most of them perform well near neutral pH conditions. Methanogens operate optimum at a range of 6.5 to 8.2 while acidogens prefer a pH range of 4 and 6.5 (Speece, 1996). The decrease in pH might lead to a reduction of the methane production rate and further accumulation of acids. The digester will only return to methanogenic activity when the pH of the reactor is restored to a value near neutral pH. In a well-operating AD, deviations of pH from desired ranges are prevented by alkalinity addition. In order to maintain the pH at or near neutral, alkalinity concentrations from 2000 to 4000 mg/L (as CaCO₃) are usually required (Tchobanoglous et al., 2003). However, in two-phase systems, the second phase of the system (methanogenic phase) does not need any alkalinity addition during the reaction, if the pH of the effluent of the first-phase is neutralized before the usage in second-phase as substrate. This is an advantage of two-phase systems in terms of process stability as also described in detail in Section 2.1.2.

2.2.8 Micro- and macro-nutrients availability

For the survival and growth of specific groups of microorganisms involved in AD processes, several macro-and micro-nutrients are necessary. Macro-nutrients are carbon, nitrogen, phosphorus, and sulfur mainly. Actually, the need of nutrients is very low and common ratio of them is C:N: P:S=100:5:1:0.5 (Speece, 1996). However, literature have reported many ratios in a large scale such as C:N: P:S=600:15:5:1 due to the fact that not much biomass is developed (Abdoun and Weiland, 2009; Jarvis et al., 1997). Trace elements like iron, nickel, cobalt, selenium, molybdenum and tungsten are important for the growth of microorganisms and must be added if, for example, energy crops are used for biogas production as the only substrate (Weiland, 1993). Nickel is generally required for all methanogenic bacteria because it is necessary for the synthesis of the cell component cofactor F430, which is involved in the methane formation. For optimal growth, the cells require cobalt to build up the Co-containing corrinoid factor III. The function of selenium, molybdenum, and tungsten is not completely clear, and the growth of only few methanogens depends on these

trace elements. The necessary concentration for the micronutrients is very low and in the range between 0.05 and 0.06 mg/L. Only iron is necessary in higher concentrations changing from 1 and 10 mg/L (Bischoff, 2009).

2.2.9 Toxic materials

A wide variety of inhibitory substances for anaerobes are the primary cause of anaerobic digester upset or failure since they are present in substantial concentrations in domestic/industrial wastes, wastewater (Speece, 1996). These are commonly ammonia, sulfide, light metal ions (Na, K, Mg, Ca, and Al), heavy metals (chromium, iron, cobalt, copper, zinc, cadmium, and nickel) and halogenated compounds. Due to the microbial consortium of anaerobic inocula, waste composition and experimental methods/conditions, it is stated in literature that inhibition is caused by specific toxicants of various types regarding with toxicant's concentration. Finding out and taking precautions against toxicants can significantly improve the treatment and methane production efficiency of any anaerobic digestion study (Chen et al., 2008).

2.2.10 Reactor Types

There are many types of reactor configurations being used for AD process such as upflow sludge blanket (UASB) reactor, Expanded granular sludge bed (EGSB) reactor, completely stirred tank reactor (CSTR), Fluidized bed reactor (FBR), Sequencing batch reactor (SBR), membrane bioreactor (MBR). All of these configurations were developed to improve treatment efficiencies and renewable energy production for various types of substrates. In Section 2.3 below, these configurations explained briefly.

2.3 Common Reactor Types Used in AD Studies

2.3.1 UASB Reactor

UASB reactor achieves AD process via blanket of granular sludge. Wastewater flows upward through the blanket and is processed (degraded) by the anaerobic microorganisms. Good settleability and solids/liquids separation can be achieved with

this reactor type by using granular sludge (Lettinga et al., 1994). UASB reactors have the advantages such as high tolerance to toxic shockloads and high OLRs. This reactor configuration has been used for many types of substrates such as cheesewhey, olive millwastewater, olive pomace leachate, potato waste leachate, thin stillage, slaughterhouse wastewater and dairy manure for both treatment and renewable energy production (Speece, 1996).

2.3.2 EGSB Reactor

EGSB reactor has similar technology with UASB reactor, but a developed one. It was developed to decrease the death spaces below 10-11% which was observed in UASB reactor. EGSB reactor provides separation of dispersed sludge and mature granule using rapid upward velocity. Then, it is possible to treat high-strength (30000 g COD/L) and low-strength wastewater (1000- 2000 mg /L) especially low temperature. By using EGSB reactor, an OLR of 3-6 times greater than a UASB reactor was accommodated with equal removal efficiency (Vallinga et al., 1986). Wastewaters containing high lipid concentration, which cause foaming and scum problems, can be treated with EGSB reactors more efficiently than UASB reactors (Speece, 1996).

2.3.3 FBR

In this type of reactor design, biomass is attached to a carrier such as sand, coal, and granular activated carbon, direct separation of liquid and biomass can be handled to avoid a process failure with total loss of biomass within short periods such as 15 minutes (Speece, 1996). Wastewaters of wine distillery and dairy industry are some of the substrates treated in FBR reactor (Arnaiz et al., 2003; Garcia et al., 1998).

2.3.4 CSTR

The CSTR is frequently used in research due to its simplicity in design and operation, but also for its advantages in experimentation. Compared to other configurations, the CSTR provides greater uniformity of system parameters, such as temperature, mixing, chemical concentration, and substrate concentration. This reactor configuration is used in a wide range such as for activated sludge and many industrial wastewaters sourced

from such as sugar beet pulp, cheesewhey, olive mill and coffee industry (Weiland, 1993; Speece, 1996; Antonopoulou et al., 2008; Koutroli et al., 2009; Dareioti et al., 2014).

2.3.5 Anaerobic MBR

MRB is the combination of a membrane process like microfiltration or ultrafiltration with a suspended growth bioreactor. MRB is used widely for domestic and industrial wastewater treatment (Cui et al., 2003). Advanced wastewater treatment could be achieved with MRBs; however, high energy cost is still problem. Successful full-scale applications of MRBs have seen for the treatment of some types of industrial wastewaters containing high strength wastes (Scott et al., 2011).

2.3.6 ASBR

ASBR was developed by Dague (1992). ASBR has not been used as widely in the wastewater treatment as other processes such as UASB. However, it does hold some advantages over other systems, including its relative ease of operation, flexibility, and the fact that it uses the same vessel for both reacting and settling. Some of the potential advantages of SBR technology could be given as;

- Ability to handle periodic flows,
- Possibility of taking tanks on and off-line to meet either short-term or seasonal variations,
- Ability to adapt to periodically changing environmental conditions in a controlled biochemical manner, thereby selecting or enriching specific microbial population; and to better ensure biomass
- Ability to retention of biomass, as supernatant withdrawal occurs in nearly ideal quiescent condition (Kennedy, 2014).

The reactor sequences through four periods; feeding, react (reaction), settling, and decanting (withdrawal). The feeding period involves the addition of the wastewater to the reactor. The feed volume is determined on the basis of a number of factors such as

desired HRT, OLR, SRT, F/M ratio, exchange ratio and expected settling characteristics of the sludge.

The reaction period is the most critical one for the conversion of organic substrates to biogas. The time required for the reaction step depends on several substrate characteristics and parameters, including effluent biomass concentration and pollution strength, required quality and temperature.

Effluent liquid and solid biomass separation occurs in the settling period. The reactor itself acts as a clarifier. The time required for settling period varies depending on the desired SRT value especially. However, the settling period typically ranges from 10 to 30 minutes. In fact, the concentration of mixed liquor suspended solids (MLSS) in the reactor could be taken as an important indicator to choose meaningful settling time. Because it has a direct effect on settling velocity of the biomass and so the ability to achieve a clear supernatant for discharge as effluent. Another important related variable with settling time is the F/M ratio. Excess amount of biomass loss causes drastic changes on F/M ratio and; thus, affect the performance of the reactor (Sung and Dague, 1995).

The decanting period takes place after settling of the solids in the reactor (following settling period). The decant volume is normally equal to the volume fed during the previous feeding step. The time required for the decant period is governed by the total volume to be decanted during each cycle and the decanting rate. When the decant step is finalized, the reactor is ready for the next cycle to be fed again (Sung and Dague, 1995). As it was mentioned before, ASBR technology has used for wide range of substrates such as glucose, sucrose, cheesewhey, olive mill and dairy industry wastewater for COD treatment and methane production (Table 2.2). Researches on the technical and economic viability of using an ASBR to treat various waste streams are still ongoing. Moreover, additional researches were done by literature to optimize the operational parameters is necessary before widespread dissemination of ASBR technology can be accomplished. In Table 2.2 below, some of the ASBR with related operational parameters from literature are given. Results of these studies showed that,

ASBR process has become effective not only for basic carbohydrates (such as glucose and sucrose), for various industrial wastewaters. ASBRs have been found to capable of achieving 68-83% COD removal for the industrial wastewaters mentioned in Table 2.2 (Ammary et al., 2005; Damasceno et al., 2007; Göblös et al., 2007).

Table 2-2 Comparison of two-phase and single-phase AD in terms of methane yield and methane content in biogas

Reactor type	Substrate	Initial COD conc. (g/L)	OLR (g/L.day)	HRT (day)	SRT (day)	COD treatment ^a (%)	Reference
ASBR	Glucose	1-6	3.2	1-2	n.g.	n.g.	Shizas and Bagley
ASBR	Glucose	10	20	0.5	6	94	Angenent and Dague
ASBR	Glucose	3.75-30	3-12	1.25-5	20-70	50-60	Cheong et al. (2008)
ASBR	Glucose	5.85	3.5	1.7	n.g.	94.3	Shimada et al. (2007)
ASBR	Glucose and VFA	1	4-5	4-5	n.g.	60-70	Brito et al. (1997)
ASBR	Sucrose and HAc	7	4.6-18.4	2.62-0.65	n.g.	40-97	Kennedy et al. (1991)
ASBR	Olive mill industry ww	2-32	5.3	0.5-3	45	83	Ammary et al. (2005)
ASBR	Cheese whey ww	1-6	2-12	0.5	n.g.	73	Damasceno et al. (2007)
ASBR	Dairy ww	n.g.	12.8	40-5	n.g.	68	Göblös et al. (2007)
^a n.g.: not given, ww: wastewater							

2.4 Molecular Tools Used in AD Process

Performance of biological treatment systems are related to the composition and activity of microbial populations they contain. The types of microorganisms present and their relative population levels in a bioreactor biomass vary with the changes in wastewater characteristics as well as operational conditions of the bioreactor (McHugh et al., 2003). For AD processes, biochemical methane production occurs in a wide variety of highly reduced anaerobic environments by degradation of organic compounds in industrial and municipal wastewater. AD systems are carried out by the syntrophic biological activity of an interdependent microbial community, composed of bacteria and archaea species. Therefore, an understanding of the microbial community structure and dynamics in AD process is a key requirement to improve and optimize the process to increase renewable energy production. For that reason, molecular biology methodologies are the essential tools to monitor and understand the microbial consortium inside an anaerobic reactor such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; 1998), fluorescent in situ hybridization (FISH), terminal restriction fragment length polymorphism (T-RFLP) (Amann et al., 1995), etc. In addition to microbial consortium analysis, some molecular methodologies such as quantitative polymerase chain reaction (qPCR) were also developed for quantitative microbial analysis to understand the relative population level of microorganisms. Thus, the role of the specific degradative function microorganisms within the treatment system can be identified to a certain extent. These methodologies make it possible to design better anaerobic treatment processes, in terms of higher degradation capacity and methane production. For example, qPCR methodology makes it possible to monitor and understand that which methanogenic species of concern has the critical role in for higher methane yields regarding with anaerobic reactor's operational conditions and environmental conditions.

2.4.1 Molecular studies to monitor microbial consortium of AD via DGGE

DGGE is a very useful molecular methodology to gain insight into the microbial consortium of AD. It is a molecular fingerprinting method that separates polymerase chain reaction (PCR) generated DNA products (Gropkbf et al., 1998). The PCR

products of environmental DNA can generate templates of different DNA sequence that represent many of the microbial organisms. During DGGE, PCR products encounter increasingly higher concentrations of chemical denaturant as they migrate through a polyacrylamide gel. Upon reaching a threshold denaturant concentration, the weaker melting domains of the double-stranded PCR product will begin to denature at which time migration slows dramatically. Differing sequences of DNA (from different bacteria or archaea specie) will denature at different denaturant concentrations resulting in a pattern of bands. Each band theoretically represents a different bacterial population present in the community. Once generated, fingerprints can be uploaded into databases in which fingerprint similarity can be assessed to determine microbial structural differences between environments or among treatments. DGGE methodology for anaerobic reactor reaction samples has been used by many researchers to clarify the population dynamics of anaerobic digesters (Embley et al., 1992; Raskin et al., 1994; Reysenbach and Pace, 1995; Nubel et al., 1996; Gropkbf et al., 1998; Casamayor et al., 2001; Roest et al., 2005; Akarsubaşı et al., 2005; Zhang et al., 2005; De Bok et al., 2006; Diaz et al., 2006; Vieria et al., 2007; Yu et al., 2008; Hook et al., 2009; Jianzheng et al., 2013).

2.4.2 Molecular studies to quantify AD consortium, in particular *Methanosarcina* and *Methanosaeta* species via qPCR

QPCR is a culture-independent and very sensitive laboratory technique of molecular biology based on PCR, which is used to amplify and simultaneously detect or quantify a targeted DNA molecule in terms of gene copy number (Shigematsu et al., 2003). Thus, it is possible to record the target gene or DNA concentration with high sensitivity, which belongs to a specific microorganism, in per gram of volatile suspended solids in biological reactor. For the AD processes, quantification and relative dominance level of *Methanosaeta* and *Methanosarcina* species are the most common methanogens studied with qPCR. There are some qPCR methods; but, TaqMan and SYBR Green qPCR are the two frequently used methods. Taqman method uses a fluorogenic probe specific to target gene to detect target gene as it accumulates during PCR. SYBR Green method uses SYBR® Green dye (a dsDNA binding dye) to detect PCR product as it accumulates during PCR. Cao and Shockey

(2012) applied both TaqMan and SYBR Green qPCR to same DNA samples and noted that both assays are reliable for determining gene expression; however, TaqMan method is more sensitive especially for the samples which has low gene copies (Cao and Shockey, 2012).

2.4.3 Factors (environmental conditions and operational parameters) affecting dominance of *Methanosarcina* and *Methanosaeta* during AD

Methanogenic microorganisms can be grouped into two as hydrogenotrophic and acetoclastic methanogens in an AD process (Demirel and Scherer, 2008). Hydrogenotrophics and acetoclastic methanogens compete for hydrogen and acetate respectively to produce methane. In an AD process, methane production is mostly carried out by acetoclastic methanogens which are responsible for the nearly 70% of total methane produced (Speece, 1996). A closer look to phylogenetic tree of acetoclastic methanogens indicated that the dominant species in an AD reactor is mostly *Methanosarcina* and *Methanosaeta* species (Demirel and Scherer, 2008). Considering that they are the major methane producers, understanding the environmental and operational conditions favouring these acetoclastic methanogens is significance to develop AD process.

Many factors such as temperature, substrate concentration, SRT, HRT, OLR affect the dominant specie and the microbial population shift (Rastogi et al., 2007; Daniels et al., 1984). Rastogi et al. (2007) reported that a change in temperature from 24 to 36 °C resulted in a microbial shift in a biogas plant. The effects of SRT and HRT change on dominant acetoclastic methanogen type have been studied in a few studies (Lee et al., 2011; Ma et al., 2013; Leclerc et al., 2004; Mladenovska et al., 2003). Short HRTs (less than 5 days) are reported to support *Methanosarcina* dominance (Leclerc et al., 2004; Mladenovska et al., 2003). A decrease in SRT from 20 days to 5 days; on the other hand, was reported to result in decrease in the dominance of *Methanosaeta* specie. SRT and HRT are two important parameters affecting the performance of AD. Therefore, the effect of SRT and HRT on the microbial population shift, dominant acetoclastic methanogens and the related performance should be researched in detail.

During quantification of *Methanosaeta* species in anaerobic bioreactors, it was found that *Methanosaeta* is the dominant acetoclastic methanogen in a variety of anaerobic

reactors at low acetate concentrations (Zheng and Raskin, 2000). The amount of *Methanosaeta* species was higher in bioreactors working with granular sludge than in those with flocculent sludge (Lee et al., 2011). Moreover, previous research has repeatedly shown that *Methanosaeta* dominance was found in anaerobic digesters, such as CSTR and UASB reactor under steady state conditions (Mc Hugh et al., 2003; Raskin et al., 1995; Schmidt and Ahring, 1999; Sekiguchi et al., 1998). Raskin et al. (1995) investigated 21 conventional sewage anaerobic digesters with a wide variation in digester design and operating conditions by means of molecular probes, and found that *Methanosaeta* specie dominated in all digesters. Their dominance was consistent with the low acetate concentrations present in all of the digesters conditions, which provided competitive advantage for *Methanosaeta* specie due to their low K_s compared to *Methanosarcina* specie.

Some of the individual biochemical characteristics of the acetotrophic methanogens direct the relative level dominance of *Methanosarcina* and *Methanosaeta* such as affinity to substrate (K_s), growth rate (μ), and substrate utilization rate in concert with environmental and operational conditions (Shigematsu et al., 2003). As Zinder (1993) noted, *Methanosarcina* are the most versatile methanogen specie offering methane production, when compared to hydrogenotrophic and other acetoclastic methanogens (*Methanosaeta*). Relatively high acetate and hydrogen concentrations form suitable conditions for the relative dominance of *Methanosarcina* which has higher in substrate utilization rate, growth rate and cell yield compared to *Methanosaeta* (Daniels et al., 1984). Thus, *Methanosarcina* is favored under conditions in which a high input of organic matter leads to rapid accumulation of acetate and hydrogen (Zinder, 1993). Consequently, digesters dominated by *Methanosarcina* are more capable of handling increased loads (Conklin et al., 2006).

CHAPTER 3

MATERIALS AND METHODS

This chapter covers the materials and methods, and experimental procedures of the experimental set-ups performed namely, Batch Reactor Set-1, Batch Reactor Set-2 and Anaerobic SBR (ASBR) study. Analytical methods involving the molecular analyses followed are also given in this chapter.

3.1. Substrate Characteristics

Four different substrates (influent types) were used in the experiments as shown in Table 3.1. As seen in Table 3.1 glucose was used as the substrate source in Batch Reactor Set-1. Sucrose, Influent A and Influent B were used as substrate source in Batch Reactor Set-2, while Influent A and B were used in the ASBR study.

Table 3-1 Substrate types used in the experiments

Experiment name	Substrate types
Batch Reactor Set-1	Glucose
Batch Reactor Set-2	Sucrose
	Influent A
	Influent B
ASBR Study	Influent A
	Influent B

Both Influent A and B are the effluents of a dark fermentative (DF) sequencing batch reactor (DF-SBR) which was studied by Ekin Güneş Tunçay in Environmental Engineering Department of Middle East Technical University, Ankara, Turkey

(Tunçay, 2015). Sucrose had been used as the substrate in this DF-SBR. Therefore, the effluents of DF-SBR were eventually fermented sucrose. Because DF-SBR run at different operational conditions, the effluents withdrawn at these different conditions were named as Influent A and B. Influent A and B were filtered immediately with 0.45 µm pore size filters (Millipore) after their withdrawal from DF-SBR and kept frozen at -20 °C in order to prevent biological activity prior to the use in this study. Moreover, the pH of the influents was adjusted to 7-7.5 at 35 °C with a 1 N NaOH solution just before the batch reactors and ASBR have been started to operate. Table 3.2 indicates the characteristics of filtered Influent A and B which were used in Batch Reactor Set-2 and ASBR study. As seen in Table 3.2, both Influent A and B had similar characteristics despite the higher sCOD and the lower total VFA content of the former. Nevertheless, both substrates of different initial VFA concentrations were used in Batch Reactor Set-2.

Table 3-2 Characteristic of the Influent A and B used in ASBR study and Batch Reactor Set-2

Parameter	Influent A	Influent B
sCOD (mg/L)	10750±254	8628±312
TAN (mg/L)	8±0.2	9±0.4
Alkalinity (mg/L CaCO ₃)	2122± 68	2467± 73
pH	6.2	6.3
Acetic Acid (mM)	21	24±0.2
Propionic Acid (mM)	4	15±0.1
Butyric Acid (mM)	17	20±0.1
Iso-butyric Acid (mM)	1	2±0.02
Total VFA (mM HAc)	35±1	51.4±0.1
sCOD: Soluble chemical oxygen demand TAN: Total ammonifiable nitrogen (NH ₄ ⁺ -N + NH ₃ -N) VFA: Volatile fatty acid HAc: Acetic acid		

3.2 Inoculum

Mixed anaerobic cultures used as inoculum (seed) in this thesis were obtained from anaerobic sludge digesters of Central Wastewater Treatment Plant of Ankara. Total suspended solid (TSS) and volatile suspended solid (VSS) values of the anaerobic seed sludge used in all experimental set-ups are given in Table 3.3. pH values of the inoculum for all experimental set-ups were around 8.5-8.6. Any pre-treatment or acclimation were not applied to the inoculum before the reactor operations.

Table 3-3 TSS, VSS and pH values of the inoculum used in the experiments

Experiment Name	TSS (mg/L)	VSS (mg/L)	pH
Batch Reactor Set-1	32267±1097	13217±443	8.5
Batch Reactor Set-2	32267±1097	13217±443	8.5
ASBR Study	42290±127	18510±240	8.6

3.3. Basal Medium (BM)

In order to supply necessary nutrients and minerals for an optimum anaerobic microbial growth, reactors were fed with basal medium, which contains the following constituents, shown in Table 3.4.

Table 3-4 Basal medium (BM) constituents (Speece, 1996)

Constituent	Concentration (mg/L)	Constituent	Concentration (mg/L)
NH ₄ Cl	300	NH ₄ VO ₃	0.5
MgSO ₄ .7H ₂ O	400	CuCl ₂ .2H ₂ O	0.5
KCl	400	ZnCl ₂	0.5
Na ₂ S.9H ₂ O	300	AlCl ₃ .6H ₂ O	0.5
CaCl ₂ .2H ₂ O	50	Na ₂ MoO ₄ .2H ₂ O	0.5
(NH ₄) ₂ HPO ₄	80	H ₃ BO ₃	0.5
FeCl ₂ .4H ₂ O	40	NiCl ₂ .6H ₂ O	0.5
CoCl ₂ .6H ₂ O	10	Na ₂ WO ₄ .2H ₂ O	0.5
KI	10	Na ₂ SeO ₃	0.5
(NaPO ₃) ₆	10	Cysteine	10
MnCl ₂ .4H ₂ O	0.5		

3.4. Analytical Methods

3.4.1 Chemical Oxygen Demand (COD)

COD determinations were carried out by EPA approved digestion method (for COD range of 0-1500 mg/L) and spectrophotometric determinations were performed by using a spectrophotometer (SN 05827, PC Multidirect). For sCOD analysis, samples were filtered through 0.45 μm pore sized filters (Millipore).

3.4.2 Biogas Production

Daily biogas production was measured with water replacement devices (Ergüder et al., 2001). In Batch Reactor Set-1 and 2 studies, a device consisting of a 50 mL burette connected to a 500 mL water reservoir was used. A needle connected to the burette via latex tubing was inserted through the rubber stoppers of the reactors to determine the produced biogas amount in the headspace of 250 mL reactors. For ASBR study, biogas production was measured with a 2 L measuring cylinder consisting of acid brine (10% NaCl w/v, 2% H₂SO₄ v/v, pH was 4.5). Thus, solubility of CO₂ was eliminated to do exact measurements (Tezel et al., 2007).

3.4.3 Biogas Composition

Biogas composition were periodically determined with a gas chromatograph (Thermo Electron Co.) equipped with a thermal conductivity detector (TCD). Produced biogas was separated as H₂, CO₂, O₂, CH₄ and N₂ by using serially connected columns (CP-Moliseve 5A and CP- Porabond Q) at a fixed oven temperature of 45 °C. Helium was used as carrier gas at 100 kPa constant pressure. The inlet and detector temperatures were set to 50 °C and 80 °C, respectively. The calibration curves prepared for the gas content analysis are given in Appendix A.

3.4.4 Volatile Fatty Acids (VFAs) Determinations

VFAs analyses were done using HPLC (Shimadzu 10A series) equipped with Alltech IOA-1000 Organic Acid Column. H₂SO₄ (0.085 M) was used as the mobile phase pumped at a flow rate of 0.4 mL/min using a low gradient pump (Shimadzu LC-10AT).

The oven temperature was kept constant at 66 °C and 10 µl sample was analyzed using a UV detector (Shimadzu FCV-10AT) with an absorbance value set at 210 nm. Prior to the liquid chromatography injections, samples were initially filtered through 0.22 µm pore-sized filters (Androga et al., 2011). The calibration curves prepared for the gas content analysis are given in Appendix B.

3.4.5 Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS)

TSS and VSS were determined according to Standard Methods (APHA, AWWA and WPCF 2005).

3.4.6 Alkalinity

Alkalinity was measured according to Standard Methods (2320-B Titration Method) (APHA 2005).

3.4.7 pH

pH analysis was performed with a pH meter (HI 8314, Hanna Instruments) and a pH probe (HI 1230, Hanna Instruments).

3.4.8 Molecular Analyses

3.4.8.1 Sampling and DNA Isolation

ASBR Study sludge samples were collected at regular intervals and stored at -20 °C during the reaction period of the ASBR. Just before the molecular analysis started, stored and frozen DNA samples were thawed prior to isolation of DNAs and centrifuging for 10 minutes at 8000 rpm. Thus, solid (biomass or microorganism) phase of the samples were separated from the liquid phase and subjected to DNA isolation.

For DNA isolation, two isolation kits, namely, OMEGA-EZNA Soil DNA Isolation and Power MBIO Soil DNA Isolation Kits were initially compared. The isolation kit giving the highest DNA concentration with highest purity (least chemical and protein

contamination) was selected and used in the following experiments throughout the thesis study. For comparison of two different sludge samples taken from DF-SBR (named as DF 1 and DF 2) mentioned in Section 3.1 and ASBR (named as ASBR 1 and ASBR 2) were used. The analyses were performed in duplicate. DNA extraction from samples were performed according to the protocol of each isolation kit. Concentration of isolated DNA samples were measured using Nanodrop 2000c Spectrophotometer (Thermo Scientific) at METU Central Laboratory. The result of this comparison study is given in Table 3.5.

In Table 3.5, 260/280 ratio is an indicator of protein contamination, and implies purity of nucleic acids for a healthy PCR application, if it is around 1.8. Moreover, 260/230 ratio between the range of 2.0 to 2.2 means that isolated DNA sample does not contain any chemical contamination due to phenol, ethanol and cell extract indicates. Such a level reveals that DNA isolation is healthy and a successful PCR application is possible with the isolated DNA samples. Referring to Table 3.5, the ratio of 260/280 for all the isolated DNA samples were around non-contaminated vicinity in terms of protein contamination for both isolation kits. Thus, all isolated DNA samples are suitable for PCR application by looking at both kits 260/280 ratio values. When the ratio values of 260/230 in Table 3.5 were observed, it could be said that not all of the isolated DNA samples are not in the range of 2.0 to 2.2. This was attributed to a potential chemical residue in all of the samples; however, the values are still not at a critical level. In other words, a healthier PCR application is still and strongly possible with the isolated DNA samples. In addition to these ratio values, nucleic acid concentrations were compared for both kits to select the one resulting in the best solution.

As it could be noticed from the Table 3.5, OMEGA-EZNA Soil DNA Isolation Kit has emerged higher DNA concentration for all sludge types compared to Power MBIO Soil DNA Isolation Kit. Thus, it was decided to use the OMEGA-EZNA Soil DNA Isolation Kit for further DNA isolation studies performed for the sludge samples taken from ASBR. For isolation, the protocol given by OMEGA-EZNA Soil DNA Isolation Kit was used. Healthy isolated DNA samples were further subjected to PCR and DGGE analyses.

Table 3-5 Comparison of OMEGA-EZNA DNA Isolation Kit and Power MBIO Soil DNA Isolation Kit

Sample No	Soil DNA Isolation Kit	DNA Concentration (ng/ μ L)	260/280	260/230
DF 1	POWER-MBIO	38.8	1.87	1.35
DF 2		36.4	1.92	1.3
ASBR 1		52.5	1.85	1.85
ASBR 2		47.3	1.85	1.9
DF 1	OMEGA-EZNA	352.5	1.88	1.41
DF 2		412.1	1.88	1.4
ASBR 1		202.1	1.87	1.54
ASBR 2		192.2	1.88	1.38
DF1 and DF2: Duplicate sludge samples taken from DF-SBR ASBR 1 and ASBR 2: Duplicate sludge samples taken from ASBR study				

3.4.8.2 PCR and Gel Electrophoresis

After DNA isolation, obtained DNAs were subjected to PCR analysis. The PCR is the process of obtaining millions or even billions of copies of DNA by in vitro amplification providing appropriate conditions of deoxyribonucleic acid (Madigo and Martinko, 2006). In other words, PCR analysis is the amplification of desired polynucleotides region on a DNA using primers. In this method, four thermal cycles are required consecutively which are listed below:

- ✓ Separation of the double strand DNA by denaturation,
- ✓ Binding of separated DNA strands with primers (annealing),
- ✓ Synthesize the target region of DNA by polymerase enzymes and

- ✓ Elongation-extension to generate new double-stranded DNA chain (Madigo and Martinko, 2006).

Before PCR analysis, suitable primer sets for all types of archaea and general bacteria were investigated from the related literature. Primer sets which were planned to use for archaea and bacteria are listed in Table 3.6. Detailed content of master mix used for PCR analysis are also presented in Table 3.7.

Table 3-6 Primer sets used for general bacteria and archaea

Target microorganisms	Primer sets and sequences	Reference
General Bacteria	<p>P338f (5'ACTCCTACGGGAGGCAGCAG'3)</p> <p>GC338F (5'CGCCCGCCGCGCCCCCGCCC CGGCCCGCCGCCCCCGCCCA CTCCTACGGGAGGCAGC3')</p> <p>(GC clamp with 40 base used for DGGE analysis)</p> <p>P518r (5'ATTACCGCGGCTGCTGG3')</p>	Boon et al. (2002)
Archaea	<p>Arch109 (T) F: 5'-ACT GCT CAG TAA CAC GT-3'</p> <p>Univ515 (GC)a R: 5'-ATC GTA TTA CCG CGG CTG CTG GCA C-3'</p> <p>a: GC clamp for Univ-515 R : 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3'</p>	De Bok et al. (2006); Roest et al. (2005)

Table 3-7 PCR mixture content

Constituent (for each PCR reaction)	Volume (μL)
Taq Buffer –MgCl ₂ (10X)	2.5
dNTP mixture (200 mM)	0.5
MgCl ₂ (15 mM)	1.5
Forward primer (0.2 μM)	0.5
Reverse primer (0.2 μM)	0.5
Taq Polymerase	0.125
Template (100 ng/ μL)	1.2
ddH ₂ O	18
Total:	24.83

PCR amplification was performed in Thermal Cycler Block 5020 (Thermo Scientific Co.) device. For general bacteria, 16S rRNA amplification primers, namely P338f and P518, were used (Boon et al., 2002; Table 3.6). PCR conditions applied for general bacteria are as follows: Initial denaturation (94°C for 5 min), and 35 cycles composed of denaturation (95°C for 1 min), annealing (53°C for 1 minute) and elongation-extension (72°C for 2 minutes) steps applied consecutively (Boon et al., 2002). Finally, elongation at 72°C for 10 minutes was applied.

For archaea, 16S rRNA amplification primers, namely Arch109f and Univ515, were used (Boon et al., 2002; Table 3.6). PCR conditions for the archeae are as follows: Initial denaturation (94°C for 5 min), and 35 cycles composed of denaturation (94°C for 30 seconds), annealing (52°C for 40 seconds), and elongation-extension (72°C for 90 seconds) steps applied consecutively (De Bok et al., 2006; Roest et al., 2007). Finally extension was applied for 5 minutes at 72°C.

PCR products obtained for general bacteria and archaea were stored at -20 °C immediately and used for DGGE analysis within 12 hours. In this way, the quality of DGGE analysis increased by using fresh formed DNA samples.

All PCR products were loaded on 1% (w/v) agarose gel mixed with 4 μ L GelRed® in 1X TAE solution and run at 80 Volt for 90 minutes in a horizontal electrophoresis (BioRad Wide Mini Sub-Cell GT). The negative and positive controls were also run with the same gel to determine whether the PCR reaction is healthy or not and to verify the amplicon size of obtained PCR products with small spaced DNA Mass Ruler (1500 to 100 base pair) in comparison (Boon et al., 2002).

3.4.8.3 DGGE analysis

In this study, as previously mentioned in Chapter 1, variation in the microbial population dynamics of the both bacteria and archaeal species of ASBR under different operational conditions were investigated. To this purpose after isolation of DNA from sludge samples, equal length gene fragments of microorganisms (for instance: 16S rRNA; 180 bp) were initially amplified by PCR and then justified in an agarose gel electrophoresis (Section 3.4.8.2). Then, DGGE analysis was applied for gene fragments of microorganisms with the base sequences via a denaturing polyacrylamide gel. In DGGE analyses different bands apparent on the gel with respect to the sequence-specific melting point of related gene sequence represents a specific microorganism. In other words, each band formed at a different denaturing gradient point across the gel indicates a different type of specie. This situation allows to compare and examine the microbial population dynamics in the culture (Tzenev et al., 2008). In addition, density owned by each band gives information about the relative density of the microorganisms also (Nikolchev et al., 2003; Nübel et al., 1999). Therefore, DGGE provides a profile creation of mixed cultures and also allows the opportunity to observe interactions between them (Sanz and Köchling, 2007).

DGGE process applied to all bacteria was carried out according to the procedure given in the study of Boon et al. (2002). In addition, study of Bok et al. (2006) was used as guidance for DGGE procedure applied for archaeal species. Before performing DGGE analyses for bacteria and archaea, an optimization study was carried out for the DGGE device used (CBS Scientific). In this optimization study, different APS (ammonium per sulphate)/TEMED ratios and concentrations were investigated and tried to obtain a healthy polyacrylamide gel. In this context, the APS/TEMED ratio of 110 μ L/10 μ L

was determined. Moreover, the amount of applied PCR product loaded to the gradient gel, and intensity and duration of the voltage applied were also investigated. During the optimization of DGGE device, different voltages (40 V, 150 V, 180 V) and execution times (such as 7.5, 9, 16 hours) combination were investigated (TUBITAK Project Report, 112 M 252). To visualize a clear separation of DNA bands applied, 180 V at 60°C for 450 minutes was set as the optimized operational conditions for both all bacteria and archaea. In addition, because PCR product volume of 45 µL applied on polyacrylamide gel resulted in more intense bands set as an optimized value. The PCR products were loaded onto wells which had 1.5 mm thickness with 8% (w/v) polyacrylamide gel and 1X TAE buffer (20 mM Tris-acetate with pH7.4; 10 mM acetate; 0.5 mM Na₂ EDTA) (Boon et al., 2002). DGGE device was run for 450 minutes at 180 V and 60°C for both bacteria and archaea. Polyacrylamide gels had a gradient 60% to 40% for all bacteria and 30% to 60% for archaea. After DGGE analyses, polyacrylamide gels were stained with a 100 mL 1X TAE buffer containing 15 µL GelRed® for 20 minutes. Then, the gels' viewed were recorded under UV light with gel imager software (Quantum Capture) and the bands were identified. Preparation of polyacrylamide gel solution for 60% to 40% GC gradient was explained as an example below step by step and consecutively in detail;

- ✓ Add 38.93 g of acrylamide and 1.07 g of bis-acrylamide and mix them with 100 mL dH₂O; mix it and get the first solution.
- ✓ Divide the solution above into two 50 mL falcon tubes in equal volumes.
- ✓ Add 16.8 g urea to one of the 50 mL solution and 25.2 g urea to other one and mix them.
- ✓ Add 16 mL formamide to the solution including 16.8 g urea; and add 24 mL formamide to the solution including 25.2 g urea. Gradient of the gels achieved by the addition of different volumes of urea and formamide. In this respect, gels with gradients of 60% and 40% are aimed to achieve
- ✓ Add 2 mL 50% TAE to each of solution in 50 mL falcon tubes to make the concentration of TAE 2% in each of them.
- ✓ Fill each falcon tube with 100 mL dH₂O and mix thoroughly (by the way TAE concentration becomes 1% in each of solutions).

- ✓ 100 mL 40% and 100 mL 60% polyacrylamide gel solutions were gathered separately.
- ✓ Divide each of the solution above into 50 mL falcon tubes again
- ✓ Taking 30 mL of 40% and 100 mL of 60% Polyacrylamide gel solutions separately in other falcon tubes and add 110 μ L APS and 10 μ L Temed in each of them and mix (to get the APS solution add 1.2 g APS into 1 mL dH₂O and dissolve it before adding to the gel solutions).

It is important to put 110 μ L APS and 10 μ L TEMED into 30-40% and 60% solutions very quickly to form the gradient gel solutions before they freeze. In addition, all the polyacrylamide gel solutions and PCR products should be prepared on the same day with DGGE analysis to get a describable images and useful bands (Section 3.4.8.3).

3.4.8.4 Sequencing the DGGE Bands

DGGE gels were used for sequence analyses. To this purpose, the (polyacrylamide) gels, where successful denaturing and clear bands were obtained and selected. These gels were placed on a UV transillumination table. Bands on the gel, which are clearly visualized under UV light, were literally out with the help of tweezers and placed in nuclease-free water. These band were further purified for sequence analyses. Purification of these bands (and in turn the DNA samples) was done with NucleoSpin Gel and PCR Clean-Up Kit (Macherey-Napel). Sequence analyses of the purified DNA samples performed by RefGen Co. via using bi-directional sequence analysis (forward + reverse sequencing) to decrease the margin for any error. After then, microbial species were identified with Sequence Analysis National Center for Biotechnology Information (NCBI) Basic Local Alignment-site (BLAST).

3.4.8.5 qPCR

3.4.8.5.1 Sampling and DNA extraction of ASBR samples

Biomass samples were taken under from ASBR reactor in different operation times for each of six operation period. One mL well-mixed representative liquid phase (ASBR reaction period content) was transferred into a 1.5 mL capacity sterilized

microcentrifuge tube for each biomass sample analyses. After centrifuging at 14000 g for 5 min to separate the solids from aqueous phase, the pellets were stored at – 20°C until just before DNA extraction. Total DNA is extracted 140 mg of centrifuged pellet. The isolation of DNA from ASBR samples were carried out using the OMEGA-EZNA Soil DNA Isolation Kit according to the manufacturer's instructions. Isolated DNA samples were stored at -20°C.

In qPCR analysis, another samples were *E. coli* cultures containing recombinant plasmids with the encoding genes of *Methanosaeta* and *Methanosarcina species* to figure out a calibration curve (standard curve) to analysis the biomass samples of ASBR study. The *E. coli* cultures containing recombinant plasmids with either *Methanosaeta* or *Methanosarcina* 16S rRNA gene segments were obtained from the University of Washington, Seattle, WA, courtesy of late Dr. John Ferguson, Emeritus Professor in the Civil and Environmental Engineering Department. Growth of the *E. coli* cultures were divided in to three steps given below, respectively;

- ✓ Firstly, *E. coli* cultures were grown in Lysogeny broth (LB) (Conda (Lennox), Cat: 1231.00) Plasmid-containing cultures were selected by adding 100 µg/mL ampicillin (Sigma- Aldrich) to the growth medium. 15 mL of LB broth was inoculated with *E. coli* culture overnight at 37°C with shaking.
- ✓ Secondly, grown samples in step 1 was inoculated in to fresh LB agar plates containing 100 µg/mL ampicillin (Sigma- Aldrich) with quadratic streak plate method to isolate a single colony. Samples were incubated overnight at 37°C with shaking for 18 h.
- ✓ Finally, single colonies for *E. coli* cultures were selected from the plates and inoculated again in a 15 mL LB broth containig 100 µg/mL ampicillin at 37°C with shaking for 18 h. Thus, non-contaminated pure *E. Coli* cultures could be gathered for the rest of the study.

The recombinant plasmids of *Methanosaeta* and *Methanosarcina* 16S rRNA gene sequences has 915 and 921 base pairs, respectively which was inserted in TOPO vector

(Invitrogen, Valencia, CA)¹⁹ and they were isolated using Thermo Scientific Plasmid DNA Isolation Kit. Insertions were confirmed by restriction enzyme ScaI (Thermo Scientific) digestion. Digestion mixture total volume was 20 µL and it contained 2 µL of extracted and purified plasmid (250-300 ng); 2 µL digestion buffer (Thermo Scientific); 2 µL ScaI enzyme; and 14 µL sterilized distilled water. The digestion reaction was 3 h at 37°C. Then, non-quantitative PCR analysis and gel electrophoresis were followed for the confirmation process.

3.4.8.5.2 PCR for *E. coli* cultures containing recombinant plasmids

A conventional, non-quantitative PCR protocol was used to amplify the 270 bp insertions that were 16S rRNA gene fragments for both *Methanosaeta* and *Methanosarcina*, using recombinant as templates. Primers and positions (all as *E. coli* positions) used were as follows (Shigematsu et al., 2003);

- ✓ For *Methanosaeta*; MS1b 585F (5'-CCGGCCGGATAAGTCTCTTGA-3'; 585-605),
Sae 835R (5'-GACAACGGTCGCACCGTGGCC-3';855-835).
- ✓ For *Methanosarcina*; Mb1b 586F (5'-CGGTTTGGTCAGTCCTCCGG-3'; 586-605),
Sar 835R (5'-AGACACGGTCGCGCCATGCCT-3'; 854-835,).

The reaction mixture in a total volume of 50 µL included:

- ✓ 100 ng of template DNA
- ✓ 25 µl of 2X ReadyMixTM Taqman PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO), and
- ✓ 30 pmole each of forward and reverse primers (1 µL for each).

Applied PCR conditions were: Initial denaturation (94°C for 2 min), 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 minute) and elongation-extension (72°C for 1 minutes) were applied consecutively. Finally, elongation at 72°C for 10 minutes was applied (Shigematsu et al., 2003). PCR products were analyzed by gel electrophoresis on 1% (w/v) agarose TAE-gels.

After confirming the target genes in *E. coli* plasmids using non quantitative PCR and agarose gel electrophoresis PCR products were measured using Nanodrop 2000c Spectrophotometer (Thermo Scientific) at METU Central Laboratory, and then diluted in 4 serial dilution method using different logarithmic ratios for both species. Solutions obtained after serial dilutions were called standard solutions for the calibration of qPCR. Thus, standard solutions for the preparation of the calibration curves of *Methanosaeta* and *Methanosarcina* species were ready for qPCR. Subsequent concentration of the serial dilutions of the samples were done according to the study of Yilmaz et al. (2014) which was given in the Section the species' gene copy concentrations (log/ μ L) were given in Section 4.3.3 (Table 4.14) in detail.

After serial dilution were done to get the standard solutions, both the standards and isolated DNA samples taken from the ASBR study in different operational periods were ready for the qPCR analysis. The primer sets for *Methanosaeta* and *Methanosarcina* were selected for qPCR analysis were the same as PCR analysis of recombinant templates mentioned. Moreover, Taq probe sets were added to analysis for both species (Shigematsu et al., 2003).

- ✓ The primer/probe set for the genus *Methanosaeta* were;
Forward primer: (5'-CCG GCC GGA TAA GTC TCT TGA-3'),
Reverse primer: (5'-GAC AAC GGT CGC ACC GTG GCC-3'), and
TaqMan® probe: (5'-ACC AGA ACG GAC CTG ACG GCA AGG-3').

- ✓ The primer/probe set for the genus *Methanosarcina* consisted of;
Forward primer: (5'-CGG TTT GGT CAG TCC TCC GG-3'),
Reverse primer (5'-AGA CAC GGT CGC GCC ATG CCT-3'), and
TaqMan® probe (5'-ACC AGA ACG GGT TCG ACG GTG AGG-3').

For absolute quantification, 5 μ l of isolated and serially diluted total genomic DNA from the biomass samples was transferred to 12.5 μ l of qPCR mixture containing: 1X TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA); 0.9 μ M each of the primers; and 0.25 μ M and 0.20 μ M probe for *Methanosarcina* and

Methanosaeta, respectively. Each qPCR batch plate included with no template controls (NTC) with 5 μ L RT-PCR grade water instead of template. Each reaction was run in triplicate for quality assurance and statistical analysis purposes. All qPCR reactions were performed using Applied Biosystems 5020 qPCR. The applied qPCR conditions were: one hold at 50°C for 2 min, one hold at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 65°C for 60 s. Negative control samples were used to check the reliability of the analysis which was not containing any DNA molecule.

3.5. Experimental Set-Ups and Procedure

3.5.1 Batch Reactor Set-1 (Single-Phase AD with Glucose)

The aim of Set-1 was to investigate the effect of initial substrate to initial microorganism ratio (S/X_0) on methane yield and productivity. The results of this study gave information about suitability of seed sludge for methane production used in Batch Reactor Set-2 also. To this purpose, batch reactors were operated at different initial S/X_0 ratios of 0.5, 1, 2, 3 and 4 (g sCOD/g VSS). Glucose was used as the substrate.

Experiments were performed in 100 mL serum bottles with effective volume of 60 mL. All reactors were inoculated with anaerobic seed sludge, establishing a VSS concentration of 4156 ± 493 mg/L. Then the substrate (glucose) solution was fed to the reactors with different amounts to achieve the S/X_0 ratios (g sCOD/g VSS) which were considered to research. Initial conditions of the reactors conducted in Batch Reactor Set-1 study are shown in Table 3.8. BM shown in Table 3.4, was added into the reactors to supply necessary macro-and micro-nutrients. In addition to BM (Table 3.4), 6000 mg/L alkalinity as NaHCO_3 was also added to the reactors to prevent any pH problem for anaerobic digestion (Speece, 1996). Control reactors, containing only anaerobic seed sludge and BM, were also conducted to determine the background gas production. All reactors were run in triplicates and presented data composed of the averaged values. Prior to incubation, headspace of those 18 reactors were purged with 100 N_2 gas for 3–4 minutes in order to strip out O_2 and maintain anaerobic conditions. Then the reactors were closed with natural rubber stoppers. Prepared reactors were incubated in a temperature controlled room at 35 ± 2 °C. Mixing was applied at 175

rpm by using a mechanical shaker for 39 days of operation. During incubation period, biogas productions and biogas compositions were daily recorded for the first week. After then, changes in biogas composition were monitored as negligible levels (1-5%) and these analyses were done periodically (in every 3 or 4 days). At the end of the incubation period, all reactors were subjected to pH and COD analyses, in order to analyse the methane production efficiency.

Table 3-8 Initial conditions of the reactors conducted in Batch Reactor Set-1

Reactor No	S/X ₀ (g sCOD /g VSS)	Initial COD concentration in reactors (g/L)	Initial pH value
Test 1	0.5	2.2	8.3
Test 2	1	4.4	8.3
Test 3	2	8.9	8.3
Test 4	3	13.3	8.3
Test 5	4	17.7	8.3
Control	-	-	8.3

3.5.2 Batch Reactor Set-2: Comparison of a Single- and a Two-Phase AD

This study was conducted to investigate the effect of preliminary dark fermentation step on methane production. To this purpose, sucrose and effluent of DF-SBR operated with sucrose (Influent B, Section 3.1, Table 3.1 and Table 3.2) were used as substrates. In other words, Batch Reactor Set-2 was conducted to investigate and compare a single-phase AD and the second phase of a two-phase AD system. Batch reactors with total volume of 250 mL and effective volume of 192 mL were used in this study. Four types of batch test reactors namely Test 1, Test 2, Test 3, Test 4 were conducted. All test reactors were initially seeded with anaerobic seed sludge. Test 1 and Test 2 reactors used Influent B as the substrate and performed as the second phase of a two-phase AD. On the other hand, Test 3 and Test 4 reactors used sucrose and performed as single phase AD. The initial conditions of all reactors conducted in this study are shown in Table 3.9. As seen in table 3.9, the initial COD concentrations of Test 1 and Test 3 and of Test 2 and Test 4 were same as 5.7 and 4.8 g/L, respectively. By this way, it was aimed to design Test 3 and Test 1 reactor couple as single-phase and

second-phase of a two phase AD, respectively. The same approach was also valid for Test 4 and Test 2 reactor couple. Blank reactors contained either Influent B or sucrose with same initial COD concentration as that of test reactors (Table 3.9). Control reactors (containing only seed sludge) were also conducted (Table 3.9). S/X₀ ratios of all test reactors were set as 1 g COD/g VSS, considering the results of Batch Reactor Set-1. Although glucose was used as substrate in Batch Reactor Set-1 (Section 3.5.1), for being one of the two monosaccharides of sucrose, S/X₀ ratio of 1 g COD/g VSS was found to result in highest methane yield (i.e. 1 g COD/g VSS) was also used in this study. Initial pH values of reactors were adjusted to 7.3. Each reactor type was run in duplicate and data given in the Results and Discussion (Section 4.2) indicate the average values. Prior to incubation, head space of all reactors were purged with 100% N₂ gas for 3–4 minutes in order to strip out O₂ and maintain anaerobic conditions. Then, the reactors were closed with natural rubber stoppers. Prepared reactors were incubated in a temperature controlled room at 35 ± 2 °C. Mixing was applied at 175 rpm by using a mechanical shaker for 30 days of operation. During incubation period, biogas productions and biogas compositions were daily recorded for the first week. After then, changes in biogas composition were monitored as negligible levels (1-5%) and these analyses were done periodically (in every 3 or 4 days). At the end of the incubation period, all reactors were subjected to pH and COD analyses.

Table 3-9 Initial conditions of the reactors conducted in Batch Reactor Set-2

Reactor No	S/X ₀ (g sCOD / g VSS)	Substrate	Initial COD concentration in reactors (g/L)	Initial pH value
Test 1	1	Influent A	5.7	7.3
Test 2	1	Influent B	4.8	7.3
Test 3	1	Sucrose	5.7	7.3
Test 4	1	Sucrose	4.8	7.3
Blank 1	-	Influent A	5.7	7.3
Blank 2	-	Influent B	4.8	7.3
Control	-	-	-	7.3

3.5.3 ASBR Study

ASBR Study was conducted to investigate the effect of HRT and SRT on methane production and archeal and general bacterial population dynamics. The change in the quantity of methane producers namely, *Methanosaeta* and *Methanosarcina* sp. with respect to the changing SRT and HRT conditions was also investigated. To this purpose, an ASBR, fed with the effluent of the DF-SBR (Section 3.1) was operated. The ASBR configuration and operational conditions were given in the following sections below.

3.5.3.1 ASBR Configuration

A representation of ASBR used in this study is shown in Figure 3.1. The laboratory-scale ASBR was a cylindrical glass with an internal diameter of approximately 15 cm and a height of 70 cm. The working liquid volume of the reactor was 1.25 L, with 1.4 L total volume. The exchange ratio was 68%. There were one inlet and three outlet ports on the reactor at different elevations vertically. One of the outlet ports was used to discharge the effluent. Other two were used for sludge and reaction phase liquid sampling. In addition, inlet port was used for influent pumping to the reactor. The reactor was incubated in a temperature controlled room at 35 ± 2 °C. Mixing was applied at 200 rpm by using a magnetic stirrer.

A peristaltic pump was used as influent pump with masterflex tubing. A 1 L feed tank was used to adjust the mesophilic temperature (35 ± 2 °C) and pH (7) of the influent just before the influent pump started to work. Effluent was discharged with an automated valve to the effluent tank. Samples periodically withdrawn for COD, TSS, VSS, pH and VFAs analyses and were stored at -20 °C.

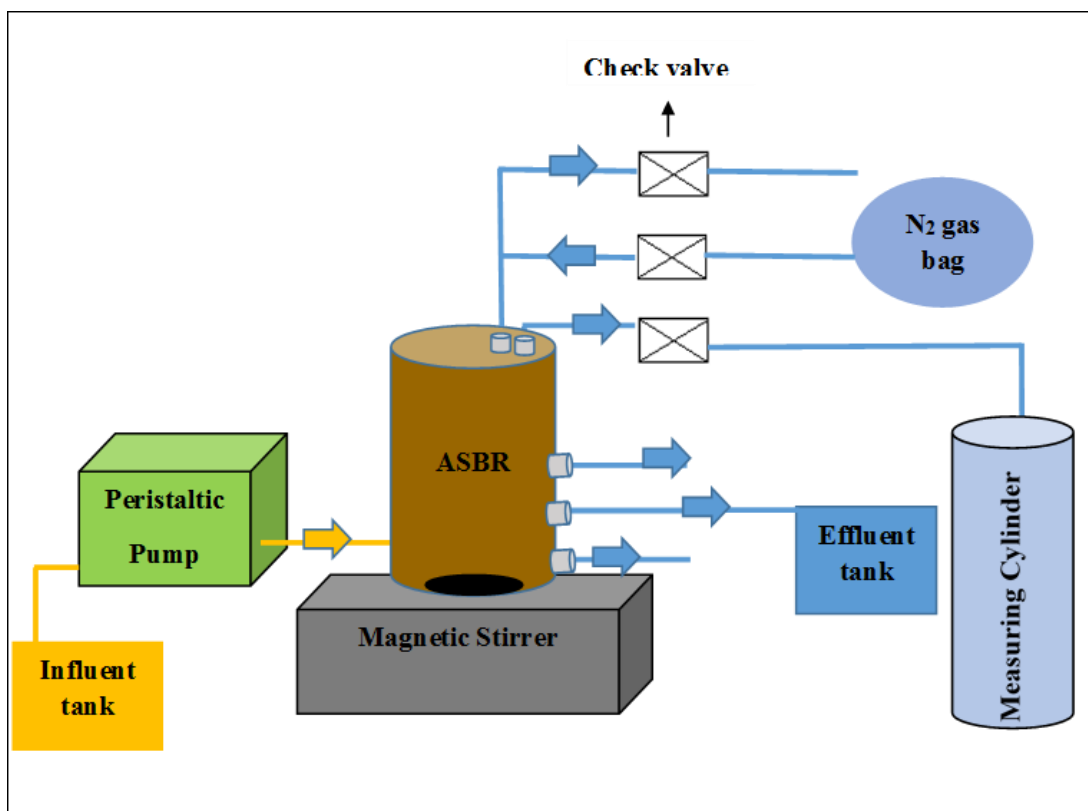


Figure 3-1 Representation of the ASBR system used in this study

Produced biogas was transferred through an automated valve and collected into a measuring cylinder and containing acid brine solution, used as water displacement device during the reaction period of the reactor. Due to vaporizations, acid brine solution was freshened periodically to keep the pH constant in order to eliminate CO₂ solubilisation. Produced biogas was analysed in terms of volume and composition at the end of each cycle. For automated operation, plug-in timers were used which automatically turned the pump, liquid and gas valves on and off at prescribed times.

3.5.3.2 Operational Conditions

ASBR was operated following the cycles composed of feeding, reaction, settling and decant periods for 157 days. The first 80 days was set as acclimation period. During this period HRT and SRT of the system were not controlled because of the methane production problem, which was discussed in detail in Section 4.3. In order to enrich methane producers, HRT of the system was initially set as 16 days and then gradually

decreased to 6 days at the end of Day 45. Due to the steady-state methane production between Days 45 and 80, the acclimation period was determined to be over.

At the end of Day 80, HRT and SRT of the ASBR were set to the previously determined values to carry out the aim of the study. In other words SRT and HRT were set as independent variables. To this purpose, six different operational periods with varying HRT and SRT combinations were applied during Days 81-157 (Table 3.10). The reason behind this was to investigate if the dominant acetoclastic methanogen species would change at different SRT and HRT values in ASBR or not. According to literature, 15 days and higher SRT values lead to *Methanosaeta specie* while SRT values lower than 10 days lead to *Methanosarcina specie* domination in AD process (Lee et al., 2011; Ma et al., 2013). Ma et al. (2013), on the other hand, reported that HRT values of lower than 4 days lead to *Methanosarcina specie* domination in AD process. Therefore, SRTs researched in ASBR were set as 10 and 20 days. In addition, HRT values ranged from 0.7 to 6 days. By this way, the relationship between methane production efficiency, SRT, HRT and acetoclastic methane producers (*Methanosaeta* and *Methanosarcina*) could be understood with six different operational conditions (periods) of ASBR study. Operational conditions of these six different periods and other parameters related to ASBR are given in Table 3.10 in detail. In fact, OLR, F/M and duration of the cycles and each period changed due to the changes made in HRT and SRT values in each operational period. Thus, OLR, F/M and duration of cycles / periods could be named as dependent variables of the system.

Table 3-10 An overview of the operational conditions of ASBR for each period

Parameters	Operation Periods (Period No and Days)					
	Period I	Period II	Period III	Period IV	Period V	Period VI
	81-96	96-108	108-121	121-136	136-146	146-157
HRT (day)	6	3	1.5	0.7	0.7	1.5
OLR (g sCOD/L/day)	1.1	2.2	4.5	8.9	8.9	4.5
SRT (day)	20	20	20	20	10	10
F/M (g sCOD/ g VSS/day)	1	1	1	1	1	1
Cycle number	4	6	13	29	23	10
Cycle time (hour)	96	48	24	12	12	24
Feeding period (minute)	2	2	2	2	2	2
Reaction period (hour)	92	44	20	5-8	8-9	20
Settling period (hour)	4	4	4	4-7	3-4	4
Decant period (minute)	2	2	2	2	2	2

Operational conditions of these six different periods are given in Table 3.10 in detail. Between Days 81-133, HRT was gradually decreased from 6 days to 3, 1.5 and 0.7 days while SRT was kept constant at 20 days for Period I, II, III, and IV. Then, SRT was decreased and kept constant at 10 days for the Periods V and VI, while HRT was set as increased 0.7 and 1.5 days, respectively (Table 3.10). Settling time has direct effect on the SRT and F/M. In addition, any changes in cycle duration results in changes in HRT values (Shizas et al., 2006). Thus, duration of both settling period and each cycle was selected carefully regarding the related literature (Table 2.2). In this respect, during the six periods, cycle duration varied between 12 to 96 hours, while settling time changed between 4 to 7 hours (Table 3.10). Feeding and decanting periods were set to the values as short as possible to increase the duration of reaction period. In literature, there are many research studies (Shizas and Bagley, 2002; Damasceno et al., 2007; Cheong and Hansen, 2008; Ndegma et al., 2008) focusing on the effect of feeding period's duration on methane production. However, this was not a prior issue within the concept of this study; therefore, feeding periods of 2 minutes was applied through the operational period (Table 3.10).

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Results of Batch Reactor Set-1

Batch Reactor Set-1 was conducted to investigate the optimum initial S/X_0 ratio leading to the highest methane production yield. Experiments lasted 39 days. Total amount of biogas and methane produced in the process was shown in Figure 4.1.

In Test 1, 2, 3, 4 and 5 reactors biogas production started almost immediately without any lag phase (Figure 4.1a). Maximum biogas was produced in Test 3 reactor (S/X_0 : 2 g COD/g VSS) as 274 mL. The ensuing highest biogas productions were recorded as 177, 152, 146 and 96 mL by Test 4 (S/X_0 : 3 g COD/g VSS), Test 5 (S/X_0 : 4 g COD/g VSS), Test 2 (S/X_0 : 1 g COD/g VSS) and Test 1 (S/X_0 : 0.5 g COD/g VSS) reactors, respectively at the end of the 39 days of incubation. It is noteworthy that more than 90 % of the biogas was produced in test reactors approximately in 25 days (Figure 4.1 a). Moreover, 19 mL biogas produced in Control reactor until the end of the incubation.

Despite the immediate biogas production in all test reactors, methane was not produced directly without any lag phase in some of the test reactors. Methane production started in Test 1 and Test 2 at the beginning of the incubation. Yet, Test 3 started to produce methane after Day 13. Moreover, Test 4 and 5 produced negligible amount of methane gas. S/X_0 ratios greater than 3 g COD/g VSS resulted in inhibition of methane production.

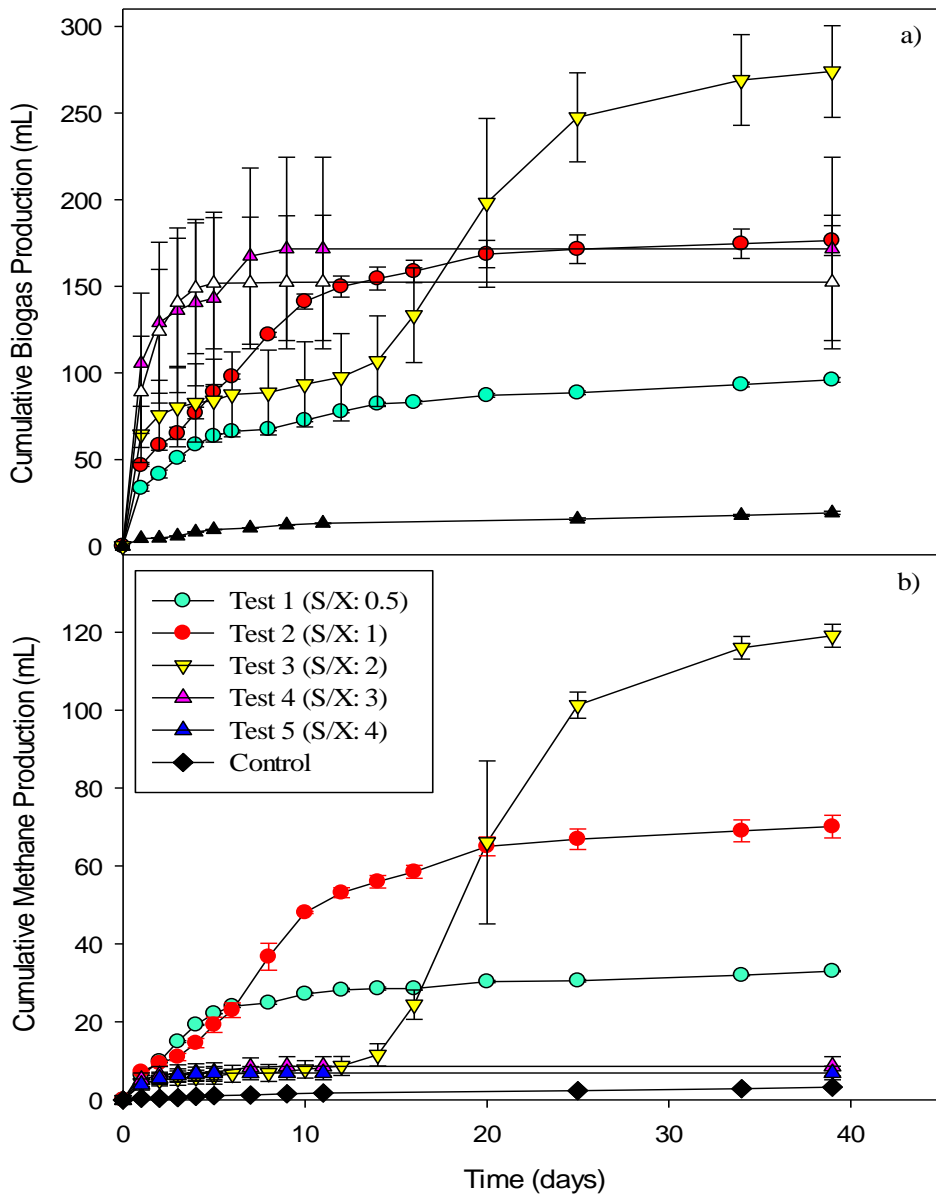


Figure 4-1 a) Cumulative biogas, b) cumulative methane production of Batch Reactor Set-1

Higher S/X_0 ratios might be an important indicator of an inhibition for AD (Raposo et al., 2006; Feng et al., 2013). This inhibition in Test 4 and Test 5 might be caused from decreased pH levels resultant of VFA production (below 6.5) which is the lower limit for the optimum growth conditions for methanogenic microorganisms (Chen et al., 1998). Although a clear inhibition monitored for the methane production in Test 4 and

5, the amount of biogas produced by these reactors were relatively high, most probably consisted of CO₂ and maybe some H₂.

Maximum methane was produced in Test 3 (S/X₀: 2 g COD/g VSS) reactor as 119 mL. The ensuing highest methane productions were recorded as 70, 33, 8 and 7 mL by Test 2, Test 1, Test 4 and Test 5 reactors, respectively. Although incubation lasted for 39 days, about 90% of the total CH₄ was produced during the first 25 days for Test 1, Test 2 and Test 3 (Figure 4.1 b) which was parallel to biogas production observation. In addition, only 3 mL methane was produced in Control (consisting of seed sludge only) reactor which was negligible compared to methane producing test reactors namely, Test 1, Test 2 and Test 3.

Methane yield and methane percent in the biogas (%) and anaerobic treatability for the test reactors are given in Table 4.1. The relationship between the methane yield and initial S/X₀ ratio is also shown in Figure 4.2.

Table 4-1 Methane production and treatability results of Batch Reactor Set-1

Reactor No	S/X ₀ (g COD/g VSS)	Methane yield (mL CH ₄ /g COD _{added})	Anaerobic treatability ^a (%)	CH ₄ percentage (%)
Test 1	0.5	248±2	63 ± 0.3	54± 0.1
Test 2	1	267±5	68 ± 2.8	66± 1.0
Test 3	2	224±9	57 ± 1.4	70± 4.8
Test 4	3	11±1	3 ± 0.8	5± 0.1
Test 5	4	-	2 ± 0.4	5± 0.0

^a (Total methane production) x (100)/(Theoretical methane potential);
1 g COD degradation leads to maximum 395 mL CH₄ at 35 °C (Speece, 1996)

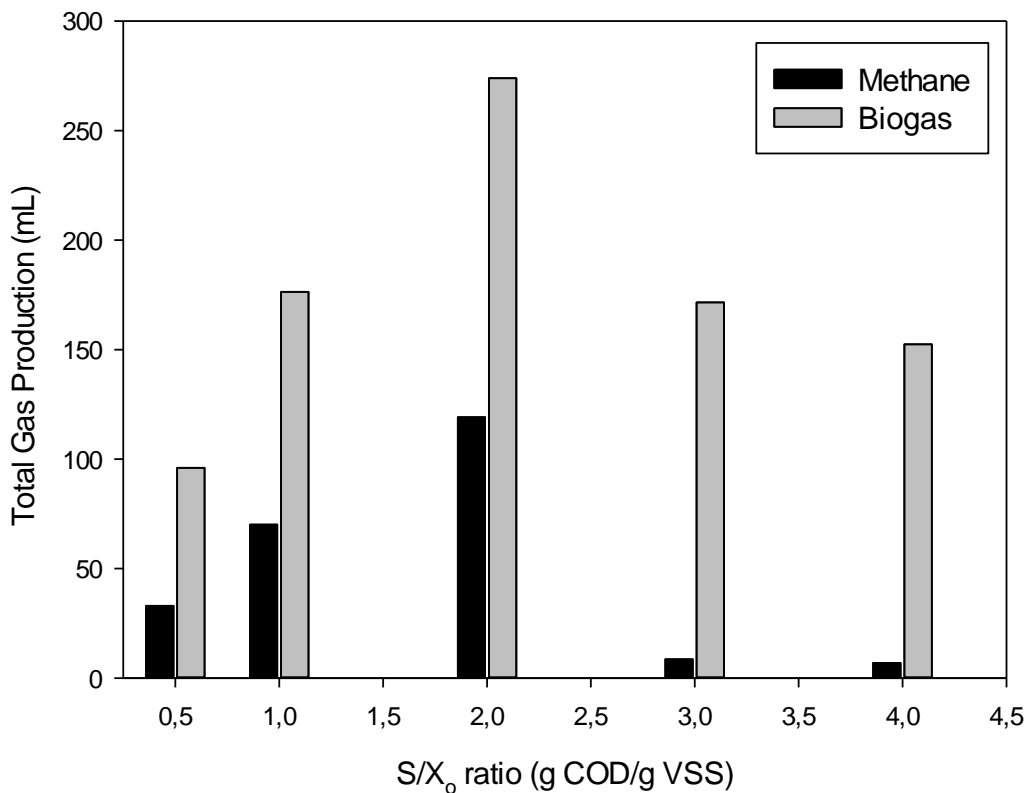


Figure 4-2 Different initial S/X_0 ratios relation to total biogas and methane production

As seen in Table 4.1, the highest methane yield was observed (267 ± 5 mL CH_4 /g COD_{added}) in Test 2 reactor, where S/X_0 ratio was 1 g COD/g VSS. In addition the highest methane percentage (%) was also recorded in Test 2 as 68 ± 2.8 %. The ensuing high methane yields were recorded in Test 1 (S/X_0 : 0.5 g COD/g VSS) and Test 3 (S/X_0 : 2 g COD/g VSS) as 248 ± 2 mL CH_4 /g COD_{added} and 224 ± 9 mL CH_4 /g COD_{added} , respectively. When the initial S/X_0 ratio increases to 3 g COD/g VSS (Test 4) and 4 g COD/g VSS (Test 5), methane production was inhibited and, accordingly anaerobic treatability percentage only remained at 2-3% (Table 4.1). This situation could be explained with the possible high accumulation of VFA in Test 4 and 5 reactors which would cause sharp decrease in pH. Thus, methanogenic microorganisms were likely to be inhibited in these reactors.

The results of this study were also supported by the related literature. Chen et al. (1995) studied S/X_0 range between 0.7 to 10.5 (g COD/g VSS) and observed decrease in methane yield correlated with the increase in S/X_0 ratio from 0.7 to 10.5 (g COD/g VSS). In another study, S/X_0 ratios of 1 to 3 (g COD/g VSS) were experienced and methane yield decreased from 233 mL CH₄ / g COD_{added} to 196 mL CH₄ / g COD_{added} while S/X_0 ratio increased from 1 to 3 (g COD/g VSS) (Raposo et al., 2006).

As a result, the highest methane production yield and anaerobic treatability were observed as 267 ± 5 mL CH₄ / g COD_{added} and 68%, respectively, in Test 2 reactor (S/X_0 : 1 g COD/g VSS). That result is comparable to the studies in the literature where the carbon source was also glucose (Table 4.2). The glucose was also used as influent carbon source in the studies of Xia et al. (2008), Giardino et al. (2010) and Chen et al. (1995) with the S/X_0 ratios of 1.07, 1.07 and 1.4 (g COD/g VSS), respectively. Methane yield records of these studies were close to the methane yield of this study as shown in Table 4.2 in detail. Recording similar methane yields with similar S/X_0 ratios does not reduce the importance of Batch Reactor Set-1 operation. Each study shown in Table 4.2 used different seed sludge which were gathered from different anaerobic digester plants and, might have different characteristics. Therefore, seed sludge used in this study should have been tested for its applicability as seed for the thesis study and to research the optimum S/X_0 ratio leading to the highest methane yield has should been tested in this study. The results of this study indicated the applicability of the anaerobic seed sludge and 1 g COD/g VSS as the optimum S/X_0 ratio for the following experiments.

Table 4-2 Methane yield comparison between Batch Reactor Set-1 and related literature

S/X_0 (g COD/g VSS)	Methane yield (mL CH ₄ / g COD _{added})	Influent	Reference ^a
1.0	267±13	Glucose	Giardino et al. (2010)
1.0	248	Glucose	Xie et al. (2008)
1.4	248	Glucose	Chen et al. (1995)
1.0	267±5	Glucose	Batch Reactor Set-1

^a All studies were performed in batch reactors.

4.2 Results of Batch Reactor Set-2

Batch Reactor Set-2 was conducted to investigate the effect of preliminary dark fermentation (DF) step on methane production. In other words, methane production difference between single and two-phase AD was analysed by the reactors conducted in Batch Reactor Set-2. Test 1 and Test 2 reactors represented the second phase of a two-phase AD, while Test 3 and Test 4 represented the single-phase AD. As previously mentioned in Section 3.5.2, Test 1 and Test 2 reactors were fed with DF-SBR effluent (Influent A and B were fermented sucrose), while Test 3 and Test 4 reactors were fed with sucrose. The difference between the reactors fed with same substrate type was the initial COD concentration (Table 3.10, Section 3.5.2). The total amount of biogas and methane gas produced in the reactors are shown in Figure 4.3.

In Test 1, 2, 3 and 4 reactors, biogas production started almost immediately without any lag phase (Figure 4.3b). Maximum biogas was produced by Test 1 reactor as 577 mL. The ensuing highest biogas productions were recorded as 520, 468, and 448 mL by Test 3, Test 2 and Test 4 reactors, respectively. Moreover, 82 mL biogas was produced in Control reactor until end of the incubation. On the other hand, contrary to the Test and Control reactors negligible amount of biogas was produced in Blank 1 and Blank 2 reactors which were 38 and 32 mL, respectively.

Methane production started after 4 days of incubation in Test 1 and Test 2 reactors, while Test 3 and Test 4 reactors started to produce methane after incubation day of 6. Thus, it can be said that, seed sludge used in the reactors was not need an acclimation period. Maximum methane was produced by Test 1 reactor as 364 mL. The ensuing highest methane productions were recorded as 311, 267, and 244 mL by Test 2, Test 3 and Test 4 reactors, respectively. After 23 days of operation, carbon sources in the reactors were probably almost depleted. In addition, no methane production detected in Blank 1 and Blank 2 reactors as it was expected. On the other hand, 25 mL methane was produced by Control reactor. However, methane produced by control reactor was not a significant amount when compared to all of the test reactors.

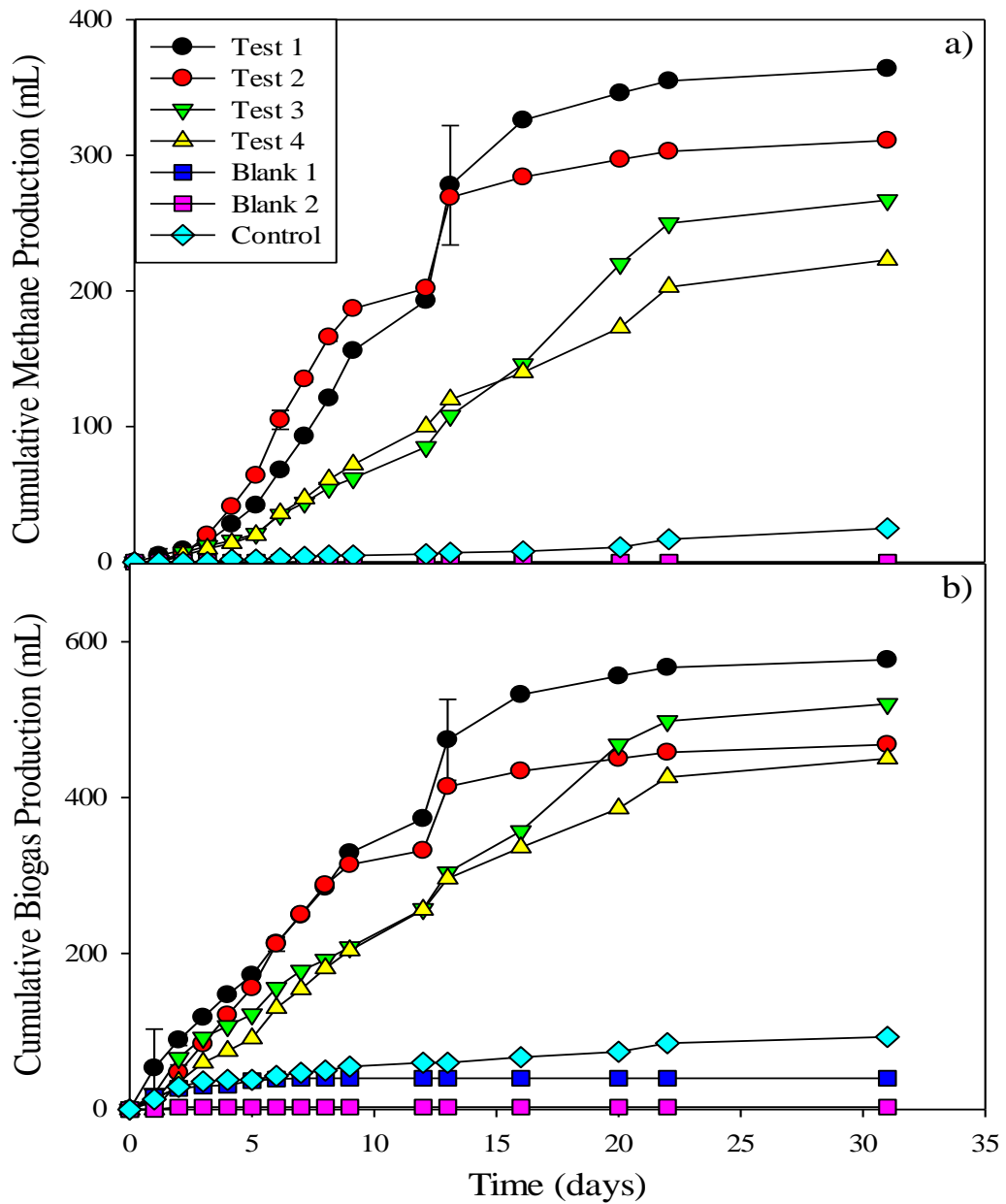


Figure 4-3 Cumulative a) methane and b) biogas production of single and two-phase anaerobic batch reactors Batch Reactor Set-2

(Test 1: Influent A, 5.7 g COD /L; Test 2: Influent B, 4.8 g COD /L;

Test 3: Sucrose, 5.7 g COD /L; Test 4: Sucrose, 4.8 g COD /L)

To analyse the results, the reactors which have the same initial sCOD concentrations were tackled firstly. Although Test 1 (second phase of a two-phase AD) and Test 3 (single-phase AD) reactors had the same initial sCOD concentrations, their methane yields were 337 ± 17 mL CH₄/g COD_{added} and 247 ± 25 mL CH₄/g COD_{added}, respectively (Table 3.10 and Table 4.3). The same situation was also observed between Test 2 (second phase of a two-phase AD) and Test 4 (single-phase AD) reactors. When 344 ± 20 mL CH₄/g COD_{added} methane yield was noted in Test 2 reactor, methane yield of Test 4 was recorded as 223 ± 6 mL CH₄/g COD_{added} (Table 3.10 and Table 4.3). Thus, methane yields of Test 1 and Test 2 reactors (second phase of a two-phase AD) were higher than the methane yields of Test 3 and Test 4 (single-phase AD). The main reason behind the methane yield differences between two-phase AD and single-phase AD is the provision of more favourable environmental conditions for both acidogenic and methanogenic microorganisms in two-phase AD system (Giordano et al., 2011; Xie et al., 2008). In other words, phase separation provides optimal environmental conditions for both acidogenesis and methanogenesis.

Table 4-3 Methane percentage and yield data of Batch Reactor Set-2

Reactor No	CH ₄ percentage (%)	CH ₄ yield ^a (mL CH ₄ /g COD _{added})
Test 1	82 ± 2	337 ± 17
Test 2	83 ± 1	344 ± 20
Test 3	78 ± 1	247 ± 25
Test 4	77 ± 0	223 ± 6
^a Methane yield was calculated at 35 °C and 1 atm		

According to the literature as seen in Table 4.3 and Table 4.4 methane yield and content (%) in biogas of two-phase AD were higher than single-phase. Bull et al. (1984) indicated that the separated phase system consistently developed the methane productivity from 268 mL CH₄/g COD to 356 mL CH₄/g COD and methane composition in biogas from 73% to 78%. Yeoh (1997) reported that methane content of the biogas generated from two-phase process was significantly higher (about 17%)

than that obtained from single-phase process. In addition, Wust (2003) remarked methane productivity 0.33 ± 0.21 L/L/day for single-phase AD and 0.45 ± 0.30 L/L/day for two phase AD.

Table 4-4 Comparison of single-phase and two-phase AD in terms of methane yield and methane percentage in biogas

Reference	Reactor Type ^c	Carbon Source	Single-Phase		Two-Phase	
			CH ₄ yield (ml CH ₄ /g sCOD _{added})	CH ₄ (%)	CH ₄ yield (ml CH ₄ /g sCOD _{added})	CH ₄ (%)
Bull et al. (1983) ^a	FBR	Glucose	268	73	356	77
Weiland (1993) ^a	CSTR	Sugar beet pulp	210	59	230	72
Nasr et al. (2012) ^b	Batch	Thin stillage	260	-	330	-
Yeoh, (1997) ^b	CSTR	Cane molasses	55	59	168	67
Wust (2003)	UASB	Cheese ww	-	55	-	66
Batch Reactor Set-2 ^a	Batch	Sucrose	223±6	77		
Batch Reactor Set-2 ^a	Batch	Influent B (Fermented sucrose)	-	-	344±20	83

^a Methane yield was calculated at 35 °C and 1 atm
^b Methane yield was calculated at STP (0°C and 1 atm)
^c CSTR: Completely Stirred Tank Reactor, FBR: Fluidized Bed Reactor

Apart from the studies in Table 4.4 Paixao (2000) used an acidogenic CSTR and a methanogenic hybrid reactor (UASB and Fixed-Bed) for the treatment of residue from the food industry. They reported that two-phase system achieved methane content of 80% in biogas. 18% higher methane yield and 13% higher methane content in biogas in two phase AD compared to single-phase AD were noticed in another study (Ghosh, 1987).

The reason of much higher methane percentage (%) and methane yield observed in the second phase of a two-phase system (Test 1 and Test 2) might be attributed to three conditions;

- ✓ Phase separation provides optimal environmental conditions for both acidogenesis and methanogenesis, removal of some portion of CO₂ in the preliminary dark fermentation step.
- ✓ When H₂ concentration is limited in the second-phase, formate+CO₂ pathway of hydrogenotrophic methanogenic archaea species leads to the increase in methane percentage and yield (Equation 2-5).
 - HCOOH (formate) → CO₂ + 2H (Equation 2)
 - 2H → H₂ (Equation 3)
 - Net: HCOOH → CO₂ + H₂ (Equation 4)
 - Finally: 4H₂ + 2CO₂ → CH₄ + 2H₂O (Equation 5)
- ✓ Homoacetogenic microorganisms might be carried out with the effluent of dark fermentation reactors (first-phase of the two-phase systems). These microorganisms might have been responsible for consuming CO₂ as substrate and improve the methane content of the biogas (Equation 6).
 - 4H₂ + 2CO₂ → CH₃COOH + 2H₂ (Equation 6).

Although a significant amount of H₂ was taken away by the DF-SBR (first phase of the two-phase system conducted by Tunçay (2015) as mentioned in Section 3.1), the residual sugar and pre-intermediate products such as butyric acid of first-phase still

remained in the effluent (i.e. influent of Batch Reactor Set-2, Influent A and B) as the sources of H₂ to be used by hydrogenotrophs for methanogenesis (Fox and Pohland, 1986). In addition, H₂ is not the only possible reductant for hydrogenotrophs to generate CH₄ by using CO₂ content of the produced biogas. Formate is the alternative electron donor for hydrogenotrophs to complete all four reduction steps of methanogenesis. Costa (2013) reported the alternative pathways of ferredoxin reduction with formate as electron donor in *Methanococcus maripaludis* that operate independently of Eha enzyme (hydrogenase) and H₂ to stimulate methanogenesis (by using molecular methods). Moreover, many studies revealed the same remarks with the data obtained from co-cultured earlier without using molecular methods (De Bok et al., 2004; Baet and McCarty, 1993; Boone et al., 1989; Thiele et al., 1988). Thus, when the H₂ concentration is limited in the second-phase, formate+CO₂ pathway might lead to increase in methane percentage of the biogas and methane yield.

Another remarkable possibility was obtained from the results of Batch Reactor Set-2. It was possible for homoacetogenic bacteria to be transferred to the second-phase of the two-phase system (Test 1 and Test 2) via the effluent of the first phase (DF-SBR). Actually, DF studies showed the potential existence of homoacetogenesis during dark-fermentative hydrogen production (Tunçay, 2015). Therefore, even if the effluent of acidogenesis reactors were centrifuged and filtered through 0.45 µm-sized pores before being used as substrate in the second phase reactors, their existence, activation and enrichment were still possible in the second phase (Test 1 and Test 2). This might have led to the production of additive acetic acid by H₂ and CO₂ consumption via homoacetogenesis. Therefore, the consumption of CO₂ in the biogas and additive acetic acid production might have resulted in a rise for methane content and yield in the Test 1 and Test 2 reactors. In addition, with the same approach, enriched hydrogen producers transferred by the influent of first-phase might have resulted in higher H₂ production, further simultaneous CH₄ production via hydrogenotrophs and in turn higher methane yield and percent.

As a result, the highest methane yield and methane percentage in biogas were observed as 344±20 mL CH₄ / g COD_{added} and 83% respectively, in Test 2 reactor which is

second phase of the two-phase AD. It was found out that, two-phase AD resulted in 39% increase in methane yield compared to its single-phase counterpart (Test 4). Yet, better comparison requires considering the retention (incubation) periods of both systems. Single-phase counterpart (Test 4) was incubated for 31 days, which was similar to that of second-phase of the two-phase AD (Test 2) (Figure 4.3). Therefore, the difference comes from the first phase of the two-phase AD, which was studied by Tunçay (2015) (Section 3.1): In the study of Tunçay (2015), a DF-SBR was operated with sucrose at 12 hours of HRT, 4 days of SRT, 22.4 g COD/L/day of OLR and 5.5 pH. The aim of that study was biological hydrogen production and hydrogen yield of 1.66 mL H₂/mol hexose was obtained as maximum yield at the operational parameters given above (Tunçay, 2015). Influent B, which was used as the substrate of Test 2 in this study, was the effluent of DF-SBR operated with operational parameters above (Section 3.1 and Table 3.2). Considering the retention time of this first phase (Tunçay, 2015), it is realized that, two-phase AD was indeed advantageous over single-phase counterpart because total HRT of the two-phase AD system is only 31.5 days. A further detailed comparison of single- and two-phase systems involving capital and operational costs (including the first-phase reactor) is not within the scope of this thesis: However, it should be noted that, despite the capital and operational costs due to the first-phase, there is significant amount of hydrogen production, which may increase the energy difference between single- and two-phase system even more than 39%. The ensuing high methane yields were recorded in Test 1 as 337±17 mL CH₄/g COD_{added}, in Test 3 as 247±25 mL CH₄/g COD_{added} and in Test 4 as 223±6 mL CH₄/g COD_{added} respectively (Table 4.3). In addition, initial sCOD concentration did not have a remarkable effect on the methane yield when the methane yields and initial sCOD concentrations of the Test 1 (5.7 mg/L sCOD, 337±17 mL CH₄/g COD_{added}) and Test 2 (4.8 mg/L sCOD, 344±20 mL CH₄ / g COD_{added}) were compared. Yet, the slightly higher methane yield obtained in Test 2 despite of the lower initial sCOD concentration was attributed to the higher HAc and VFA content of Influent B used in Test 2 (Table 3.10, Section 3.2.1).

In Table 4.5, the methane yield results of the second phase of two-phase studies were given to compare the results of Test 2 (the reactor with the highest methane yield) with

the related studies in literature (344 ± 20 mL CH₄/g COD_{added}). Especially the studies of Nasr et al. (2012), Giordano et al. (2010) and Xie et al. (2008) should be taken into consideration for being conducted in some reactor types. Table 4.5 and this study's results indicate that among the batch reactor studies considered the highest methane yield was achieved in this study.

Table 4-5 Comparison of different two-phase mesophilic AD reactor types conducted with different carbon sources in terms of methane yield and methane content in biogas

Substrate	Reactor type ^b	CH ₄ (%) ^c	CH ₄ yield	Reference
Glucose	Batch	-	248 mL/g COD _{added}	Xie et al. (2008)
Glucose ^a	Batch	-	267 mL/g COD _{added}	Giordano et al. (2010)
Thin stillage ^a	Batch	68	310 mL/g COD	Nasr et al. (2012)
Cheese whey	PBR	75	310 mL/g COD	Antonopoulou et al. (2008)
Grass waste	-	79		Yu et al. (2002)
Sugar beet pulp	-	72	280 mL/g COD _{added}	Hutnan et al. (2000)
Fruit and Vegetable	ASBR	-	320 mL/g COD _{added}	Bouallagui et al. (2004)
Food waste	UASB	76	0.21 m ³ /kg VSS _{added}	Han and Shin. (2004)
Spent tea leaves ^a		73	330 mL/g COD _{added}	Goel et al. (2001)
Coffee waste	CSTR	80	-	Houbron et al. (2003)
Distillary waste	UASB	75	-	Blonskaja et al. (2003)
Influent B (Fermented Sucrose)	Batch	83	344 mL/g COD _{added}	Batch Reactor Set-2
^a Methane yield and productivity were calculated at STP (0 °C and 1 atm) ^b ASBR: Anaerobic Sequencing Batch Reactor, PBR: Packed Bed Reactor, CSTR: Completely Stirred Tank Reactor, UASB: Up-flow Anaerobic Sludge Blanket ^c Peak CH ₄ content in biogas.				

4.3 Results of SBR Study

4.3.1 Methane Production Efficiency

ASBR was operated for 157 days (103 cycles). During this period six different operational periods with different HRT and SRT combinations were applied. The operational conditions (cycle time, HRT, SRT, OLR), effluent sCOD concentration, methane yield, volume of biogas and methane produced with respect to each cycle (103 cycles at the total) are given in Figure 4.4. The operational conditions, methane yield, cumulative biogas and methane production were also depicted with respect to time in Figure 4.5.

As previously mentioned, the first 80 days of operation was described as an acclimation period. After seeding of reactor, the ASBR was subjected to an initial HRT of 3 days and cycle time of 2 days for the first 5 days (Figure 4.4, 4.5). The substrate was the effluent of DF-SBR with a COD concentration of 10750 ± 254 mg COD/L and total VFA (tVFA) concentration of 35 ± 1 mg/L HAc (Table 3.2). During these first 5 days (2 cycles), methane yield ranged between 14-20 mL CH₄/g COD_{added} which is very low compared to the theoretical value of 395 mL CH₄/g COD (Speece, 1996). Seed sludge gathered from Central Wastewater Treatment Plant of Ankara is actually used for digestion of activated sludge; thus, not enriched with fermented sucrose. Considering literature review, HRT of the system was increased from 3 days to 16 days (Cycle 3) (Figure 4.3, 4.4) and so cycle time (from 3 to 4) as it can be seen from Figure 4.4a-b and 4.5a to support and shortened the acclimation period (Göblös et al., 2007; Cheong et al., 2008; Nakasaki et al., 2013; Table 2.1).

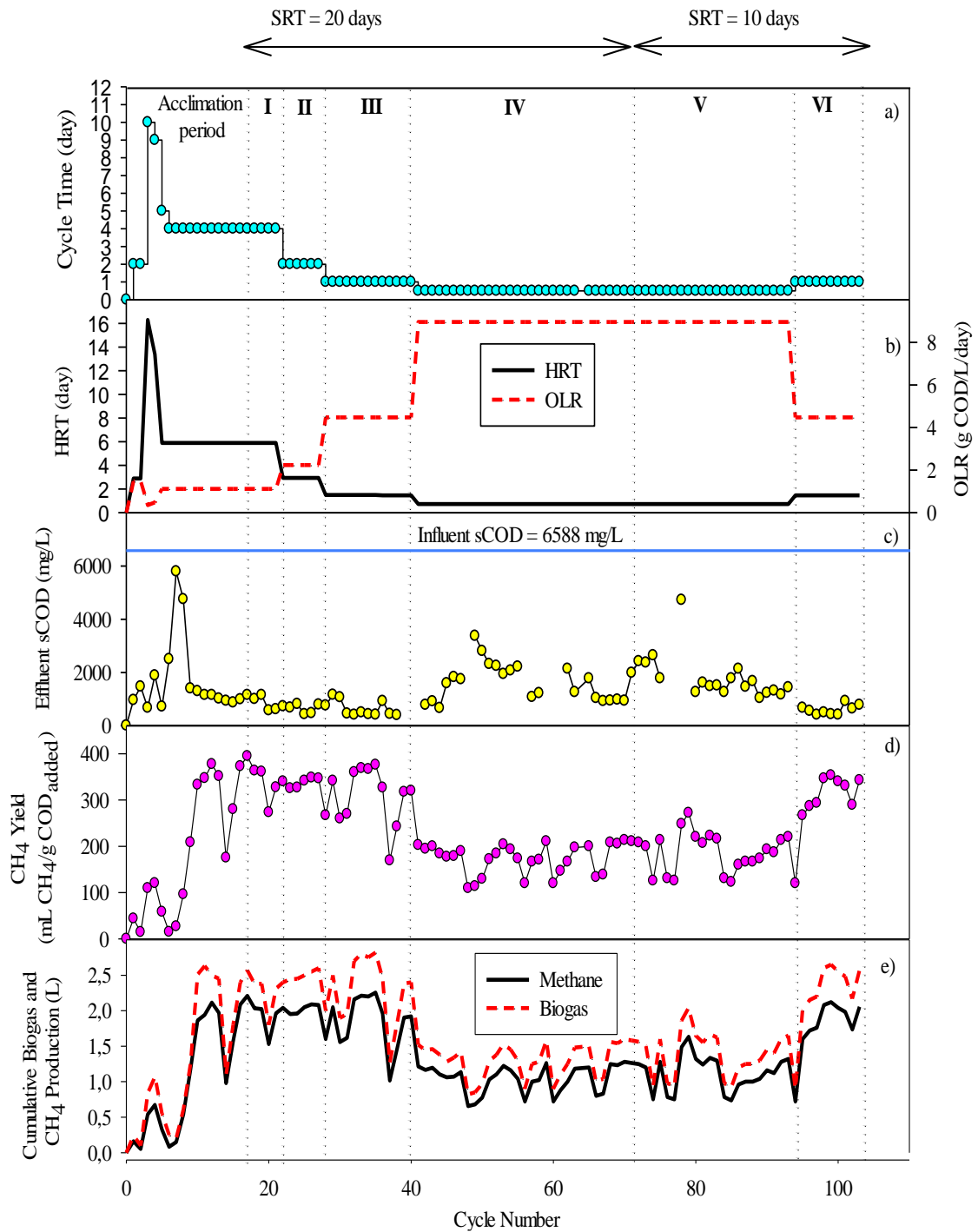


Figure 4-4 a) Cycle time, b) HRT and OLR, c) Effluent sCOD concentration, d) Methane yield, e) Cumulative biogas and methane production changes with respect to cycles (The first 17 cycles (80 days) indicate the acclimation period during which SRT was not controlled. Six different operational periods were indicated with the dash lines after the end of Day 80) (pH: 7-7.5, 35 °C)

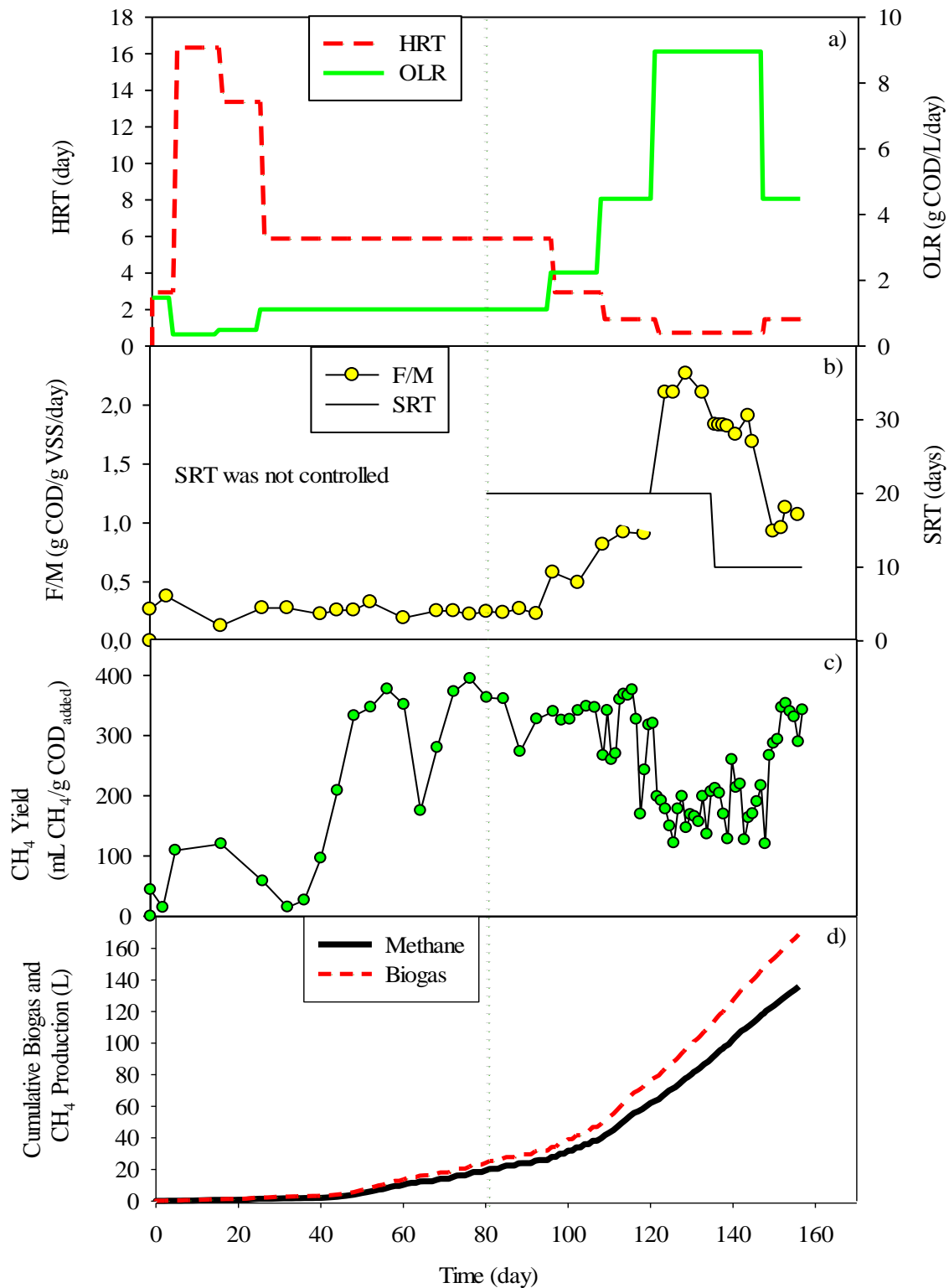


Figure 4-5 a) HRT and OLR, b) F/M and SRT, c) Methane yield, d) Cumulative biogas and methane production changes by time (SRT control was started after Day 80 which is indicated by a vertical dash line) (pH: 7-7.5, 35 °C)

HRT was periodically decreased for the following days from 16 to 14 days on Day 17 (Cycle 4), then to 6 days on Day 27 (Figure 4.5a). Until the operation day of 45 (between the cycles 1-9) methane yield ($<208 \text{ mL CH}_4/\text{g COD}_{\text{added}}$) was very low when compared with the theoretical value which is $395 \text{ mL CH}_4/\text{g COD}$ (Speece, 1996). According to Koutroli et al. (2009) and Salomoni et al. (2011) high OLRs and hydraulic shocks (low HRTs) may cause the loss of system stability and washout of microorganisms. It could be speculated that similar problems were experienced in this study. However, progressing with 6 days of HRT, methane yield significantly displayed an increasing trend. Methane yield value of $98 \text{ mL CH}_4/\text{g COD}_{\text{added}}$ on Day 41 increased to $380 \text{ mL CH}_4/\text{g COD}_{\text{added}}$ by Day 47 (Figure 4.5c).

Between the Days 57 and Day 80 (HRT of 6 days (OLR of 1.1 gCOD/L/day), the methane yield ranged between 280 and $395 \text{ mL CH}_4/\text{g COD}_{\text{added}}$, except the $175 \text{ mL CH}_4/\text{g COD}_{\text{added}}$ observed on Day 65 (cycle 14) which might be attributed to the washout of methanogens which could not be explained and required detailed research.

During the acclimation period, SRT was not controlled. It was aimed to washout the microorganisms not acclimating to the substrate and operational conditions. As seen in Figure 4.4 and 4.5, the high and almost stable biogas and methane production and high methane yield values between Days 49 and 80 indicated that seed sludge acclimation was obtained. Therefore, for the following days (and cycles), ASBR operation was processed considering the SRT of the system.

After operational day of 80, the rest of the incubation time until the operation day of 157 divided into six different operational periods. In these six different operational periods, ASBR was operated at various HRT and SRT combinations which were indicated in Table 4.6 clearly. In addition, both operational conditions and the resultant maximum and average methane yield, productivity as well as average effluent quality corresponding to each period are given in Table 4.6.

Two different substrate types (Influent A and Influent B) were used in ASBR study which are mentioned in Section 3.1 (Table 3.1 and Table 3.2). Firstly, Influent A was used until the end of Day 128. Then, Influent B was used between the Days 129 (middle of the operation period IV) and 157. In Table 4.7, operational conditions, ranges of methane yield and productivity data corresponding to each period are given with respect to usage periods of Influent A and Influent B. In addition, operational conditions of DF-SBR (first phase of the two-phase AD) are also given in Table 4.7 to clarify the total HRT of the two-phase AD.

Both Influent A and B had similar characteristics (Table 3.2) despite the higher sCOD and the lower tVFA content of the former. Influent A and B had sCOD and tVFA concentrations of 10750 ± 254 , 8628 ± 312 mg sCOD /L and 35 ± 1 , 51.4 ± 0.1 mg/L HAc, respectively. Thus, their effect on methane production can be compared by looking at the methane yield and productivity data.

To do that comparison, it is needed to take closer look at period IV in Table 4.7 where both the substrates were used in at some operational conditions. As it could be clearly observed in Table 4.7 that methane yield and productivity ranged between 109-204 mL CH₄/g COD_{added} and 1069-1890 mL CH₄ /L/day respectively, while using Influent A was used as substrate. After depletion of Influent A, Influent B was started to be used in period IV, and methane yield, productivity ranged between 120-211 mL CH₄/g COD_{added} and 1280-1830 mL CH₄/L/day, respectively. Therefore, it could be said that there was no significant effect of substrate type (Influent A and Influent B) on methane yield and productivity of ASBR.

Table 4-6 Operational conditions, methane production and effluent quality data of ASBR

Combinations corresponding to different six operational periods	Period I	Period II	Period III	Period IV	Period V	Period VI
Operation days	81-96	96-108	108-121	121-136	136-146	146-157
HRT (day)	6	3	1.5	0.7	0.7	1.5
SRT (days)	20	20	20	20	10	10
OLR (g COD/L/day)	1.1	2.2	4.5	8.9	8.9	4.5
F/M (g COD/g VSS/day)	0.3±0.0	0.5±0.1	0.9±0.1	2.1±0.1	1.8±0.1	1.0±0.1
Cycle time (hour)	96	48	24	12	12	24
Maximum methane yield ^a (mL CH ₄ /g COD _{added})	395	348	367	211	272	347
Average methane yield ^a (mL CH ₄ /g COD _{added})	343±17	338±19	307±31	172±24	188±22	297±14
Average methane productivity ^a (mL CH ₄ /L/day)	924±194	1599±56	1487±302	1617±251	1794±279	1437±300
Maximum methane productivity ^a (mL CH ₄ /L/day)	1231	1664	1805	1891	2362	1696
Average effluent sCOD (mg/L)	735±21	495±17	631±19	1666±44	1662±57	591±13
Average effluent tVFA (mg/L HAc)	3.9	2.7	2.6	16.6	12.1	2.7
^a Methane yield and productivity were calculated at 35 °C and 1 atm						

Table 4-7 Range of methane yield, productivity of ASBR obtained at different operational conditions and influent substrates and operational conditions of DF-SBR (first phase of the two-phase AD)

Substrate Type	Period No	Operational Conditions (day)	Methane Yield (mL CH ₄ /g COD _{added})	Methane Productivity (mL CH ₄ /L/day)	DF-SBR Operational Conditions (Tunçay, 2015)
Influent A	Period I	HRT: 6	273-395	734-1231	HRT: 12 hours SRT: 9 days OLR: 22.4 gCOD/L/day pH: 5.5
		SRT: 20			
	Period II	HRT: 3	325-348	1536-1664	
		SRT: 20			
	Period III	HRT: 1.5	169-367	812-1805	
		SRT: 20			
	Period IV	HRT: 0.7	109-204	1069-1890	
		SRT: 20			
Influent B	Period IV	HRT: 0.7	120-211	1280-1830	HRT: 12 hours SRT: 4 days OLR: 22.4 gCOD/L/day pH: 5.5
		SRT: 20			
	Period V	HRT: 0.7	123-272	1356-2362	
		SRT: 10			
	Period VI	HRT: 1.5	267-347	1280-1696	
		SRT: 10			

As seen in Table 4.6 and Figure 4.4 Period I was started on operational Day 81. It lasted 15 days (Days 81-96 and Cycles of 17-21) and SRT value was kept constant at 20 days by withdrawal of predetermined volume of the reactor content during the reaction phase in certain days. HRT was set as 6 days during this period. Maximum methane yield was recorded as 395 mL CH₄/g COD_{added}; thus, the maximum theoretical value (1 g COD = 395 mL CH₄) was achieved during this period (Speece, 1996). In addition, maximum methane productivity was recorded as 1231 mL CH₄/L/day. Reaction time was 92 hours. Average effluent sCOD and tVFA concentrations were measured as 735±21 mg/L and 3.9 mM as HAc, respectively (Table 4.6). To understand the other sCOD source in the effluent additional to tVFA related sCOD sources (3.9 mM as HAc = 501 mg/L COD), effluent liquid samples were analysed by HPLC in METU Central Laboratory. Butanol, ethanol and methanol were expected in the effluent considering the related literature (Damon and Pettitt, 1980; Xu et al., 2013). However, none of them was monitored as a result in the effluent of ASBR in Period I.

In Period II (Days 96-108 and Cycles of 22-27), HRT was decreased by half (3 days) and SRT was kept constant at 20 days. During twelve days of the ongoing operation (Cycles 22-27), average methane yield and maximum methane productivity were recorded as 338±19 mL CH₄/g COD_{added} and 1664 mL CH₄/L/day respectively (Table 4.6). Reaction time was 44 hours. Effluent sCOD was measured as 495±17 mg/L. tVFA of 2.7 mM (HAc) was detected in effluent sCOD (Figure 4.4c, and Table 4.6). The next step was continued under another reduced HRT trial.

In Period III (Days 108-121 and Cycles 28-40), HRT and SRT were set as 1.5 and 20 days respectively. OLR of this period was 4.5 g COD/L/day. Reaction time was reduced from 44 hours to 20 hours. Maximum methane production yield and productivity were recorded as 367 mL CH₄/g COD_{added} and 1805 mL CH₄ /L/day, respectively. Average effluent COD concentration of 631 mg/L and 2.6 mM average tVFA (HAc) concentration were recorded during this period (Table 4.6).

HRT of Period IV (Days 121-135 and Cycles 41-70) was 0.7 days. According to the literature, reactor was at the borderline of hydraulic stability limits for a methanogenesis study (Koutrol et al., 2009; Salomon et al., 2011). That means there is not enough time for the utilization of substrate and therefore, wash-out of microbial cultures is a strong possibility (Speece, 1996). In this period, SRT was set as 20 days similar to the previous periods. However, 4 hours of settling period was not been able to keep SRT constant at 20 days. This situation might be caused from decreasing HRT to 0.7 days. While effluent VSS concentration in Period II and III varied between 150-300 mg/L, effluent VSS concentration (1630-2100 mg/L VSS_{effluent}) were recorded at the beginning of Period IV (Days 122-123) (Appendix C). Thus, as it was mentioned above, microorganisms were washed out from the reactor (Koutrol et al., 2009; Salomon et al., 2011). Consequently, settling time period was extended to 5 and 6 hours respectively. However, it was monitored that 5 and 6 hours settling time was also not enough to keep SRT at 20 days. Therefore, settling time was further increased to 7 hours and it was sufficient enough to set SRT at 20 days. During Period IV, methane yield dropped by almost half (172 ± 24 ml CH_4/g COD_{added}) comparing with the previous periods (Table 4.6 and Figure 4.4d). Average effluent sCOD was calculated as 1666 ± 44 mg/L, containing 17 mM tVFA as HAc. Only the methane productivity was not affected and recorded as 1891 mL $CH_4/L/day$ (Table 4.6).

In Period V (Days 136-147 and Cycles 71-93) SRT was decreased from 20 days to 10 without changing the cycle time (12 hours) and HRT (0.7 day). The settling time was re-decreased to 4 hours. It was enough to keep SRT at 10 days with 4 hour settling period which was found adequate to sustain the hydraulic limits of the reactor by contrast with Period IV. Average methane yield was obtained as 188 ± 22 mL CH_4/g COD_{added} . Average effluent COD and tVFA concentrations were 1662 ± 57 mg/L and 12.1 mM (HAc), respectively. During this period, the maximum methane production rate of 2362 mL $CH_4/L/day$ was recorded, which was the highest productivity level observed during the whole 157 days of operation time (Table 4.6).

Period VI (Days 148-157 and Cycles 94-103) was the last period of the ASBR study. SRT was kept constant at 10 days as it was in Period V; however, HRT was increased to 1.5 days. Average methane yield increased to 297 ± 14 mL CH₄/g COD_{added} and maximum methane productivity was recorded as 1696 mL CH₄/L/day. Effluent sCOD concentration was measured as 591 ± 13 mg/L containing 2.7 mM tVFA as HAc. With a closer look, it could be easily realized that effluent data of Period VI showed a significant similarity with the effluent results of Period III. The main common point of these periods were the HRTs. In both, reactor was operated at 0.7 days of HRT and OLR of 4.5g COD/L/d. In addition, VFA compositions of the all periodical effluents of the ASBR Study are given at Appendix D.

To summarise, SRT and HRT combinations of the ASBR study ranged between 10-20 and 0.7-6 days, respectively. The highest methane yield was achieved at 20 days of SRT and 6 days of HRT as 395 mL CH₄/g COD_{added} in Period I (by using Influent A as substrate). HRT value of DF-SBR was 12 hours for the same first and second phase sequence of AD. Thus, total HRT of the two-phase AD system was 6.5 days for the maximum methane production. The values of highest methane productivity was observed as 2362 mL CH₄/L/day at SRT and HRT values of 10 and 0.7 days, respectively in Period V (by using Influent B as substrate). HRT value of DF-SBR was 12 hours for the same first and second phase sequence of AD. Thus, total HRT of the two-phase AD system was 6.5 days for the highest methane productivity also. It was noticed that as HRT decreased from 6 to 0.7 days at constant SRT (20 days), average methane yield also decreased (Table 4.6). Similarly, as HRT increased from 0.7 to 1.5 days at constant SRT (10 days), methane yield also increased. Different SRTs with constant HRT of 0.7 days resulted in similar methane yields (Table 4.6, Periods 4 and 5). Thus it was concluded that methane yield had directly proportional relationship with the HRT and no significant relationship with SRT changes. Low HRT values (0.7 days) were also found to increase methane productivity. Yet, increase in effluent sCOD and tVFA concentrations should be also taken into consideration. Results showed that larger HRTs resulted in improved performance of ASBR, while SRT changes did not cause any significant change in reactor performance.

The comparison of this study with related literature is presented in Table 4.8 based on methane yield and methane productivity. Maximum methane productivity of Period V (2362 mL CH₄/L/day at 8.9 gCOD/L/day of OLR and 0.7 days of HRT), where the productivity had highest value along six different operational periods, was compared with the literature. While composing Table 4.6, mesophilic conditions and similar substrate characterization were used as the filters for comparison. Kyazze et al. (2007) studied in laboratory scale CSTR to monitor the performance of a two-phase AD with sucrose as substrate. In that study, methane productivity was recorded as 1800 mL CH₄/L/day and this data is far below the productivity obtained in this study (2362 mL CH₄/L/day). Biochemical structure of the carbon source used in studies of Cooney et al. (2007) and Park et al. (2010) were similar (glucose and molasses) with that of this study. Park et al. (2010) used a column reactor packed with porous polyurethane foams (Packed bed reactor) for methanogenic process and found methane productivity as 1940 mL CH₄/L/day at 6 day of HRT and 4.6 g/L/day of OLR. Cooney et al. (2007) used CSTR as the methanogenic reactor which was the second phase of a two-phase system and recorded 1300 mL CH₄/L/day at 13.3 g COD/L/d of OLR and 3 days of HRT. These productivity values are lower than that obtained in this study. However, only remarkable and higher methane productivity data was recorded in the Liang (2009) 's study which was noted as 3380 mL CH₄/L/day. The higher productivity obtained in Liang et al. (2009)'s study was attributed to almost 9 times greater OLR applied and potentially the use of natural organic source, that is molasses, instead of sucrose.

Average methane yield of Period I (343±17 mL CH₄/L/day at 1.1 gCOD/L/day of OLR and 6 days of HRT), where the yield had highest average value along six different operational periods in this study, was also compared with the literature. Nasr et al. (2012) reported a yield of 360 mL CH₄/g COD_{added} studying with thin stillage in batch reactors. Bull et al. (1983) used glucose as sole carbon source in fluidized bed reactor and noted 356 mL CH₄/g COD_{added} as methane yield. These data are very close to the methane yield of this study (343 mL CH₄/g COD_{added}, which was reached in Period I as an average value and the highest average yield in different operational conditions of ASBR study). In addition, Xie et al. (2008) and Giardino et al. (2010) also used

highly similar carbon source (glucose), in batch reactors, with study of Bull et al. (1983). However, methane yield records were 248 mL CH₄/g COD_{added} and 267±13 mL CH₄/g COD_{added} and could not scattered the methane yield of this study.

Table 4-8 Comparison of this study with related literature

Reactor Type ^a	Substrate	Methane Yield	Methane Productivity (L CH ₄ /L/day)	Reference
Batch	Glucose	248 mL CH ₄ /g COD _{added}	n.g.	Xie et al. (2008)
Batch	Glucose	267 ±13mL CH ₄ /g COD _{added}	n.g.	Giardino et al. (2010)
FBR	Glucose	356 mL CH ₄ /g COD _{added}	n.g.	Bull et al. (1983)
CSTR	Glucose	n.g.	0.13	Cooney et al. (2007)
CSTR	Sucrose	n.g.	1.80	Kyazze et al. (2007)
PFR	Molasses	n.g.	1.94	Park et al. (2010)
UASB	Molasses	n.g.	3.38	Liang et al. (2009)
Batch	Molass	461 mL CH ₄ /g COD _{added}	n.g.	Koç et al. (2014)
CSTR	Molass and thin stillage mix	190 mL CH ₄ /g COD _{added}	n.g.	Yeoh (1997)
CSTR	Olivemill, chesewhey and dair ww mix	316 mL CH ₄ /g COD _{removed}	0.50	Dareiotti et al. (2014)

Table 4-8 (continued)

CSTR	Sugar beetpulp ww	230 mL CH ₄ /g COD _{added}	n.g.	Weiland (1993)
Batch	Thin stillage	360 mL CH ₄ /g COD _{added}	3.78 mL CH ₄ /hour	Nasr et al. (2012)
CSTR	Thin stillage ww and glycerol mix	n.g.	1.3	Luo et al. (2011)
Reactor Type ^a	Substrate	Methane Yield	Methane Productivity (L CH ₄ /L/day)	Reference
CSTR	Food industry ww	n.g.	1.75	Han and Shin (2004)
UASB	Food industry ww	n.g.	1.83	Han et al. (2005)
CSTR	Cheesewhey ww	310 mL CH ₄ /g COD	n.g.	Antonopoulou et al. (2008)
UASB	Cheesewhey ww	n.g.	5	Georgia et al. (2008)
CSTR	Olivemill ww	n.g.	0.16	Gavala et al. (2005)
Batch	Sugar beetpulp ww	227 mL CH ₄ /g COD	0.44	Guo et al. (2014)
CSTR	Food industry ww	140 mL CH ₄ /g COD _{added}	1.13 ± 0.08	Koutrouli et al. (2009)
ASBR	Sucrose	343 mL CH ₄ /g COD _{added}	2.4	This study (ASBR)
^a FBR: Fluidized bed reactor, ASBR: Anaerobic sequencing batch reactor, CSTR: Completely stirred tank reactor, UASB: Upflow anaerobic sludge blanket, PFR: Packed Bed Reactor, ww: wastewater, n.g: not given				

4.3.2 Change in the Population Dynamics

Sludge samples taken from ASBR were used to characterize the composition and dynamics of the microbial community in terms of archaea and general bacteria. The sampling date of the sludge was selected considering the achievement of steady-state conditions for each different operational conditions of the ASBR and shown in Table 4.9. As described in Section 3.4.8.2; firstly, DNA isolation of the samples obtained from ASBR study (Table 4.9) were performed and resulting isolated DNAs were amplified for both bacterial and archaeal species with PCR. Verification of amplicon size of PCR products were done by loading the amplified DNAs on gel electrophoresis and compared with small spaced DNA Mass Ruler (Section 3.4.8.2).

Table 4-9 Notation of sludge samples and operation time when they were taken for molecular studies

Sludge samples	Operation day	HRT (day)	SRT ^a (day)
Seed Sludge	1	3	N.C.
Acclimation period	26	6	N.C.
Acclimation period	52	6	20
End of acclimation period	80	6	20
End of Period I	97	6	20
End of Period II	107	3	20
End of Period III	117	1.5	20
End of Period IV	136	0.7	20
End of Period V	147	0.7	10
End of Period VI	157	1.5	10
^a NC: not controlled			

Because DGGE analysis also provides the confirmation of PCR products, gel electrophoresis analysis was performed only for the PCR products of archaea as an example (Figure 4.6). Then, DGGE process was applied to all bacteria and archaea species. After DGGE analysis, DNA samples purified from polyacrylamide gel were used for sequence analysis to identify the microorganisms (Section 3.4.8.4).

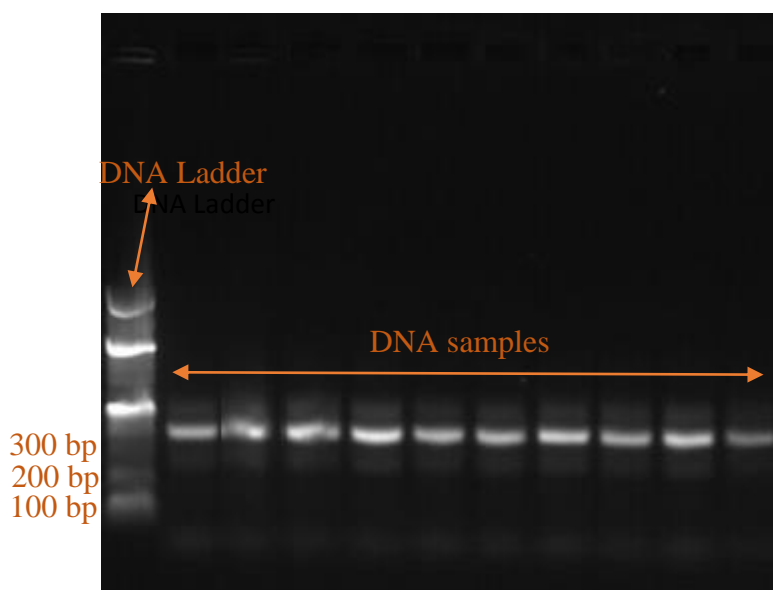


Figure 4-6 Gel electrophoresis image of PCR products of archaea

DGGE analysis was firstly performed for archaea species. DGGE analysis gel image for archaea is shown in Figure 4.7. It is assumed that each band observed in the different vertical position on the DGGE gel represents to a different kind of microbial specie. Intensity owned by a band provides information on the population density of the microorganisms (Nübel et al., 1999; Nikolchev et al., 2003). Increasing the intensity of a band means the increase in the population of microorganism represented by the band. It can be understood from Figure 4.7 that some of the archaea species (MRA 5, MRA 6, MRA 7) remained inside the ASBR during the whole operation time (Days 1-157, Table 4.10). On the other hand, some of the bands such as MRA 2 and MRA 4 remained in the ASBR for a time duration and then disappeared. MRA 2, MRA 3, MRA 4, MRA 8, MRA 9 and MRA 10 were the bands that did not exist in the reactor at the beginning. Bands MRA 2 and MRA 3 appeared on Day 26 and bands MRA 8,

MRA 9 and MRA 10 appeared on operation day 80. Each band appeared in DGGE analysis did not have the same intensity (Figure 4.7). While some bands were observed with a high intensity (MRA 6), intensity of some of them varied within time (MRA 5 and MRA 2), which might indicate the change in the concentration of the specie represented by the related band.

As it can be seen in Figure 4.8, some of the bands were named and selected for sequence analysis. Variations on intensity and appearance for a while were set as the selection criteria for the bands. Sequence analyses were performed for the named bands and are shown in Table 4.9. According to taxonomic affiliation of the partial 16S rRNA sequences' bands, MRA 3, MRA 4, MRA 5, MRA 7, MRA 8, MRA 9, and MRA 10 were most similar to 16S rRNA of *Methanosaeta concilii* (Table 4.9). Sequences of bands MRA 1 and MRA 2 were similar with *Methanospirillum hungatei*. Identity of band MRA 6 could not be determined during sequencing. This situation might be due to a problem during the sequencing of this band. For being on the same row, bands MRA 1 and MRA 2 were expected to be the same specie. For confirmation, sequence analyses were nevertheless performed for both of these bands which was further confirmed.

Methanospirillum hungatei is known as a type of methanogen consuming hydrogen and formate (Quinn et al., 2014). It also grows easily in environmental conditions containing 80% H₂ and 20% CO₂. Acetate and formate can be used as carbon source by this methanogen specie (Ferry et al., 1974). Ekiel et al. (1983) reported that 99% of the methane produced by *Methanospirillum hungatei* is related to CO₂. According to Figure 4.7, *Methanospirillum hungatei* did not appear in the seed sludge at the beginning of the reactor operation. However, it became apparent during acclimation period (Days 26, 52, 80). In addition, *Methanospirillum hungatei* maintained its existence during the operation of both 20 days of SRT (Days 81-135) and 10 days of SRT (Days 136-157). It also survived for all HRT conditions studied. Despite its existence for all SRT and HRT combinations, domination of this specie was observed when SRT was 20 days and HRT was 3 days due to increase of the corresponding band (Period II, Day 107, Figure 4.7).

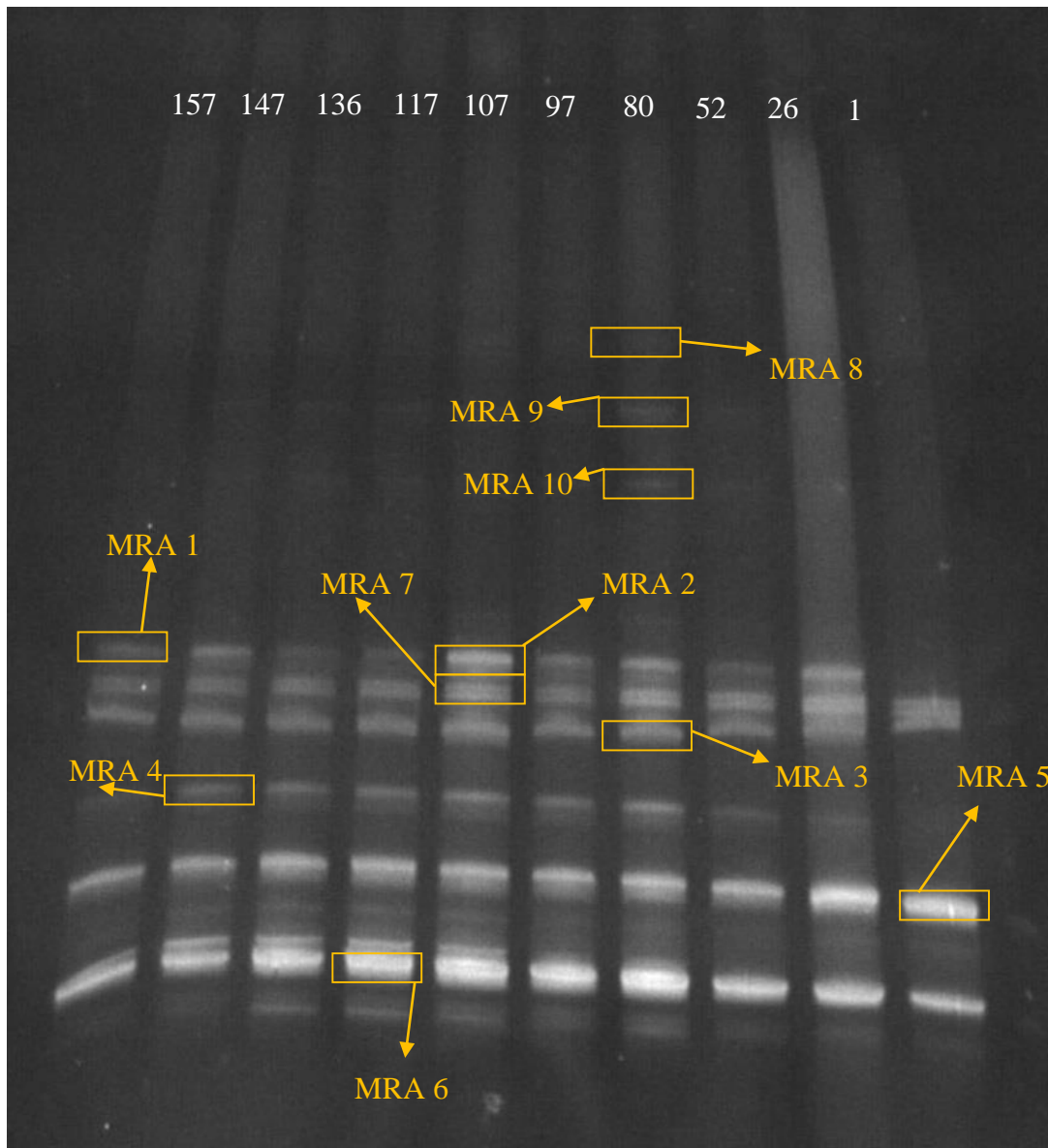


Figure 4-7 DGGE analysis image of Archaea

(1-157 indicates the operational day when the sludge samples were taken and the bands marked with “” used for sequence analysis. MRA: Archaea DGGE band of ASBR)

Furthermore the intensity of the bands MRA 1 and MRA 2, which represent *Methanospirillum hungatei*, began to decrease by day 177 (HRT of 1.5 days, SRT of 20 days, Table 4.9). The intensities of the bands for the following days were also low indicating the potential decrease in the concentration of the *Methanospirillum hungatei*. The operational conditions corresponding to these mentioned days (Day

117-157) were SRTs of either 20 or 10 days and HRTs of 0.7 or 1.5 days. Thus, it was concluded that *Methanospirillum hungatei* did not have tendency to be dominant at HRTs values of 0.7 to 1.5 days.

As seen in Table 4.9, the sequences of the bands except for MRA 1, MRA 2 and MRA 6 were all identified as *Methanosaeta concilii*. In other words, the most dominant methanogenic archaea identified by DGGE was found as *Methanosaeta concilii* in the ASBR (Figure 4.7 and Table 4.10). Isolated DNAs from DGGE bands of MRA 3, MRA 4, MRA 5, MRA 7, MRA 8, MRA 9, and MRA 10 showed 98% to 99% similarity with *Methanosaeta concilii*. As it is known from the literature, *Methanosaeta concilii* use acetate as the only carbon source (Shigematsu et al., 2003). Thus, this specie is a typical acetoslastic methanogen. In general, acetoclastic methanogens are more abundant than hydrogenotrophic methanogens. There are two common acetoclastic methanogen species known as *Methanosatea* and *Methanosarcina*. Considering Figure 4.7, *Methanosaeta concilii* appeared both in the seed sludge at the beginning of the reactor operation (MRA 5) and also in the periods with different SRTs (20 days to 10 days) and HRTs (6 days to 0.7 days).

According to Lee et al. (2011), 15 days and lower SRT values resulted with the dominance of *Methanosarcina* species in an anaerobic reactor. In addition, when SRT value is higher than 15 days in an anaerobic environment (and often in a conventional anaerobic sludge digester), *Methanosaeta* is found as the dominant specie. *Methanosarcina* has a higher maximum growth rate but a lower affinity for acetate (maximum growth rate: 0.21 day⁻¹; K_s: 4 mM acetate) than *Methanosaeta* (maximum growth rate: 0.11 day⁻¹; K_s: 0.44 mM acetate) (Wandrey and Aivasidis, 1983; Zehnder et al., 1980). In the light of this information, it was expected that the operating conditions specified for ASBR system would have led to the existence of *Methanosarcina* when SRT was reduced from 20 days to 10 days and HRT was reduced to 0.7 days. However, DGGE analyses of this study showed that *Methanosaeta concilii* existed in the ASBR for each of the operational conditions studied (10-20 days of SRT and 0.7 to 6 days of HRT). Kalyuzhnyi et al. (1996) observed that increasing OLR (>11.2 g COD/L/day) shifts the tendency of microbial

domination from *Methanosaeta* to *Methanosarcina* species. As seen in Table 4.5, the maximum OLR applied to the ASBR was 8.9 g COD/L/day. So, it was speculated that the OLR values supporting the dominance of *Methanosarcina* was also as high as 9 g COD/L/day. To indicate the shift from *Methanosaeta* to *Methanosarcina*, higher OLRs should be studied, as Kalyuzhnyi et al. (1996) mentioned.

Table 4-10 The results of sequencing analyses for DGGE analysis of Archaea

Sequenced band ^a	Operation day	Archaea Specie ^b
MRA 1	157	<i>Methanospirillum hungatei</i>
MRA 2	107	<i>Methanospirillum hungatei</i>
MRA 3	80	<i>Methanosaeta concilii</i> (98%)
MRA 4	147	<i>Methanosaeta concilii</i> (99%)
MRA 5	1	<i>Methanosaeta concilii</i> (99%)
MRA 6	117	ND
MRA 7	107	<i>Methanosaeta concilii</i> (99%)
MRA 8	80	<i>Methanosaeta concilii</i> (98%)
MRA 9	80	<i>Methanosaeta concilii</i> (98%)
MRA 10	80	<i>Methanosaeta concilii</i> (99%)
^a MRA: Archaea DGGE band of ASBR		
^b ND: not determined		

As a result, decreasing the SRTs from 20 days to 10 days, did not lead to change in dominant acetoclastic methanogenic specie type and *Methanosaeta concilii* dominated the ASBR among the archaeal consortium during all operating conditions and combinations (10-20 days of SRT and 0.7 to 6 days of HRT). Additionally *Methanospirillum hungatei*, methane producer from H₂-CO₂ couple and formate was also detected in ASBR study, especially when the HRT was equal and greater than 3 days.

It is important to be aware of in these molecular analyses that it may not be possible to observe all methanogenic species with any archaeal primers. Another archaeal primer set can be used instead of the primers used in this study and the results might be the same or not. Thus, besides the *Methanosaeta concilii* and *Methanospirillum hungatei*, other methanogen types might be also existed in the reactor.

Sludge samples taken from ASBR system were also investigated for all bacterial species as mentioned before. After the isolation of DNA samples all bacterial 16S rRNA gene fragments were amplified with primer sets as stated in Table 3.6. PCR products were subjected to DGGE analyses. DGGE gel images obtained for all bacteria in the sludge samples withdrawn at different operational days is shown in Figure 4.8.

When Figure 4.8 is analysed, it could be monitored that some of the bands were spanned in DGGE gel. This is likely due to the characteristics of the chemical used to stain the DGGE gel which is called GelRed. It was believed that chemical features of GelRed caused non-clear image. In addition, this spanning problem could be also correlated to the operational problems during the running and staining of the DGGE gel and just before taking the image under UV light. Nevertheless, Figure 4.8 indicates that the number of bacteria species is quite limited. Some bands with less intensity (MRB 4) has almost appeared through whole operation, and some bands such as MRB 5 just became apparent towards the end of operation. Sequence analysis of general bacteria results were given in Table 4.10.

As seen in Table 4.10, MRB 1 and MRB 5 bands could not be sequenced. MRB 2 and MRB 3 bands were expected to be the same for locating in the same horizontal line (row). In other words, both MRB 2 and MRB 3 were sequenced for control purposes, with the similar approach as mentioned before for the archaea species sequencing (MRA 1 and MRA 2). Indeed both bands indicated the similar species. MRB 2 and MRB 3 bands were defined as the *Clostridium saccharobutylic DSM 13864* with 99% similarity. Clostridium species are spore-forming hydrogen producers. Main activity of this specie is to produce acetone, butanol and ethanol from various sugars such as

sucrose, fructose, mannose. Therefore, it was meaningful to face with this specie in an ASBR study which used the effluent of a sucrose-fed dark fermentation system (DF-SBR) as the carbon source. Referring to Figure 4.8, *Clostridium saccharobutylic* were found in the reactor regardless of SRTs and HRTs studied. MRB 2 and MRB 3 bands were also associated with hydrogen producing specie *Clostridium acetobutylicum* with 98% similarity. Sequencing result of the bacterial strains identified by band MRB 4 showed 98% similarity with *Gammaproteobacteria-Enterobacteriales* belonging to the family of *Escherichia coli str. K-12 substr.* It was also defined as facultative anaerobic bacteria *MG1655* (Table 4.11). This bacterium has appeared at the beginning of the reactor operation; and, the related band became more intense when HRT was increased from 0.7 to 1.5 days (Days 109-157, Figure 4.8).

Table 4-11 The results of sequencing analyses for DGGE analysis of all bacteria

Sequenced band ^a	Operation Day	Bacteria Species ^b
MRB 1	1	ND
MRB 2	107	<i>Clostridium saccharobutylicum</i> DSM 13864 (99%) <i>Clostridium acetobutylicum</i> (97%)
MRB 3	117	<i>Clostridium saccharobutylicum</i> DSM 13864 (96%) <i>Clostridium acetobutylicum</i> (96%)
MRB 4	136	<i>Escherichia coli str. K-12 substr. MG1655</i> (98%)
MRB 5	147	ND
^a MRB: Bacterial DGGE band of ASBR		
^b ND: Not determined		



Figure 4-8 DGGE analysis image of all Bacteria
(1-157 on each lane indicates the operational days when the sludge samples were taken and the bands marked with “” and “MRB” indicated the bacterial species (bands) used for sequence analyses)

4.3.3 Quantitative Analysis of *Methanosaeta* and *Methanosarcina* species

In this part of the thesis, biomass samples obtained in different operational periods from the ASBR were analysed with qPCR to investigate the population concentration and relative dominance of acetoclastic methanogens, *Methanosarcina* and *Methanosaeta*. In this way, firstly, enriched *E.coli* cultures containing recombinant plasmids with either *Methanosarcina* or *Methanosaeta* 16S rRNA target gene segments were used to obtain standard curves for the absolute quantification of each acetoclastic specie as mentioned before in Section 3.4.9.1. In Table 4.12, Nanodrop analyses results of the isolated DNAs from biomass samples of ASBR study and enriched *E.coli* cultures containing recombinant plasmids with either *Methanosarcina* or *Methanosaeta* 16S rRNA gene segments were given prelusively.

Table 4-12 Results of nanodrop analyses performed for biomass samples of ASBR study and *E.coli* cultures

Sample No ^a	Average DNA Concentration (ng/μL)	260/280	260/230
ASBR 1	109.5±4.6	1.94±0.02	1.87±0.03
ASBR 2	102.1±5.1	2.01±0.03	2.16±0.01
ASBR 3	55.2±2.3	2.03±0.02	2.27±0.02
ASBR 4	123.5±4.2	1.90±0.04	1.93±0.03
ASBR 5	33.2±1.7	1.89±0.02	1.97±0.04
ASBR 6	33.9±1.8	2.16±0.04	2.24±0.02
ASBR 7	145.5±3.9	1.95±0.03	1.85±0.03
ASBR 8	127.6±3.2	1.84±0.01	1.91±0.02
Methanosaeta	44.6±2.7	1.81±0.02	2.09±0.01
Methanosarcina	23.6±1.3	1.88±0.01	2.07±0.02
^a ASBR: Biomass sample taken from ASBR study for qPCR analysis			

In Nanodrop analyses, 260/280 ratio implies purity of nucleic acids for a healthy PCR application, if it is around 1.8. Moreover, 260/230 ratio value in the range of 2.0 to 2.2 means that isolated DNA sample does not contain chemical contamination (such as phenol, ethanol and cell extracts) such a level that a PCR healthier PCR application is possible (Section 3.4.8.1). When the ratio of 260/230 in Table 4.12 were analysed, it can be said that all the 260/230 ratios of the isolated DNA samples were almost in the range of 2.0 to 2.2. In fact, for PCR process, isolated DNA samples may be fine, even if 260/280 and 260/230 ratios are not strictly between the related ratios mentioned above (around 1.8 and between 2.0-2.2). However, since qPCR process is more sensitive than PCR process and optimized for absolute quantification of target encoding genes, 260/280 and 260/230 ratios of environmental samples should be around 1.8 and between the ranges of 2.0-2.0 respectively as much as possible. As mentioned before all of the samples gathered from ASBR have been qualified for the qPCR process.

After plasmid DNA isolation and Nanodrop analyses, plasmids were linearized with the restriction enzyme ScaI as described in Section (3.4.9.1). Then, a non-quantitative PCR protocol was used to amplify the 270 bp insertions that were 16S rRNA gene fragments for both *Methanosaeta* and *Methanosarcina*, using recombinant plasmids as templates (Section 3.4.9.2). The products of PCR process were confirmed with gel electrophoresis. Then second Nanodrop analyses were done to note the plasmid DNA concentrations. Thus, target gene copy concentration for each specie could be calculated by using the equations below (Equation 7 and 8) and the results were given in Table 4.13.

$$\frac{MW \text{ of ds DNA of } \frac{Methanosarcina}{Methanosaeta} (g/mol)}{Avagadro's \text{ Number } (1molecule/mol)} = Gene \text{ copy number } \left(\frac{g}{gene \text{ copy}} \right)$$

(Equation 7)

$$\frac{DNA \text{ concentration after PCR } (ng/\mu L)}{Gene \text{ copy number } (g/gene \text{ copy})} = Concentration \text{ of gene copy copy}/\mu L$$

(Equation 8)

Table 4-13 Gene copy concentration for *Methanosaeta* and *Methanosarcina*

Sample	Molecular weight of ds DNA (g/mole or	Copy number for (g/molecule or g/copy)	DNA concentration after PCR (ng/ μ L)	Working stock (1/100 dilution)	Concentration of plasmid DNA in working stock
<i>Methanosarcina</i>	167628.4	2.8×10^{-19}	378.1 ± 16.5	3.8	1.36×10^{10}
<i>Methanosaeta</i>	168246.8	2.8×10^{-19}	377.7 ± 7.2	3.8	1.35×10^{10}
Avagadro's Number: 6.02214×10^{23} ds: double-strand					

The precision of microbial quantification using qPCR relies on the assumption that the ASBR sample and the standard solutions share the same PCR efficiency. It is thus crucial to check the qPCR efficiencies in both standard solutions and ASBR samples. Quantitative standard curves were constructed using the plasmids that contained the partial-length 16S rRNA gene sequences from the representative strains of the target methanogenic groups as previously described (Shigematsu et al., 2003) (Section 3.4.9.1). After determining the concentration of gene copy for *Methanosaeta* and *Methanosarcina*, serial dilution step was ready to carry out the standard curves for both *Methanosaeta* and *Methanosarcina*. Serial dilutions were done to obtain the standard solutions to figure out the standard curves according to the study of Yılmaz et al. (2014) in logarithmic values and given in Table 4.14 below.

Table 4-14 Serial diluted Log Gene Copy/ μ L values for *Methanosarcina* and *Methanosaeta*

Log Gene Copy/ μ L for <i>Methanosarcina</i>	Log Gene Copy/ μ L for <i>Methanosaeta</i>
3.5	3.6
4.5	4.6
5.5	5.6
6.5	6.6
7.5	7.6

After preparing standard solutions with serial dilutions, qPCR analyses were run with the isolated DNA samples taken from ASBR at different operational periods in triplicate at the same time together with the standard solutions of *Methanosarcina* and *Methanosaeta*. The cycle threshold (CT) values, which is the cycle number of fluorescence generated within a reaction crosses the fluorescence threshold, were determined by plotting against the logarithm of *Methanosarcina* and *Methanosaeta* target gene copy concentrations. CT is inversely proportional to the original relative expression level of the gene of interest. The related CT and the concentration values were given in Table 4.15 and Table 4.16 for standard solutions of *Methanosarcina* and *Methanosaeta*. In addition, standard curves for each specie can be seen in Figure 4.9 and Figure 4.10

Table 4-15 CT and Log Gene Copy/ μ L values for standard curve of *Methanosarcina*

Log Gene Copy/ μ L	Average CT Value
3.5	34 \pm 0.6
4.5	31.4 \pm 0.1
5.5	27.8 \pm 0.1
6.5	24.5 \pm 0.1
7.5	21 \pm 0.2

Table 4-16 CT and Log Gene Copy/ μ L values for standard curve of *Methanosaeta*

Log Gene Copy/ μ L	Average CT Value
3.6	33.7 \pm 0.4
4.6	31.1 \pm 0.2
5.6	27.4 \pm 0.2
6.6	24 \pm 0.1
7.7	20.6 \pm 0

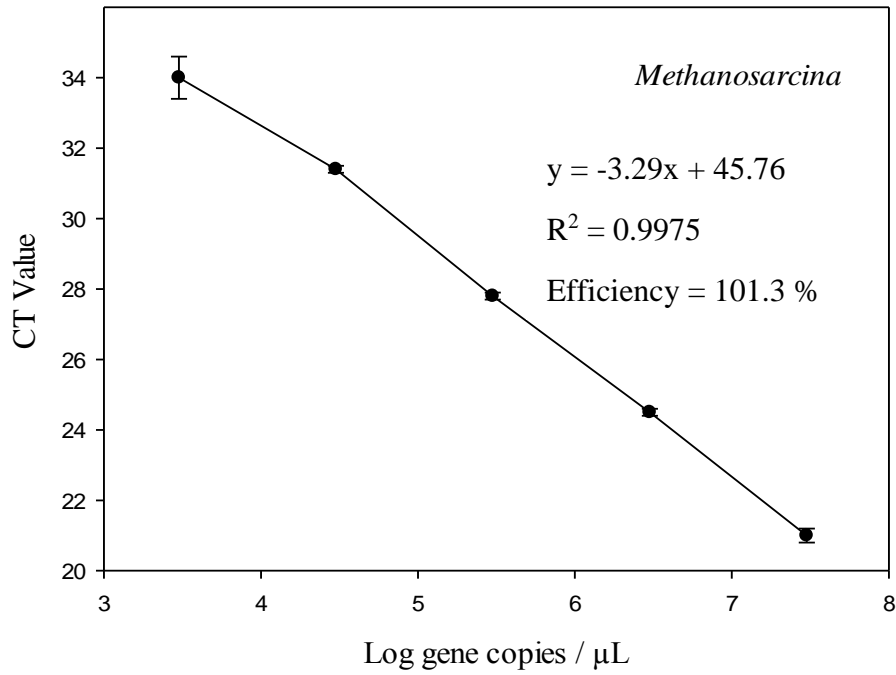


Figure 4-9 Standard curve for *Methanosarcina*

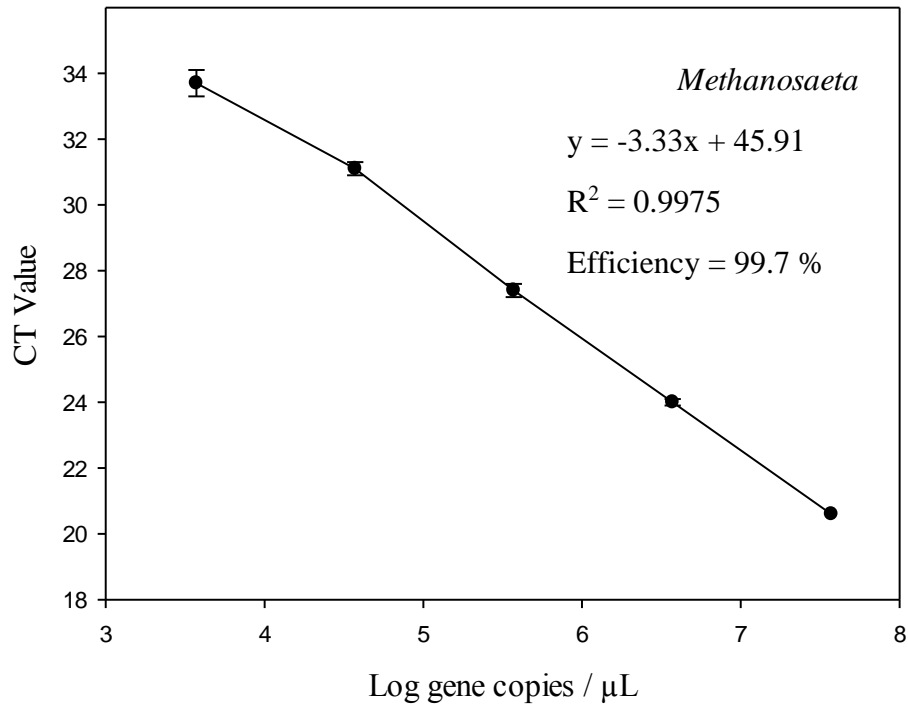


Figure 4-10 Standard curve for *Methanosaeta*

As it can be seen in Figure 4.9 and Figure 4.10 the resulting slopes, correlation coefficients, and efficiencies (Appendix E, Equation 9) were -3.33, 0.9975, 99.7% for *Methanosaeta*; and -3.27, 0.9975, 101.3% for *Methanosarcina*. In practice, a reliable standard curve should have an R^2 value of more than 0.95 and a slope between -3.0 and -3.9 corresponding to PCR efficiencies of 80–115 % (Yılmaz et al., 2014). Thus, these values are indicating high precision of qPCR analysis carried out in this study.

For getting reliable standard curves for *Methanosaeta* and *Methanosarcina*, qPCR results of isolated DNA samples of ASBR study could be discussed which were run with the standard solutions in the same qPCR batch analysis. But, before doing that discussion, an important information should be given about qPCR analysis of isolated DNA samples of ASBR. PCR inhibitors present in the environmental samples might affect the precision and accuracy of qPCR analysis. Thus, serial dilution is also useful for the ASBR samples to assess whether samples contain PCR inhibitors or not. For this reason, each sample was serially diluted to get the DNA concentration in the range of 0.5-0.7 (ng/μL) for *Methanosarcina* and 0.14-0.18 (ng/μL) for *Methanosaeta*. In this way, PCR inhibitors were eliminated and CT value of each sample were obtained

in the CT value range of each specie's standard curve. Therefore, the results of these samples could be analysed reliably. Serial dilution ratios of the isolated DNA samples of ASBR study were given below in Table 4.17.

Table 4-17 Serial dilution ratios of the isolated DNA samples of ASBR study for *Methanosarcina* and *Methanosaeta* qPCR analyses

Sample No	DNA conc. For quantification of <i>Methanosarcina</i> (ng/ μ L)	End of serial dilution ratio of main sample for quantification of <i>Methanosarcina</i>	DNA conc. for quantification of <i>Methanosaeta</i> (ng/ μ L)	End of serial dilution ratio of main sample for quantification of <i>Methanosaeta</i>
ASBR 1	0.55	1/200	0.14	1/800
ASBR 2	0.50	1/200	0.13	1/800
ASBR 3	0.55	1/100	0.14	1/400
ASBR 4	0.62	1/200	0.15	1/800
ASBR 5	0.55	1/60	0.14	1/240
ASBR 6	0.57	1/60	0.14	1/240
ASBR 7	0.73	1/200	0.18	1/800
ASBR 8	0.64	1/200	0.16	1/800

The resulting CT values and corresponding Gene Copy/ μ L were given for *Methanosarcina* and *Methanosaeta* in Table 4.18 and Table 4.19, respectively. The figures of qPCR analysis performed for both ASBR samples and standard solutions are given in Appendix F, G, H and I. The volume-based concentrations (gene copies/ μ L) were converted into the biomass-based concentration (copies/g VSS) using the VSS_{reaction} concentration of each ASBR biomass sample. Average VSS_{reaction} concentrations for the acclimation period and the other six periods are given in Appendix C.

Table 4-18 The resulting values of qPCR analysis of ASBR biomass sample for *Methanosaeta*

Sample No	Operation day	CT Value	Gene Copy/ μ L	VSS _{reaction} period of ASBR (g/L)	Gene Copy/g VSS	Average Methane Yield (mL CH ₄ /g COD _{added})
ASBR 1	26	23.7 \pm 0.1	3.7 \times 10 ⁹	3.8	9.8 \times 10 ¹⁴	120 \pm 11
ASBR 2	80 (end of acclimation Period)	23.5 \pm 0.1	4.2 \times 10 ⁹	4.5	9.5 \times 10 ¹⁴	363 \pm 14
ASBR 3	97 (end of Period I)	24.5 \pm 0.1	1.1 \times 10 ⁹	3.9	2.8 \times 10 ¹⁴	343 \pm 17
ASBR 4	107 (end of Period II)	24.8 \pm 0	1.8 \times 10 ⁹	5.5	3.2 \times 10 ¹⁴	338 \pm 19
ASBR 5	121 (end of Period III)	23.3 \pm 0.3	1.5 \times 10 ⁹	4.9	3.1 \times 10 ¹⁴	307 \pm 31
ASBR 6	136 (end of Period IV)	23.4 \pm 0.1	1.4 \times 10 ⁹	4.9	2.8 \times 10 ¹⁴	172 \pm 24
ASBR 7	147 (end of Period V)	25.4 \pm 0.1	1.2 \times 10 ⁹	5.3	2.2 \times 10 ¹⁴	188 \pm 22
ASBR 8	157 (end of Period VI)	25.2 \pm 0.1	1.3 \times 10 ⁹	4.2	3.2 \times 10 ¹⁴	297 \pm 14

Table 4-19 The resulting values of qPCR analysis of ASBR biomass sample for *Methanosarcina*

Sample No	Operation day	CT Value	Gene Copy/ μ L	VSS _{reaction} period for ASBR study (g/L)	Gene Copy/g VSS	Average Methane Yield (mL CH ₄ / g COD _{added})
ASBR 1	26	35.4 \pm 0.2	3.0 \times 10 ⁵	3.8	7.9 \times 10 ¹⁰	120 \pm 11
ASBR 2	80 (end of acclimation Period)	28.4 \pm 0.1	4.2 \times 10 ⁷	4.5	9.4 \times 10 ¹²	363 \pm 14
ASBR 3	97 (end of Period I)	27.8 \pm 0.3	3.2 \times 10 ⁷	3.9	8.4 \times 10 ¹²	343 \pm 17
ASBR 4	107 (end of Period II)	25.5 \pm 0.3	3.3 \times 10 ⁸	5.5	6.0 \times 10 ¹³	338 \pm 19
ASBR 5	117 (end of Period III)	24.2 \pm 0	2.5 \times 10 ⁸	4.9	5.1 \times 10 ¹³	307 \pm 31
ASBR 6	136 (end of Period IV)	24.2 \pm 0.3	2.5 \times 10 ⁸	4.9	5.1 \times 10 ¹³	172 \pm 24
ASBR 7	147 (end of Period V)	25.2 \pm 0.1	4.1 \times 10 ⁸	5.3	7.7 \times 10 ¹³	188 \pm 22
ASBR 8	157 (end of Period VI)	25.4 \pm 0.1	3.5 \times 10 ⁸	4.2	8.4 \times 10 ¹³	297 \pm 14

Although *Methanosarcina* species were not find in sequence analysis, qPCR results revealed the existence of *Methanosarcina* species in acclimation period and all HRT and SRT combinations. It should be noted that different archaeal primer sets were used in DGGE and qPCR analyses. In fact, in qPCR analyses, primers specific to *Methanosarcina* was used, while in DGGE analyses, primers archaeal specific were

used. Thus it can be said that detecting all archaeal species may not be possible by using any archaeal primer set.

While seen in the Table 4.18 and 4.19, 16S rRNA encoding gene copies of *Methanosaeta* ranged from 2.2×10^{14} to 9.8×10^{14} per gram of VSS, *Methanosarcina* specie ranged from 7.9×10^{10} to 8.4×10^{13} for the biomass samples of ASBR. Thus, it can be clearly said that *Methanosaeta* culture had the population domination over *Methanosarcina* in all the biomass samples of ASBR study. As it was mentioned before (Section 4.3.1), until the Day 45, methane yield was very low (<208 mL CH₄/g COD_{added}) when compared with the theoretical value which is 395 CH₄/g COD (Speece, 1996). As it can be seen in Tables 4.18 and 4.19, biomass sample ASBR 1 represents the microbial culture in the middle of this period (Day 26) and average gene copy numbers of *Methanosaeta* and *Methanosarcina* were 9.8×10^{14} and 7.9×10^{10} per gram VSS, respectively. 9.8×10^{14} gene copy/g VSS value is the highest value of the gene copy numbers of *Methanosaeta* for the whole ASBR operation (Table 4.18). 9.5×10^{14} gene copy of *Methanosaeta*/g VSS concentration was found at end of the acclimation period, where methane yield was 363 mL CH₄/g COD_{added} (Table 4.18). In addition, *Methanosaeta* concentration decreased and remained nearly constant, the Periods of I, II and III, in the range of 2.8×10^{14} - 3.2×10^{14} gene copy/g VSS where the methane yield was in the range of 327-347 mL CH₄/g COD_{added}. For the following periods (Periods IV, V and VI), *Methanosaeta* concentration in the reactor did not change significantly and varied in the range of 2.2×10^{14} - 3.2×10^{14} gene copy/g VSS (Table 4.18).

Methanosarcina concentration in the reactor was increased from 7.9×10^{10} to 9.4×10^{12} gene copy/g VSS by the end of the acclimation period from, while methane yield increased from 120 ± 11 to 363 ± 14 mL CH₄/g COD_{added} at the same time interval. The reason of the increase in the methane yield might be attributed to *Methanosarcina* concentration *Methanosarcina* concentration further increased to 6.0×10^{13} gene copy/g VSS end of the Period II (338 ± 19 mL CH₄/g COD_{added}). Following acclimation period *Methanosarcina* concentration increased ten fold and reached 6.0×10^{13} gene copy/g VSS by Day 107 (end of period II, Table 4.19). Yet, this ten fold increase did

not lead to any significant change in methane yield. Moreover, while gene copies of *Methanosarcina* ranged from 5.1×10^{13} (Period III) to 7.7×10^{13} (Period V) per gram of VSS, methane yield of the reactor decreased dramatically from 338 ± 31 to 188 ± 22 mL $\text{CH}_4/\text{g COD}_{\text{added}}$. Therefore, it was noticed that there was not a strong relationship between the concentration of *Methanosarcina species* and methane yield values of the ASBR Study. Similarly, for the last Period VI, methane yield of the reactor increased to 297 ± 14 mL $\text{CH}_4/\text{g COD}_{\text{added}}$, but gene copy of *Methanosarcina/g VSS* was around 8.4×10^{13} which was close to the concentration in Period V (7.7×10^{13} gene copy/g VSS).

To understand the effect of SRT and HRT on the change in the quantity of *Methanosaeta* and *Methanosarcina*, Figure 4.11 was given to analysis below. SRT was kept constant at 20 days and HRT decreased from 6 days to 0.7 days (Section 4.3.1, Table 4.6 and Figure 4.11) in a planned order from the beginning of the acclimation period (Day 45) to the end Period III (Day 121). In this time interval, *Methanosaeta* concentration in the reactor decreased from 9.5×10^{14} to 3.1×10^{14} gene copy/g VSS. In the same time interval, *Methanosarcina* concentration increased from 9.4×10^{12} to 5.1×10^{13} gene copy/g VSS (Table 4.18). On the other hand, SRT was decreased to 10 days with the beginning of the Period V (Day 137) till end of the Day 157 (end of the ASBR Study) HRT was increased from 0.7 days to 1.5 days (Table 4.19 and Figure 4.11). In this time interval (operational days of 118-157), *Methanosaeta* concentration changed between 2.2×10^{14} and 3.1×10^{14} gene copy/g VSS which was not significant (Table 4.18). Similarly *Methanosarcina* concentration varied between 5.1×10^{13} and 8.4×10^{13} gene copy/g VSS at the same time interval and not showed a significant change in concentration also.

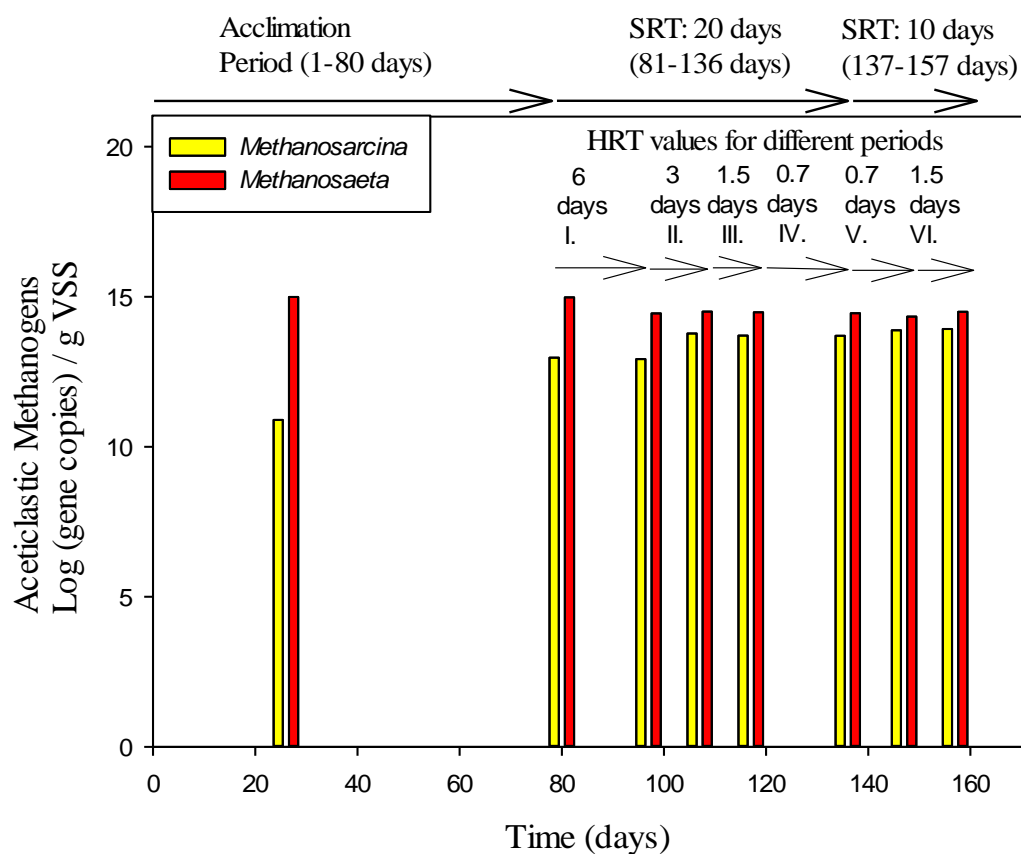


Figure 4-11 *Methanosarcina* and *Methanosaeta* densities in the biomass samples of ASBR study

Therefore it can be clearly said that *Methanosaeta* was the dominant specie in this study agreeing with the related studies stating that SRTs greater than 15 days favour *Methanosaeta* species in AD (Lee et al., 2011; Ma et al., 2013). Yet, *Methanosaeta* concentration tended to slightly decrease at 20 days of constant SRT, while *Methanosarcina* concentration increased at the same time interval in this study (Figure 4.11). Thus, another operational parameter's effect should be prioritized (HRT) for this study. Decrease in HRT from 6 days to 0.7 days (Days 45-121 and constant 20 days of SRT) resulted three fold decrease in *Methanosaeta* (9.5×10^{14} to 3.1×10^{14} gene copy/g VSS) and nearly five fold increase in *Methanosarcina* concentration (9.4×10^{12} to 5.1×10^{13} gene copy/g VSS) in the ASBR study (Table 4.18 and 4.19). Increase in *Methanosarcina* concentration with the decrease in HRT (6 to 0.7 days) in that time interval (Days 45-117) was also supported by the study of Lee et al. (2011). In addition, change in the SRT from 20 days to 10 days (Days 136-157) did not affect both

Methanosaeta and *Methanosarcina* concentration while HRT varied between 0.7 to 1.5 days (Table 4.18, 4.19 and Figure 4.11).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Methane production efficiency of single-phase and two-phase AD in batch reactors and ASBR were investigated in this thesis study. Also population dynamics and change in the quantity of acetoclastic methane-producers, namely, *Methanosaeta* and *Methanosarcina* of the second-phase of a two-phase ASBR were monitored under varying HRT and SRT conditions.

The starting point was to investigate the initial S/X_0 ratio effect on methane yield in Batch Reactor Set-1 operated with glucose as substrate. The highest methane yield was observed as 267 ± 5 mL CH_4/g COD_{added} with the batch reactor in which S/X_0 ratio was 1 g COD/g VSS. The results indicated the applicability of the anaerobic seed sludge and 1 g COD/g VSS as the optimum S/X_0 ratio for the following experiments.

In Batch Reactor Set-2, a clear methane production difference between single-phase and two-phase AD was observed. The results of Batch Reactor Set-2 are as follows;

- ✓ Batch Reactor Set-2 confirmed the advantage of the two-phase AD system in terms of methane production over single-phase AD system.
- ✓ The highest methane yield and methane percentage in biogas were observed as 344 ± 20 mL CH_4/g COD_{added} and 83% respectively in the second phase of the two-phase AD system.
- ✓ The use of the two-phase AD system instead of its single-phase counterpart resulted in 39% increase in methane yield when the substrate was sucrose.
- ✓ The higher methane yield and content in biogas of the second phase of two-phase AD system compared to its single-phase counterpart might be speculated as follows;

- When H₂ concentration is limited in the second-phase, formate+CO₂ pathway of hydrogenotrophic methanogenic archaea species might lead to the increase in methane percentage and yield.
- Homoacetogenic microorganisms might be carried with the effluent of dark fermentation reactors (first-phase of the two-phase systems). These microorganisms might have been responsible for consuming CO₂ as substrate and improve the methane content of the biogas.

In ASBR study, the optimum operational conditions leading to the highest methane production were determined among six different HRT and SRT combinations (0.7-6 days of HRT and 10-20 days of SRT). Moreover, DGGE and qPCR analyses threw a light on microbial population dynamics and relative dominance of *Methanosaeta* and *Methanosarcina species* in ASBR. The results of ASBR study are summarized as follows;

- ✓ The highest average methane yield was achieved at 20 days of SRT and 6 days of HRT as 343±17 mL CH₄/g COD_{added}.
- ✓ The highest average methane productivity was observed as 1794±279 mL CH₄/L/day, at an SRT and HRT of 10 and 0.7 days, respectively.
- ✓ Methane yield had directly proportional relationship with the HRT and no significant relationship with SRT within the values studied.
- ✓ While methane productivity increase at low HRT values (0.7 days), effluent sCOD and tVFA concentrations increase also.

According to the DGGE and qPCR analyses, the main operational conditions which has a significant effect on *Methanosaeta* and *Methanosarcina* concentration in the ASBR was HRT. The outcomes of DGGE and qPCR analyses are given below;

- ✓ According to the DGGE and sequence analyses, *Methanosaeta concilii* dominated the ASBR among the archaeal consortium during all HRT and SRT

combinations (10-20 days of SRT and 0.7 to 6 days of HRT). This was also verified with qPCR analyses.

- ✓ *Methanospirillum hungatei* a methanogen consuming H₂, CO₂ and formate for methane production was also detected at HRTs of 3 and 6 days. Therefore, existence of this specie might have increased the methane yield and percent in biogas. When HRT was decreased to values equal to and less than 1.5 days, the concentration of *Methanospirillum hungatei* was likely to decrease independent of the SRT values studied (10 or 20 days)
- ✓ *Clostridium saccharobutylicum* DSM 13864, *Clostridium acetobutylicum* and *Escherichia coli* str. K-12 substr. MG1655 were the bacterial species found in the ASBR.
- ✓ According to qPCR results, *Methanosaeta* concentration dominated ASBR during all HRT and SRT combinations as it was also expected regarding the results of DGGE and sequence analyses.
- ✓ Although *Methanosarcina* species were not found in sequence analysis, qPCR results revealed the existence of *Methanosarcina* species during whole operational time of varied HRT and SRT combinations. It should be noted that different archaeal primer sets were used in DGGE and qPCR analyses. In fact, in qPCR analyses, primers specific to *Methanosarcina* was used, while in DGGE analyses, archaeal specific primers were used. Thus it can be said that detecting all archaeal species may not be possible by using any archaeal primer set.
- ✓ While *Methanosaeta* concentration did not change significantly *Methanosarcina* concentration increased nearly a hundred fold in the acclimation period. At the same time interval, average methane yield increased from 120 to 363 mL CH₄/g COD_{added}. Therefore, increase in methane yield might be attributed to the increase in *Methanosarcina species*' concentration in the acclimation period.
- ✓ *Methanosarcina* and *Methanosaeta* concentration remained nearly constant, while passing through from Period III (20 days of SRT and 1.5 days of HRT) to Period IV (20 days of SRT and 0.7 days of HRT). However, average methane yield decreased from 307±31 mL CH₄/g COD_{added} to 172±24 mL

CH₄/g COD_{added}. In addition, no wash-out was observed. Therefore, concentrations of both of these species could not explain the possible archaeal population shift behind the decreasing methane yield. As a result, decreased methane yield might be attributed to hydrogenotrophic archaea species which could not be detected with the primers used in DGGE and qPCR performed in this study.

- ✓ Decrease in HRT from 6 days to 0.7 days resulted in three fold decrease in *Methanosaeta* and nearly five fold increase in *Methanosarcina* concentration at a constant SRT of 20 days.
- ✓ Decrease in SRT from 20 days to 10 days did not lead to any significant change in the concentration of *Methanosaeta* and *Methanosarcina* in the ASBR.

To better understand the relationship between the methane yield and microbial population dynamics of AD systems, more detailed DGGE and qPCR analyses may be done with different and specific archaeal primer sets. Thus, the species which might not be detected in this study can be observed including hydrogenotrophic archaea species. In this respect, the species that are responsible for the decrease in methane yield can be detected. Such a result might be useful to optimize the operational conditions and to achieve a properly working AD.

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APPENDIX A

CALIBRATION CURVES FOR BIOGAS COMPOSITION ANALYSIS

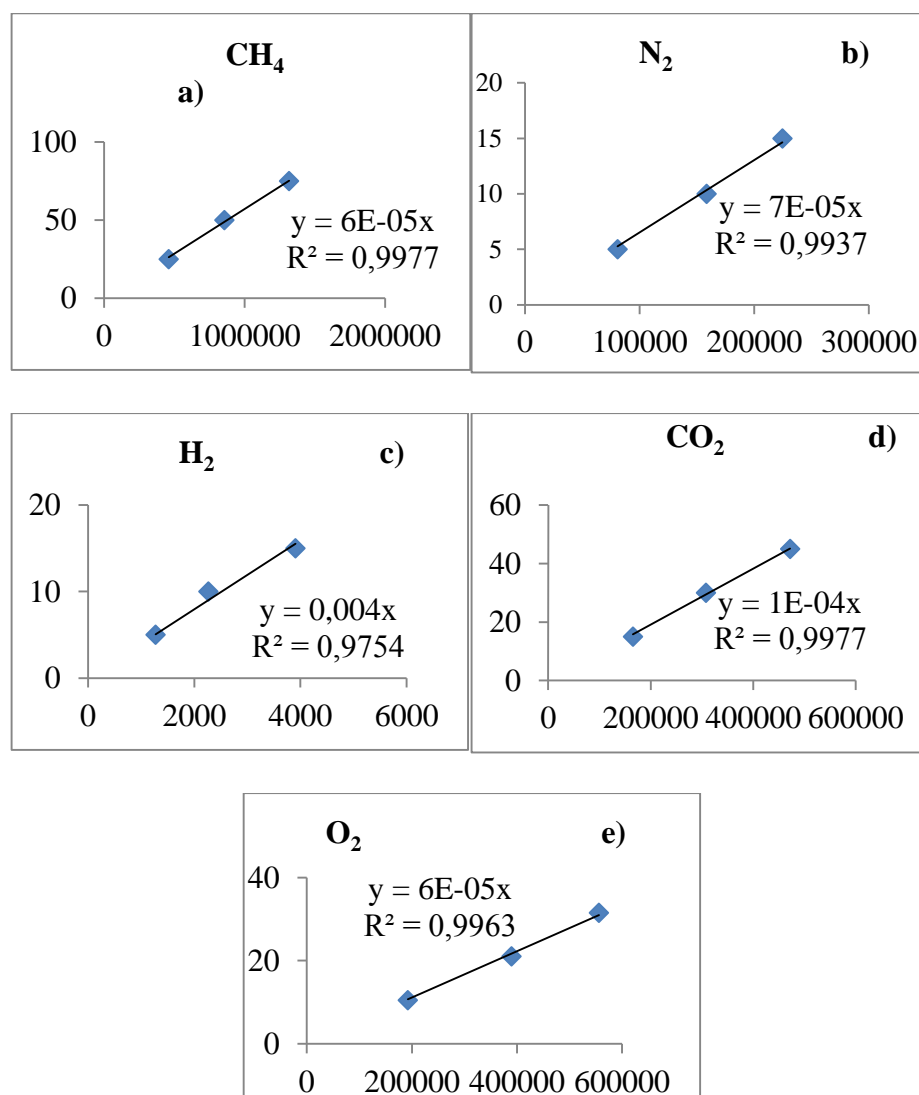


Figure A-1 Calibration curves of a) CH₄, b) N₂, c) H₂, d) CO₂, e) O₂ (for headspace gas analyses of Batch Reactor Set-1, Batch Reactor Set-1 and ASBR study)

APPENDIX B

CALIBRATION CURVES FOR VOLATILE FATTY ACIDS ANALYSIS

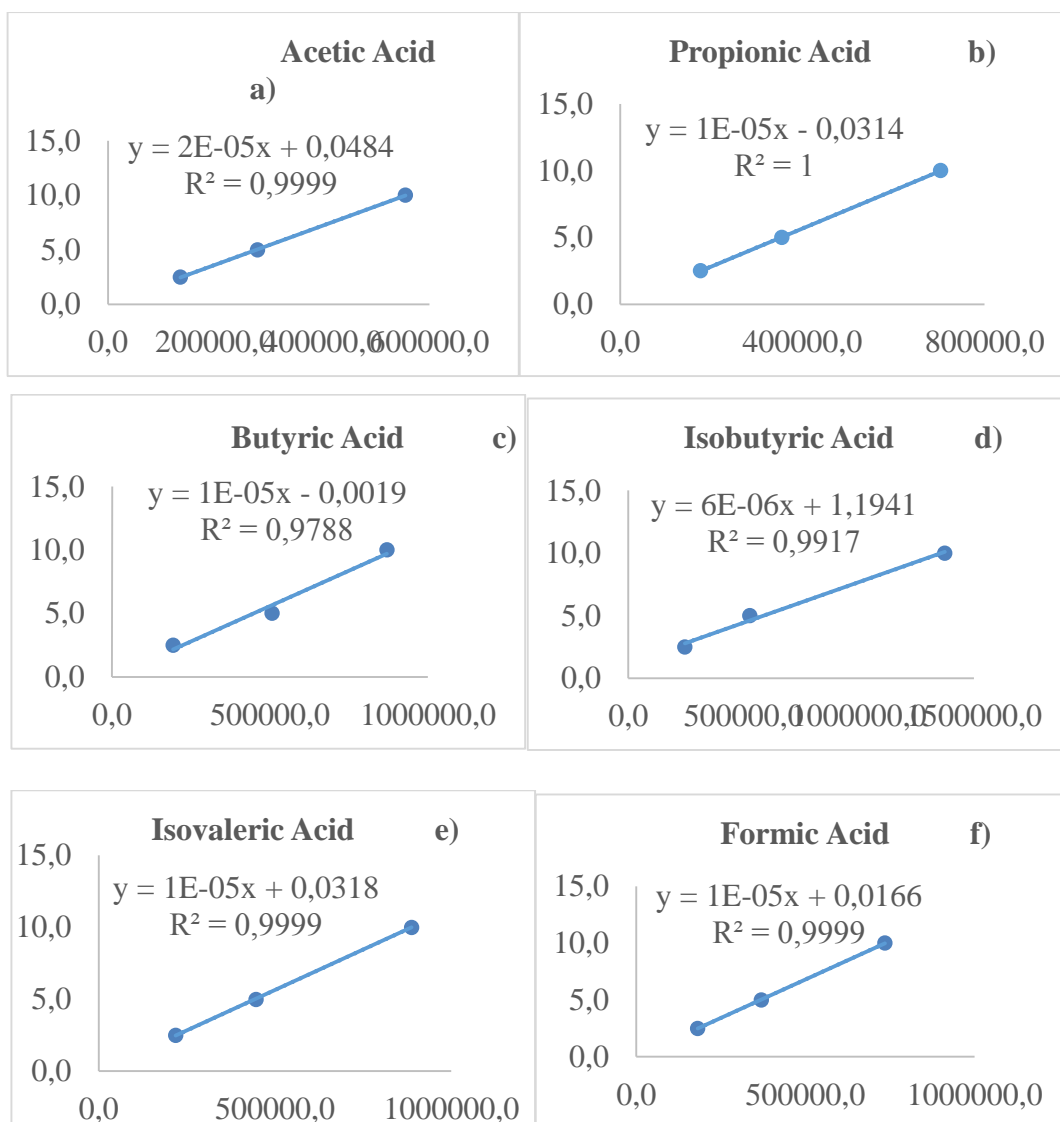


Figure B-1 HPLC calibration curves used for VFAs analyses a) Acetic acid, b) Propionic acid, c) Butyric acid, d) Isobutyric acid, e) Isovaleric acid, f) Formic acid

APPENDIX C

VSS REACTION CONCENTRATIONS FOR ASBR STUDY

Table C-1 VSS concentrations in the reaction period and the effluent of ASBR study

Operational time interval	VSS _{reaction} (mg/L)	VSS _{effluent} (mg/L)
Acclimation period (Days 45-80)	4730±787	621±311
Period I	4574±309	583±126
Period II	4365±343	180±52
Period III	4971±291	239±177
Period IV	4295±256	305±199
Period V	4975±209	390±74
Period VI	4550±312	654±67

APPENDIX D

VOLATILE FATTY ACID CONCENTRATION OF THE PERIODICAL EFFLUENTS OF ASBR STUDY

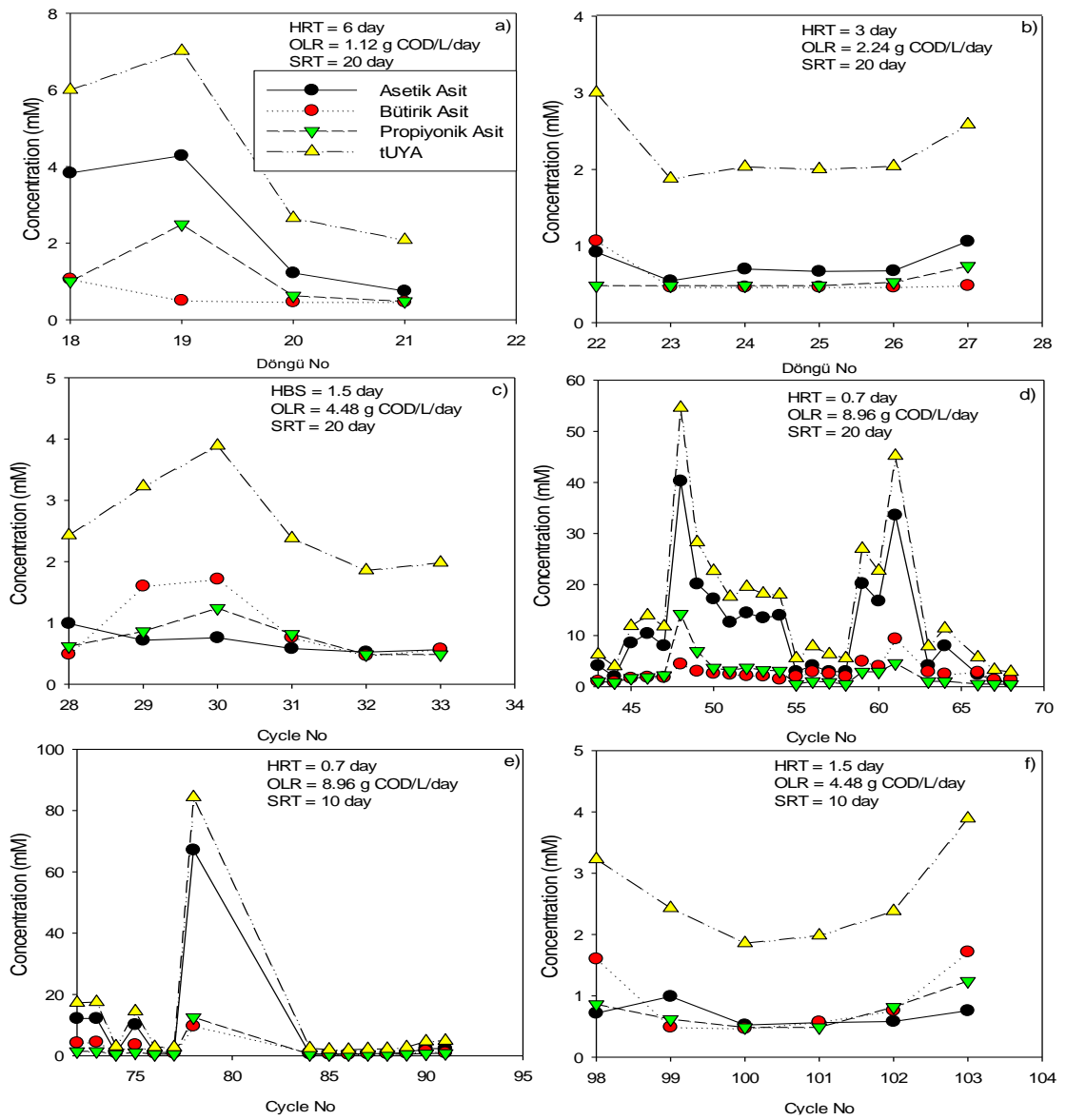


Figure D-1 Volatile Fatty Acid Composition of the Periodical Effluents of ASBR Study

APPENDIX E

EFFICIENCY DESCRIPTION OF qPCR

The qPCR efficiency can be defined as the increase in amplicon per cycle. The formula used to determine the qPCR efficiency is given in Equation 9.

$$\text{Efficiency (E)\%} = [(10^{-1/\text{slope}}) - 1] * 100$$

(Equation 9)

APPENDIX F

qPCR STANDARD CURVE ANALYSIS PICTURE FOR *METHANOSARCINA sp.*

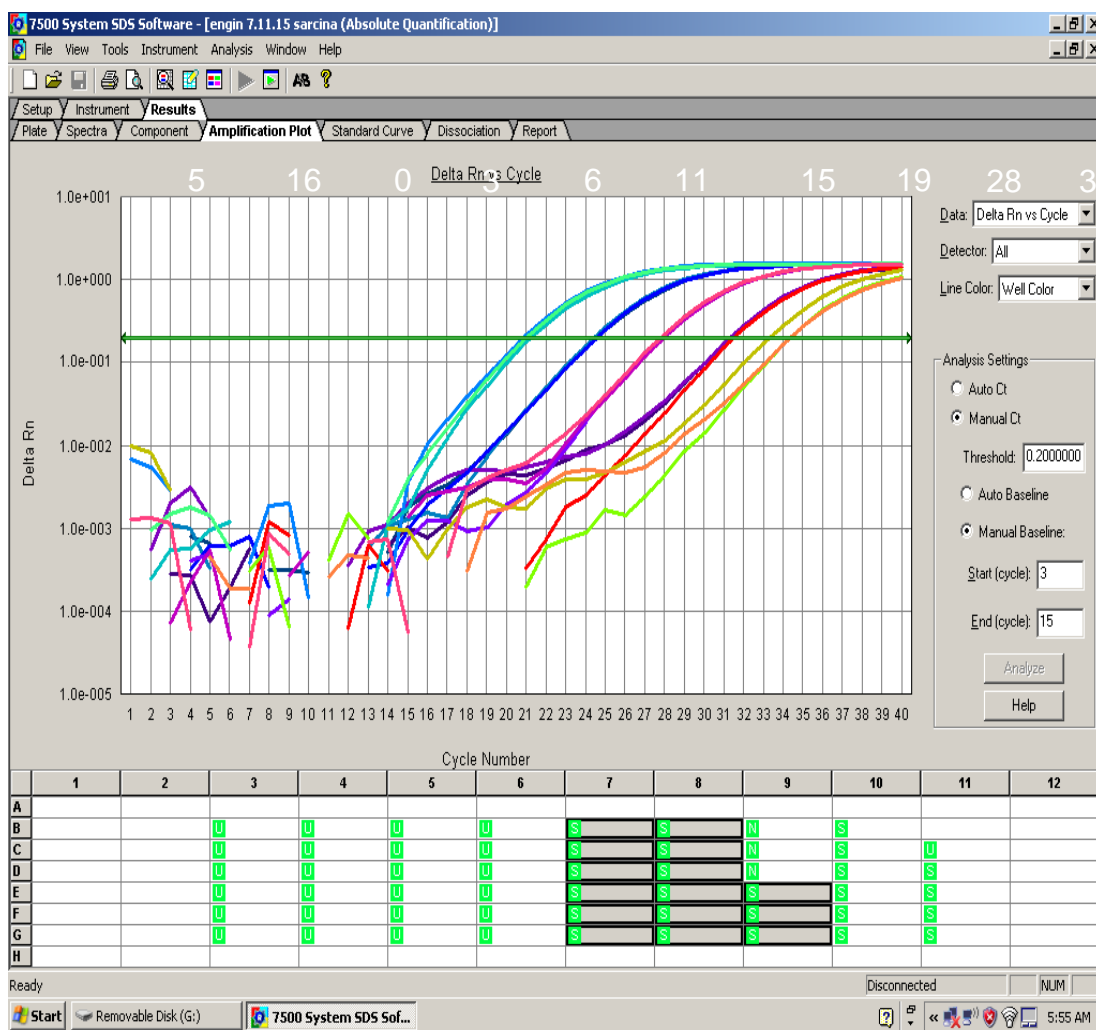


Figure F-1 qPCR standard curve analysis picture for *methanosarcina sp.*

APPENDIX G

qPCR STANDARD CURVE ANALYSIS PICTURE FOR *METHANOSAETA sp.*

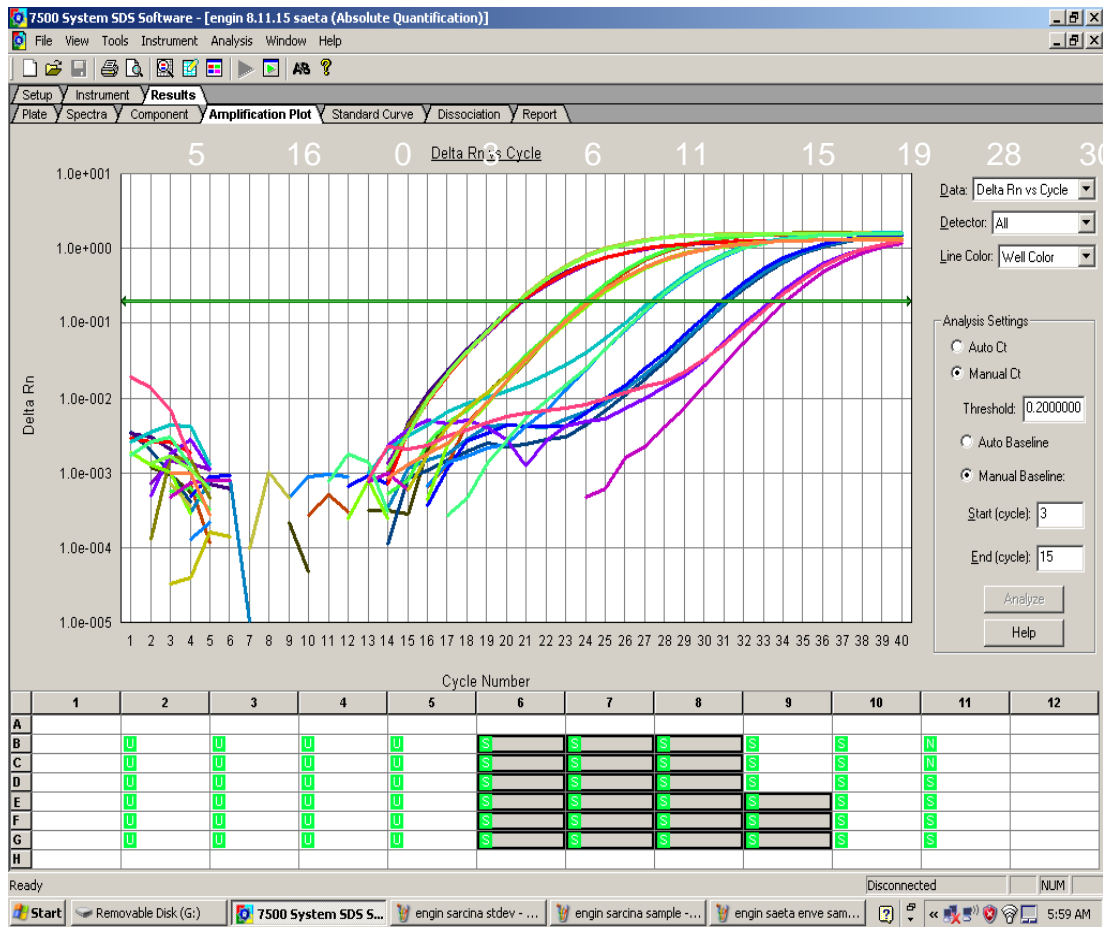


Figure G-1 qPCR standard curve analysis picture for *methanosaeta sp.*

APPENDIX H

qPCR ANALYSIS OF ASBR SAMPLES PICTURE FOR *METHANOSARCINA sp.*

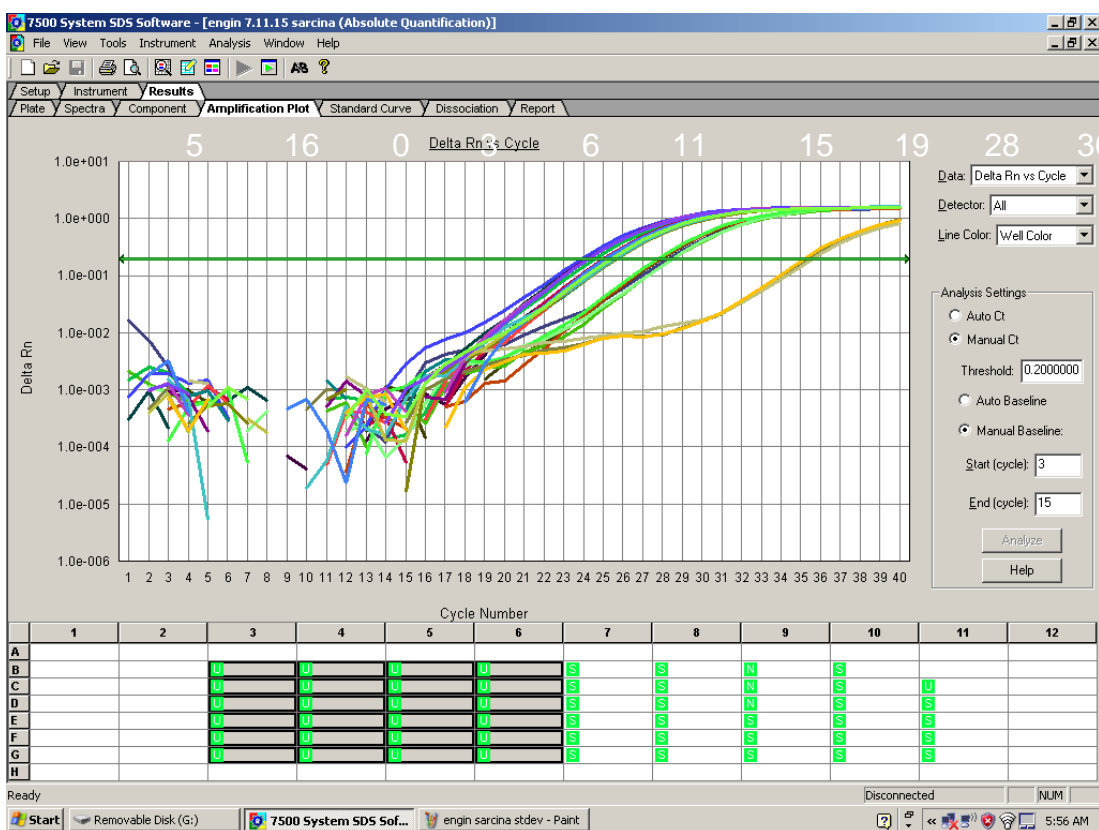


Figure H-1 qPCR analysis of ASBR samples picture for *methanosarcina sp.*

APPENDIX I

qPCR ANALYSIS OF ASBR SAMPLES PICTURE FOR *METHANOSAETA sp.*

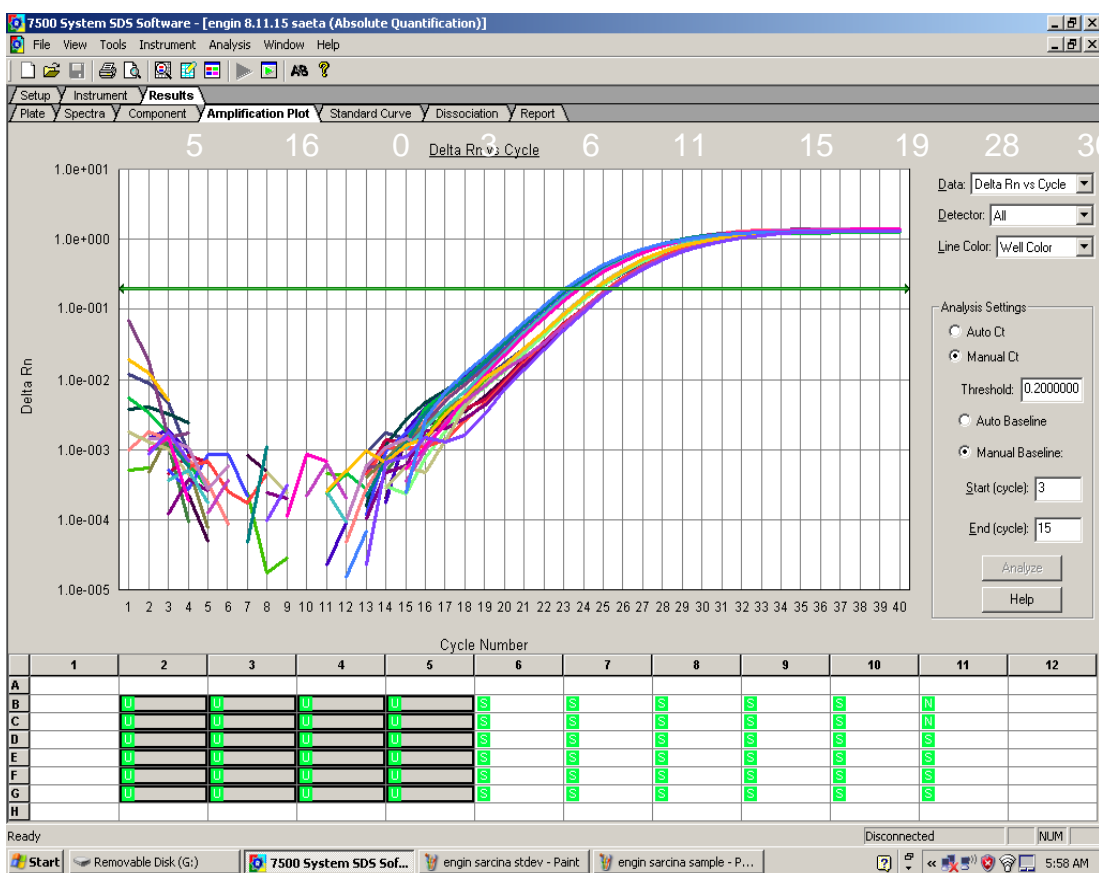


Figure I-1 qPCR analysis of ASBR samples picture for *methanosaeta sp.*