ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IMPACTS OF OXYTETRACYCLINE ON PERFORMANCE OF THERMOPHILIC ANAEROBIC MANURE DIGESTERS AND ACTIVE MICROBIAL POPULATION

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

OKSİTETRASİKLİNİN TERMOFİLİK ANAEROBİK DIŞKI ÇÜRÜTÜCÜLERİNİN PERFORMANSI VE AKTİF MİKROBİYAL POPÜLASYON ÜZERİNE ETKİLERİ

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Fulya Süzen, a M.Sc. student of ITU Graduate School of Science Engineering and Technology student ID **501081819**, successfully defended the thesis entitled "**IMPACTS OF OXYTETRACYCLINE ON PERFORMANCE OF THERMOPHILIC ANAEROBIC MANURE DIGESTERS AND ACTIVE MICROBIAL POPULATION**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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(Biologist)

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ABBREVIATIONS

AD : Anaerobic Digestion
ATP : Adenosine Triphosphate
CHP : Combined Heat and Power Station
CSTR : Continious Stirred Tank Reactor
CTC : Chlorotetracyline
DAPI : 4,6-Diamine Phenylindol
DGGE : Denaturating Gradient Gel Electrophoresis
DNA : Deoxyribonucleic acid
EDTA : Ethylenediaminetetraacetic Acid
FISH : Fluorescent *in situ* Hybridization
GHG : Green Hause Gasses
HRT : Hydraulic Retention Time
OTC : Oxytetracycline
PCR : Polymerase Chain Reaction
PBS : Phosphate Buffer Solution
PFA : Paraformaldehyde
Q-PCR : Quantative Polymerase Chain Reaction
rDNA : Ribosomal DNA
RNA : Riboxynucleic Acid
rRNA : Ribosomal RNA
SRT : Solid Retention Time
SSCP : Single Strand Conformation Polymorphism
TC : Tetracycline
TPAD : Temperature-Phased Anaerobic Digestion
TRFLP : Terminal Restriction Fragment Length Polymorphism
TS : Total Solids
TVS : Total Volatile Solids
VA : Veterinary Antibiotic
VFA : Volatile Fatty Acids

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IMPACTS OF OXYTETRACYCLINE ON PERFORMANCE OF THERMOPHILIC ANAEROBIC MANURE DIGESTERSAND ACTIVE MICROBIAL POPULATION

SUMMARY

Antibiotics used in veterinary practice are poorly metabolized and excreted within manure; hence posing a threat to biogas production from substrates as such, for they are possibly detrimental to microflora of anaerobic digestion processes. In this study, effects of a commonly used veterinary antibiotic, OTC (oxytetracycline), on biogas production in thermophilic anaerobic digestion of cattle manure was investigated. Effects of changing operational parameters such as mixing rate and solid content on biogas yield,active microbial population and eliminaton of oxytetracycline was also studied. In this study, two sets (Set1 and Set2) each containing four digesters (D1, D3, D4 and D5) were setwith both blank (as control) and medicated cow manure. Set1 was maintained under thermophilic conditions (55 ^{0}C±1), with 90 rpm mixing rate and two different total solid content (5-5.5 % and 7.5-8 % TS content). Set2 was similar to Set1 except mixing rate which was 120 rpm. Seed from a lab-scale manure digesters was added at a ratio of 1:10.HRTs were set to 20 days in both Set1 and Set2 and samples were taken for analytical, molecular and physicochemical analysisfor every 5 days. Digesters were monitored for biogas production, total solid (TS) reduction, biogas and volatile fatty acid concentrations.In *situ* Hybridization (FISH) and RNA based Q-PCR were used to monitor of active microbial populationsdynamics.

In this study, maximum biogas yields of 132-134 L/kg TVS were found in control digesters, in Set1 and Set2, respectively. Results showed that 90 and 120 rpm mixing rates did not effect biogas production performance significantly. Inhibitions in cumulativebiogas production after 20 days were between 10-18% for the digesters containing OTC concentrations of 1.5-4.7 mg/L. Maximum TS reduction reached to 30% at the end of 20 days digestion period. Although acetic and propionic acids were dominantly detected in slurries; their concentrations were not at inhibitory level at the end of 20 days. OTC concentration showed a decreasing trend during operational time and half life of OTC was evaluated to be 14 days for thermophilic anaerobic digestion.

Dynamics of active populations were investigated by molecular methods such as FISH and RNA based Q-PCR. According to FISH results, active bacterial population decreased in all digesters at the end of operation whereas active *Methanomicrobiales* population increased. *Methanobacteriales* was most abundant methanogen in all digesters as reaching percentage of 48%. *Methanobacterialesspp*. also decreased in OTC containing digesters.Active *Methanosarcina* population oscillated in digesters

showing no spesific trend. According to Q-PCR results, bacterial, *Methanobacteriales, Methanomicrobiales, Methanosaeta ve Methanosarcinales* gene copy numbers decreased with operational time in all digesters in Set1. *Methanobacteriales* and *Methanosarcinales* gene copy numbers decreased in Set1 digesters where mixing rate was 90 rpm whereas gene copy numbers increased in Set2 digesters where 120 rpm were maintained. Bacterial, *Methanomicrobiales*, *Methanosaeta* gene copy numbers decreased in all digesters during operational time. *Methanosaeta* gene copy numbers were low and did not show significant decreasing trend within operational time. Both activity and gene copy number of *Methanosaeta spp.* was low in number indigesters suggesting methanogenesis was performed mainly by *Methanosarcinalesspp*. and hydrogenotrophic archaea like *Methanobacteriales and Methanomicrobiales.*

OKSİTETRASİKLİNİN TERMOFİLİK ANAEROBİK DIŞKI ÇÜRÜTÜCÜLERİNİN PERFORMANSI VE AKTİF MİKROBİYAL POPÜLASYON ÜZERİNE ETKİLERİ

ÖZET

Son yıllarda hızla gelişen endüstri ve nüfus artışıyla birlikte bütün dünyada enerji ihtiyacı da hızla artmaktadır. Enerji kaynağı olarak fosil yakıtların fazlaca tüketilmesi sera gazı etkisi yaratması nedeniyle iklim değişikliğine sebep olmaktadır. Bu kaynakların çevre üzerinde yarattığı olumsuz etkileri minimum seviyeye indirgemek için alternatif ve çevre dostu enerji kaynakları kullanılması öngörülmektedir. Bu anlamda, anaerobik çürütme yoluyla biyogaz üretimi yenilenebilir enerji üretimi açısından diğer enerji formlarına göre önemli avantajlar sunmaktadır. Bu süreç, atmosferdeki sera gazı (SG) emisyonlarını azaltabilmekte ve bu prosesin son ürünü olan digestate bitkiler için gübre olarak yeniden kullanılabilmektedir. Bu nedenlerle, anaerobik çürütme prosesi biyoenerji üretimi için en tasarruflu ve çevreye faydalı teknolojilerden biri olarak kabul edilmektedir.

Organik atıkların herhangi bir türü anaerobik çürütme işlemi için substrat olarak kullanılabilmektedir. Elde edilmesi son derece kolay olan hayvansal atıklar (hayvan dışkısı) en yaygın olarak kullanılan substratların başında yer almaktadır. Diğer taraftan, bu atıkların kullanılması anaerobik çürütücü sistemlerinde bazı sorunlar yaratabilmektedir. Veteriner hekimliğinde kullanılan antibiyotikler, zayıf metabolize olma özellikleriyle, vücuttan gübre olarak atılarak hayvan dışkısında sıklıkla tespit edilmişlerdir. Bu antibiyotikler havasız çürütme sistemlerindeki hassas mikroflaraya zararlı olduklarından, hayvan gübresinin biyogaz üretiminde substrat olarak kullanılması sırasında problem teşkil edebilirler.

Ayrıca, antibiyotik içeren hayvan atıkları toprakla şartlandırıldığında çevre üzerinde bu antibiyotik kalıntılarının potansiyel etkisi dikkate alınması gereken önemli bir diğer husus haline gelmektedir. Antibiyotiklerin sık kullanımı mikroorganizmaların antibiyotiklere karşı direnç geliştirmeleri ile ilgili sorunları önemli ölçüde arttırmaktadır. Bunlara ek olarak, OTC hayvanların sindirim sistemlerinde indirgenerek metabolitlerine ayrışmaktadır. Oluşan bu metabolitler mikrobiyal popülasyon üzerine etki eden inhibitörlerdir. Orijinal molekül tarafından üretilen inhibisyondan ziyade bu antibiyotik metabolitleri bakteriyel faaliyetler üzerine inhibisyon etkisi yaratmaktadır.

Bu maddelerin biyogaz üretimi üzerindeki etkileri mühendislik açısından incelenmiş olmasına rağmen, mikrobiyal popülasyon üzerindeki olası etkileri ile ilgili çalışmalar oldukça azdır. Bu nedenle, bu çalışmanın amacı yaygın olarak kullanılan bir veteriner antibiyotiği olan OTC'nin, aktif mikrobiyal popülasyon üzerine ve

termofilik gübre çürütücülerdeki biyogaz üretimi üzerine olan etkilerinin değerlendirmesidir.

Bu çalışmada, sık kullanılan bir veteriner antibiyotiği olan oksitetrasiklinin (OTC), intramusküler enjeksiyon yoluyla aşılanmış olan büyükbaş hayvanın gübresinin termofilik havasız çürütücülerinde biyogaz ve metan üretimleri ile mikrobiyal populasyon üzerine olan etkisi farklı katı oranları ve karıştırma hızları denenerek gözlemlenmiştir. Çalışma doğrultusunda, Set1 ve Set2 olmak üzere iki deney seti kurulmuştur ve bu setlerin her biri 2 tane kontrol ve 2 tane de OTC içeren olmak üzere 4 (D1, D3, D4 and D5) reaktör içermektedir. D1 ve D4 kontrol (OTC içermeyen), D3 ve D5 ise OTC içeren reaktörlerdir. Set1, termofilik koşullarda (55 ± 1^0C), 90 rpm karıştırma hızında ve farklı katı oranlarıyla (5-5.5 %TS and 7.5-8 %TS) kurulmuştur. Set2, sadece Set1'den farklı olarak 120 rpm karıştırma hızıyla kurulmuştur diğer koşullar Set1 ile aynı olacak şekilde kurulmuştur. Bu çalışmada, mikroorganizmaların yüksek sıcaklığa adapte olabilmelerini sağlamak adına sıcaklık 1. gün 37 °C, 2. gün 40 °C, 3. gün 45 °C, 4. gün 50 °C ve 5. gün 55 °C olacak şekilde kademeli olarak ayarlanmıştır.

Termofilik çürütücülerin mezofilik çürütücülere kıyasla, yüksek metabolizma hızı, prosesin daha hızlı olması ve daha yüksek verimde biyogaz üretimi, düşük hidrolik bekleme sürelerinin olması, daha yüksek organik yükleme oranları ile beslenme yeteneği ve patojenlerin daha yüksek verimle ölmelerinin sağlanması gibi birçok avantajı bulunmaktadır. Ayrıca, bu çalışmaya göre, termofilik koşullarda mezofiliğe oranla daha yüksek metan oranı bulunmuştur ve OTC'nin yarı ömrü de daha düşük olarak saptanmıştır.

Bu çalışma kapsamında, 3.5 yaşındaki dişi Holstein ırkı, 440 kg vücut ağırlığındaki süt ineğinin vücudunun her iki tarafına, musculus semitendinosus ve musculus semimembranosus kaslarına, eşit miktarda ve standart uygulama dozunda 50 ml solusyon (20 mg/kg) oksitetrasiklin (OTC) enjekte edilmiştir. 5 gün boyunca her 24 saatte bir hayvandan dışkı örnekleri toplanmış ve homojen bir numune elde etmek için bu 5 numune karıştırılmıştır ve bu karışım deney boyunca anaerobik çürütücüler için substrat olarak kullanılmıştır. Bu çalışmada, çürütücülere laboratuvar ölçekli dışkı çürütücülerinden alınan aşı çamuru 1:10 oranında eklenmiştir. Her iki set için de hidrolik bekletme süresi (HRT) 20 gün olarak belirlenmiştir ve her 5 günde bir analitik, moleküler ve fizikokimyasal analizler için numune alınmıştır. Çürütücülerdeki biyogaz üretimi, TS giderimi, biyogaz ve VFA konsantrasyonları izlenmiştir. Fluorescent in *situ* Hybridization (FISH) and RNA tabanlı Q-PCR yöntemleri kullanılarak mikrobiyal populasyonun çeşitliliğine bakılmıştır.

Bu çalışmada, Set 1 ve Set 2'de maksimum biyogaz verimi 132-134 L/kgTVS olarak bulunmuştur. Sonuçlar, 90 ve 120 rpm karıştırma hızlarınnın biyogaz verimi üzerine önemli bir etki yaratmadığını göstermiştir. 20 günün sonunda, 1,5-4,7 mg/L değerler aralığındaki OTC konsantrasyonunun toplam biyogaz üretimi üzerine inhibisyonu %10-18 oranında olmuştur. 20 günlük işletme sonunda maksimum TS giderimi %30 oranında bulunmuştur. Asetik ve propiyonik asit baskın olarak gözlenmekle beraber inhibisyon etkisi yaratabilecek konsantrasyonlarda VFA birikimi gözlenmemiştir. İşletme süresi boyunca OTC azalma eğilimi göstermiştir ve termofilik anaerobik çürütücülerde yarı ömrü 14 gün olarak hesaplanmıştır.

Aktif populasyon dinamiği FISH ve Q-PCR gibi moleküler yöntemler kullanılarak incelenmiştir. FISH sonuçlarına göre, aktif bakteri populasyonu işletmenin sonunda bütün çürütücülerde azalırken aktif *Methanomicrobiales* populasyonu yükselmiştir. Bütün çürütücülerde en fazla bulunan metanojen (%48 oranında) *Methanobacteriales*

olmuştur.Ayrıca *Methanobacteriales spp.* kontrol çürütücülerinde değişme göstermemekle birlikte OTC içeren çürütücülerde azalma göstermiştir. Aktif *Methanosarcina* populasyonu dalgalanmalar göstermiştir ve spesifik bir trend izlenmemektedir.

Q-PCR sonuçlarına göre, Set 1 çürütücülerinde bakteriyal, *Methanobacteriales, Methanomicrobiales, Methanosaeta ve Methanosarcinales* gen kopya sayısı zaman içinde azalmıştır. *Methanobacteriales* and *Methanosarcinales* gen kopya sayısı 90 rpm karıştırma hızıyla işletilen Set 1'de azalırken 120 rpm karıştırma hızıyla işletilen Set 2'de artma göstermektedir. Bakteriyal, *Methanomicrobiales* and *Methanosaeta* gen kopya sayıları bütün çürütücülerde azalmıştır. *Methanosaeta* gen kopya sayısı oldukça düşük konsantrasyonlarda tespit edilmiştir ve zaman içinde önemli bir azalma göstermemiştir. *Methanosaeta* türlerinin aktivite ve gen kopya sayıları setlerin bütün çürütücülerinde düşük konsantrasyonlarda gözlenmiştir ve bu da metan oluşumunun özellikle *Methanosarcinales* türleri ve hidrojenotrofik metanojenler grubundaki *Methanobacteriales* ve *Methanomicrobiales* türleri tarafından gerçekleştirildiğini göstermektedir.

1.INTRODUCTION

With the industrial developments and population increase in the world energy consumption has increased (Hassan, 2003).Currently, 88% of the world‘s energy demand is provided by fossil fuels. Extensive use of fosil fuels has caused in a series of environmental problems, including local air pollution, acid rain, the threat of climate changes and discharges to soil and water (Palm et al., 1999). Over consumption of these sources have led authorities to search for alternative and environmentally friendly energy sources (Weiland, 2010) to prevent the climate change which occurs due to the greenhouse effect (El-Mashad et al., 2004). The production of biogas through anaerobic digestion presents significant advantages over other forms of renewable energy production. This process can reduce the greenhouse gas (GHG) emissions to the atmosphere and the digestate can be reused as fertilizer for the crops. That‘s why, it is considered as one of the most energy-efficient and environmentally beneficial technology for bioenergy production (Weiland, 2010).

Biogas production from agricultural originated waste is very important used one in Europe and in the world. Germany is the most biogas producing country in Europe, having 4000 agricultural biogas plants operated at the end of 2007 (Weiland, 2010). This kind of extent utilization of biogas may provide the additional of future energy need and bring independence from fossil fuels. Manure can be considered as one of the most important substrate for anaerobic digestion because although it has lower methane value compared to energy crops, it is easier to obtain (Cavinato et al., 2010)and has lower production costs (Walla and Schneeberger, 2005). Animal manure is also a very rich source by means of organic matter. Especially in countries where livestock farming is extensively applied, like Turkey, biogas production from manure is likely to bring high economic benefits.

Unfortunately, despite all the benefits of agriculture originated wastes used for biogas production, some substances such as veterinary antibiotics used in animal

husbandry cause a problem in digester systems. Their low metabolisation is the main problem with the veterinary antibiotics (Sarmah et al., 2006; Karcı et al., 2009). Type and amount of antibiotics given to animals are thought to have an effect on the animal manure. The fraction of antibiotics in the manure may generate a toxic matter and inhibit the digestion step (Massé et al., 2000).

Tetracyclines are known to be the most widely used veterinary antibiotics in the world. They exhibit broad range antimicrobial activity against a variety of pathogen bacteria and used in human therapy and livestock industry (Mellon, 2001; Thrile-Bruhn, 2003; Arıkan 2006). Metabolisation and absorption of tetracyclines by the organisms are known to be in very small portions so that, most of the tetracyclines can be detected in the excreta without any change (Sarmah et al., 2006). As a member of tetracyclines family, oxytetracycline is a common antibiotic used in livestock animals due to the broad spectrum of activity and low cost.

In additon to inhibitory substances like antibiotics, there are some operational conditions that effect the anaerobic digestion system and biogas production yield in a variety of ways. Temperature is one of the most important factor which effects the microbial activity and the performance of the anaerobic digestion process significantly in many ways such as ionization equilibrium, solubility of substrates, substrate removal rate and other constants such as specific growth rate, decay biomass yield, and half saturation constant (Vindis et al.,2009). One of the factors that affects the biogas production yield and anaerobic digestion performance is mixing (Gerardi, 2003). Mixing enhances substrate contact with the microbial community, ensures the uniformity of pH and temperature, prevents stratification and scum accumulation (Ong et al., 2002; El-Mashad and Zhang, 2010), facilitates the removal of biogas from the digestant, aids the reduction in particle size and provides rapid dispersion of any toxic materials coming in the tank as it means toxicity minimizing (Hassan, 2003; Karim et al. 2005; Hoffmann et al., 2008; Kaparaju et al., 2008; Pandey 2011). Another operational factor is organic loading rate (OLR) or solid content which effects the performance of anaerobic digestion such as start-up performance, retention time, biogas production yield, and conversion ratio of total and volatile solids. Generally, with increasing the solid loading rate, the total CH_4 yield increases, while efficiencies of both the solid reduction and the solid conversion to CH_4 gas decrease (Wu et al., 2009; Li et al., 2011).

In order to benefit at the highest level from this technology, insights of the process should be well understood. Although the general processes occurring in anaerobic biological digesters, such as hydrolysis, acidogenesis, acetogenesis, methanogenesis, are well known, the complex microbial ecology of the organic substrate, symbiotic relationships, the effect of microbial diversity on performance of the anaerobic digestion systems are still needed to be enlightened. Understanding of the microbial ecology of the anaerobic digesters plays an important role in the controlling and operation of the biogas systems (Narihiro and Sekiguchi, 2007; Kim et al., 2010).

Most of the studies which are related to effect of the oxytetracycline on the anaerobic digestion of manure were based on the gas outputs. However, identification of the microbial community and effects of antibiotics on them should be studied. The main problem in identification of community was unavailability of the microorganisms to be cultured from the gastrointestinal track or feces with classical *in vitro* methods. Since the growth conditions are not defined. However, recent molecular methods, such as polymerase chain reaction (PCR) based amplification of 16S rDNA have significantly expanded the availability of identification from the fecal materials. Experiments were successful in the identification of microbial species in human feces (Matsuki et al., 1999). These methods should be applied in the determination and identification of the microbial community in the manure.

Effect of antibiotics and some operational factors such as mixing rateandsolid content on anaerobic digestion processes have been studied many times, however the main focus of these studies have generally been physical and chemical aspects; microbiological data have unfortunately been overlooked. Therefore, in this study, the inhibitory effect of oxytetracycline was evaluated both in terms of its effects on microbial community structure and biogas production in thermophilic manure digesters. In addition to that, the effect ofchangingoperationalparameters such as mixing rateandsolid contenton the performance ofthermophilic systems were examined. In this aspect, single and multiple effects of these parameters on the system performance were determined.

2. THEORETICAL BACKGROUND

2.1 Energy Crisses and Renewable Energy

Energy is an obligatory component of society. Our modern society depends on energy for almost everything ranging from home apparatuses, lighting, transportation, heating/cooling, communication, to industrial processes to supply goods for our daily needs. We surrently consume around 500 Quadrillion Btu (QBtu) of energy, and about 92% of this consumption comes from non-renewable sources such as petroleum, coal, natural gas and nuclear (Khanal, 2010). All production and use of energy have an impact on the natural environment anywise. The energy consumption of today's generations should not exceed and it is also necessary to ensure an adequate quality of life for coming generations. Therefore, the available energy must be used more effectively (Palm et al., 1999). In today's energy demanding life style need to explore and use new sources of energy which are clean and renewable as well as eco-friendly is a must (Yadvika et al.,2004; Khanal, 2010).This mentioned renewable energy which is called biogas can be generated from the anaerobic digestion of the biomass.

Biogas, a clean and renewable form of energy, could very well substitute (especially in the rural sector) for conventional sources of energy such as fossil fuels, oil, etc. which are causing ecological-environmental problems and at the same time exhausting at a faster rate (Yadvika et al.,2004; UTES, 2008; Weiland, 2010).Therefore, biogas production from biomass like wastes, residues, and energy crops will play a vital role in future. Biogas production from agricultural originated waste is a very fast growing market in Europe and in the world. The European energy production from biogas reached 6 million tons of oil equivalents in 2007 (Weiland, 2010). Germany is the most biogas producing country in Europe (Öztürk, 2005), having 4000 agricultural biogas plants operated at the end of 2007 (Weiland, 2010).Averagely 15% of global energy use is covered by biomass. This percentage can be higher in developing countries up to 38%. In Turkey, energy is generated only

from hydraulic and thermal reactors and alternative energy production is almost zero. About 12 million cattle and 30-40 million sheep is present in Turkey and digestion of manures defecated by these animals can generate 25% of annual energy production. Biomass energy is expected to increase the energy use per capita in Turkey which is very low comparing to European countries (Öztürk, 2005).

2.2 Fundamentals of Anaerobic Digestion

Anaerobic digestion process as a means of biogas production is becoming increasingly appealing as a topic and it is considered to be an efficient way of producing renewable energy (Monteiro et al., 2011). Anaerobic digestion is the biochemical process conducted by the several groups of microorganisms which degrade the organic matter into a mixture of gas that consist of methane and carbon dioxide (biogas) in the absence of oxygen (Clark, 2011). In general, biogas that is generated by anaerobic digestion consists of 40-70% methane (CH_4), 30-60% carbon dioxide (CO_2), moisture and small amounts of hydrogen sulfide (H_2S) (Ilkılıc et al., 2011). The biogas production process effectively increases energy and nutrient recovery, with the additional advantage of preventing pollution from agricultural and industrial operations. At the same time, it also provides offsetting the operations' usage of fossil fuels. (Chen et al., 2008; Monteiro et al., 2011).

Anaerobic digestion as one of the most efficient waste and wastewater treatment technologies, has been widely used for the treatment of municipal sludge and limited application in the treatment of organic industrial wastes including fruit and vegetable processing wastes, packinghouse wastes, and agricultural wastes. Anaerobic digestion presents many significant advantages as listed below:

- Renewable energy production
- Cheap and environmentally healthy organic waste recycling
- Reducing the greenhouse gas emission
- Pathogen reduction through sanitation
- Improving the fertilization efficiency
- Lower sludge production
- Lower energy requirement
- Less nuisance from odors and flies

- Economical advantages for the farmers(Demirer and Chen, 2004; Kim et. al., 2006; Holm-Nielsen et al.,2009).

2.2.1 Biochemistry of anaerobic digestion

The anaerobic digestion process in other words methane fermentation is a complex process, which can be divided up into four phases: hydrolysis, acidogenesis, acetogenesis/dehydrogenation, and methanation. Each individual degradation steps are carried out by different consortia of microorganisms. They partly stand in syntrophic interrelation, have different growth rates and place different requirements on the environment (Weiland, 2010; Clark, 2011).

AD is a synergistic process of a consortium of microbes which can be classified along with a series of metabolic pathways. The main reactions of the AD process are shown in Figure 2.1. At the beginning of the AD process, hydrolysis occurs to reduce complex organic matters to simple soluble molecules by extracellular enzymes. Proteins, lipids and carbohydrate polymers are hydrolyzed to amino acids, long-chain fatty acids, and sugars, respectively. These reduced compounds are then converted to a mixture of short chain volatile fatty acids (VFAs) and other minor products such as carbon dioxide, hydrogen and acetic acid by fermentative bacteria (Liu et al., 2002). The organic acids are converted to acetate, carbon dioxide, and hydrogen by acetogenic bacteria and these products are the direct substrates for methane production. The final step of AD is methanogenesis, in which a variety of methanogenic bacteria utilize acetate, carbon dioxide, and hydrogen to produce methane. (Liu et al., 2002; Ahring et al., 2003; Weiland, 2010; Li et al., 2011). Methanogenesis is the focus of many AD studies due to its sensitivity to feedback inhibition by acidic intermediates (Li et al., 2011).

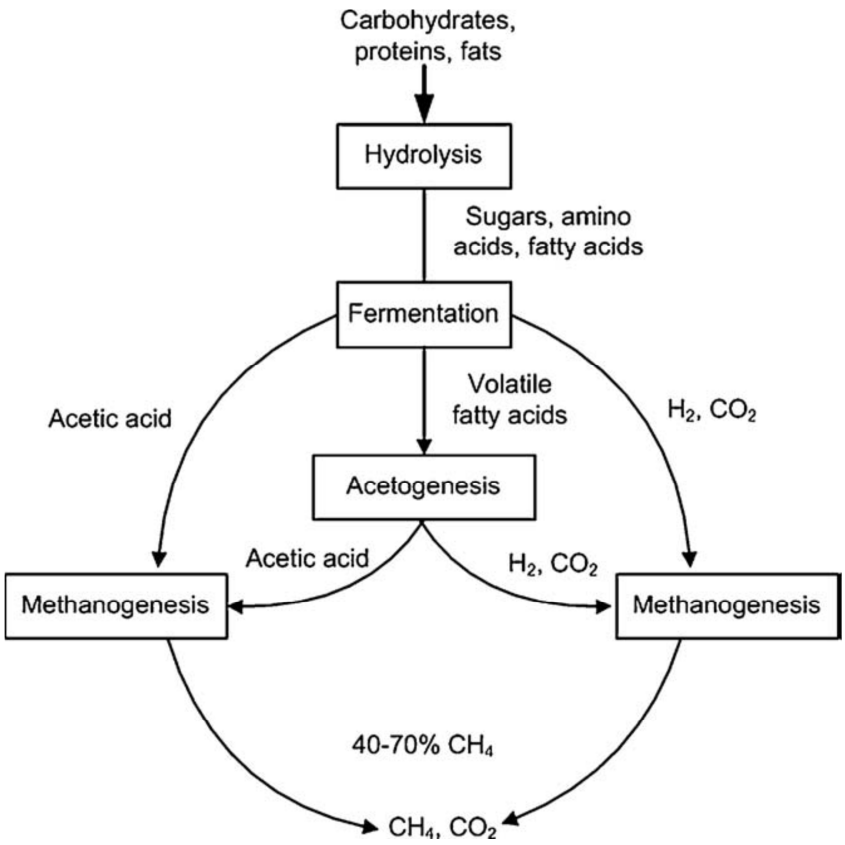


Figure 2.1 : Process flow of the degradation of organic material through anaerobic digestion. (Li et al., 2011).

Hydrolysis

First step in anaerobic degredation of organic matter is the hydrolysis. Complex wastes are required to be hydrolyzed into units as an initial step for taking up by the microbial cells. The hydrolysis of macromolecules such as lipids, proteins and carbohydrates into simple monomers such as aminoacids, sugar and long-chain fatty acids under anaerobic conditions is carried out by specific extracellular enzymes (Hassan, 2003; Khanal, 2010). The reaction rates are affected by some factors like pH, temperature, cell residence time and the waste constituents in the digester produced by hydrolytic bacteria. The hydrolysis process is especially critical in digesters operated with particulate matter like manure and may even be considered the rate limiting step. (Öztürk, 2010; Coat et al., 2011; Li et al., 2011). Hydrolysis was shown to be a rate-limiting step for digestion of high particulated substrate like swine waste, cattle manure and sewage sludge while methanogenesis is the rate-limiting step for readily degradable substrate (Boe, 2006).

Acidogenesis/Fermentation

Following the hydrolysis stage, solubilized monomers are taken up by facultative and obligatory anaerobic bacteria and converted to short chain organic acids, alcohols, hydrogen and carbon dioxide in acidogenesis (Hassan, 2003; Gerardi,2003; Li et al., 2011). The most important of the acids is acetate since, it is the main organic acid/volatile acid utilized as a substrate by methanogenic bacteria (Gerardi,2003), and about two thirds of biologically generated CH_4 is coming from acetate (Schmidt et al., 2000; Reay, 2010).

Acetogenesis

Acidogenesis state is followed by acetogenesis. Overall, two different types of acetogenic mechanisms can be pronounced. First of them is acetogenic hydrogenation which involve the production of acetate as an end product, either from the fermentation of hexoses or from CO_2 and H_2. Second of them is the acetogenic dehydrogenation which refers to the anaerobic oxidation of long and short chain volatile fatty acid (Kun, 2006).

Acetic acid is produced from propionic acid by *Methanobacterium bryantii, Desulfibrio,* and *Synthrophobacter wolinii*. The common organisms which convert butyric, caproic and valeric acids to acetic acid are *Synthrophomonas wolfei* and *Syntrophus buswellii* (Chynoweth, 1987; Pandey, 2011).

Methanogenesis

Methanogenesis is the final and may be the most important step of the anaerobic digestion process which is carried out by a group of strictly anaerobic microorganism called *Archaea* (Hassan, 2003). In the methanogenic stage, methane is formed mostly from acetate, carbon dioxide and hydrogen gas (Gerardi, 2003). Approximately 70 % of the fermenter methane comes from acetate and the remainder from CO_2 reduction to CH_4 (Hassan, 2003; Schön, 2010). Methanogenesis stage is considered to be the rate-limiting step in the whole anaerobic digestion process due to the slow growth rate of the methanogens (Kobayashi et al., 2009) comparing to acidogens and accordingly, the performance of anaerobic system and the quality of the digestate depend on the methanogenic activity (Gerardi, 2003; Montero et al., 2009; Shin et al., 2011).

To maintain the stability of anaerobic treatment system, the nonmethanogenic and methanogenic bacteria must be in a state of dynamic equilibrium (Hassan, 2003). When methanogenesis does not work effectively due to some upset, volatile fatty acids (VFAs) accumulate, which may cause to decrease in the pH and a cessation of the methane production(Schoen et al., 2009).

2.2.2 Process microbiology

The mechanism behind anaerobic degradation of organic matter and biogas production is needed a complex microbiological process requiring the activity of several different groups of microorganisms, both bacteria and archaea, with a variety of metabolic capacities. These groups consist of hydrolytic and fermentative bacteria, hydrogen-producing acetogenic bacteria, homoacetogens, hydrogenotrophic methanogens and aceticlastic methanogens (Khanal, 2008).

Hydrolytic and fermentative bacteria

In the hydrolysis phase, undissolved compounds like cellulose, proteins and fats are degraded into their monomers by extracellular enzymes of facultative and obligatory anaerobic bacteria, or through physicochemical reactions (Ivanov, 2011). The community that works in the hydrolysis step is quite heterogenic. For example: it was claimed that *Clostridium* is responsible for degradation of compounds which contain cellulose and starch while *Bacillus* play role in the degradation of proteins and fats. The name of the hydrolytic microorganisms are stated as the cellulytic (*Clostridium spp*.), proteoytic *(Peptococcus spp., Bacteroides spp*., *Peptostreptococcus spp*.), lipolytic (genera of clostridia and micrococci) and aminolytic (*Clostridium butyricum*, *Bacillus subtilis*) bacteria (Kun, 2006). The hydrolytic microorganisms are also able to break down some intermediate products to simple volatile fatty acids (VFAs), carbon dioxide, hydrogen and ethanol (Khanal, 2008; Ivanov, 2011). Some of these acid-forming bacteria are *Lactobacillusspp*., *Bifidobacteriumspp*., *Butyrivibriospp*. (Kun, 2006).

Hydrogen-producing acetogenic bacteria

These group of bacteria metabolize higher organic acids such as propionate, butyrate and ethanol into acetate, H_2 and CO_2. These bacteria are *Enterobacterspp*., *Citrobacterspp*., *Serratiaspp*., *Syntrobacterspp*. (Kun, 2006).

In a coculture of hydrogen-producing acetogenic bacteria and hydrogen-consuming methanogenic bacteria, it exists a symbiotic relationship between these two groups of bacteria. Hydrogen-consuming methanogenic bacteria rapidly use the hydrogen and keep the level of hydrogen partial pressure extremely low. This provides a thermodynamically appropriate condition for the hydrogen-producing acetogenic bacteria to break down the aforementioned organic compounds into acetate, H_2 and CO_2 (Khanal, 2008; Deublein and Steinhauser, 2008).

Homoacetogens (hydrogen consuming acetogens)

Homoacetogenesis has attracted much attention in recent years because of its final product, acetate is an important precursor to methane generation. Homoacetogens utilize mixture of hydrogen and CO_2 as a substrate. This group of bacteria include *Clostridiumspp*., *Acetobacteriumspp*. (Kun, 2006). Some homoacetogens use CO as a substrate and some use organic substrate such as formate and methanol to produce acetate as the end product (Khanal, 2008).

Hydrogenotrophic methanogens and aceticlastic methanogens

Hydrogenotrophic methanogens produce methane from CO_2 and H_2 while aceticlastic methanogens convert acetate to methane (Ivanov, 2011). Acetate utilizing methanogens produce methane by the way of acetate decarboxylation and this accounts for 70 % of the methane generated. Remain 30% of the methane is produced by hydrogen removing methanogens using H_2 and CO_2 (Kun, 2006; Deublein and Steinhauser, 2008; Clark, 2011). Acetate removing methanogens include *Methanosarcinaspp*., *Methanothrixspp*., *Methanosaetaspp*., *Methanolobusspp*.. Hydrogen removing methanogens include *Methanomicrobiumspp*., *Methanobacteriumspp*., *Methanobrevibacterspp*., *Methanococcusspp*., *Methanogeniumspp*., *Methanospirillumspp*. (Kun, 2006).

Several environmental factors influence the selection of the microorganisms involved in a specific stage of anaerobic digestion process. Thus, different processes harbour different microbial compositions. For example, the composition of the microbial community in a specific anaerobic digestion process is strongly influenced by temperature, through its effects on both growth and survival of microorganisms in the system (Leven et al., 2007). In addition to temperature, in the anaerobic digestion

process, acidogenic and methanogenic microorganisms differ not only in terms of their nutrition and pH requirements, but also with respect to their physiology, growth, and nutrient uptake kinetics, and in their particular ability to withstand environmental changes (Montero et al., 2009).

It is repoted that the growth rate of acidogens is quite high (doubling time of the order of one hour or even less) and low pH resistant (pH: 5-6) (Clark, 2011). Methanogens are slow-growing with a generation time of 2-3 days at 35^0C as compared with 2-3 hours for acid-formers (Kun, 2006; Clark, 2011) and also sensitive to pH and grow in the narrow range of 6.5-8.0. Methanogens are also sensitive to O_2 and oxygen as low as 0.01 mg/l is toxic to methanogens (Kun, 2006). Conditions that enhanced activity of both acid- and methane-forming bacteria include; an oxygen-free environment, a relatively constant temperature, a pH between 6.5 and 7.5, a consistent supply of organic matter to feed upon.

Characteristics of methanogens

The methanogenic archaea are strictly anaerobes and they use hydrogen, carbonmonoxide, formate and a few alcohols to gain energy and produce CH_4 and CO_2. Alternatively, methanogens reduce methyl groups to methane. Some methanogens are able to use hydrogen as a substrate to reduce the methyl.
The substrates utilized by methanogens for methane fermentation can be divided into three groups (Khanal, 2008; Deublein and Steinhauser, 2008; Madigan et al., 2009):

- CO_2 type:

In the first substrate type, carbon dioxide, formate and carbon monoxide are reduced to methane. The general equation for this conversion was given in Eq **2.1**. This conversion is mostly hydrogen dependent, however, other substrates in this class can supply the electrons for CO_2 reduction. This class is also known as hydrogenotrophic methanogens. These microorganisms include *Methanobacteriales, Methanomicrobiales, Methanococcales* and *Methanosarcinaceae.*

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

(Deublein and Steinhauser, 2008; Madigan, 2009).

- Methyl type:

The second substrate group is methyl substances. The organisms responsible for methane formation using methyl substances are called ormethylotrophic methanogens and are limited to *Methanosarcinales*, except for *Methanosphaera spp*., which belong to the order *Methanobacteriales*. CH_4 production from methyl compounds can occur with an external electron donor such as H_2 (Equation **2.2**) and also without H_2 (Equation **2.3**).

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

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

(Deublein and Steinhauser, 2008; Madigan, 2009).

- Acetate type:

Acetate is the last type of substrate used by methanogens. Since acetate is the major product of fermentation (about 70%), it is found in anaerobic digesters commonly, and represent a large portion of methane production (Gerardi 2003). Equation **2.4** shows the conversion of acetate to methane.

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G° = -31kJ/mol \qquad \textbf{(2.4)}$$

(Deublein and Steinhauser, 2008; Madigan, 2009).

For this reaction, only two genera are known to use acetate for methanogenesis or methane production: *Methanosarcina* and *Methanosaeta*.

For the acetate conversion process, acetate must first be converted to acetyl coenzyme A (acetyl-CoA) before convertion to methane. Acetyl-CoA can interact with carbon monoxide dehydrogenase and methyl group of acetate is transferred to the corronoid enzyme to yield CH_3-corronoid. CoM-mediated terminal step of methanogenesis follows this reaction. Methyl-coenzyme M reductase is found to be unique to methanogens and is required in the final stage of methanogenesis causing reduction of methyl group. In the last step of methane production, the methyl group is reduced to methane (Rastogi et al., 2008) with electrons derived from oxidation of the carbonyl group of acetyl-CoA to CO_2 (Ince et al., 2011).

Taxonomy of methanogens

Phylogenetically, methanogens participate in the domain archaea. Archaea is consisted of microorganisms different from Bacteria (Eubacteria) and Eukarya as shown in figure 2.2. Archaeal cells possess of membrane lipids composed of isoprenoids ether-linked to glycerol or other carbohydrates, lack of peptidoglycan, and a distinctive RNA sequence (Garcia 2000; Khanal,2008). rDNA based 3 domain system is given in Figure 2.2. (Bauman, 2007).

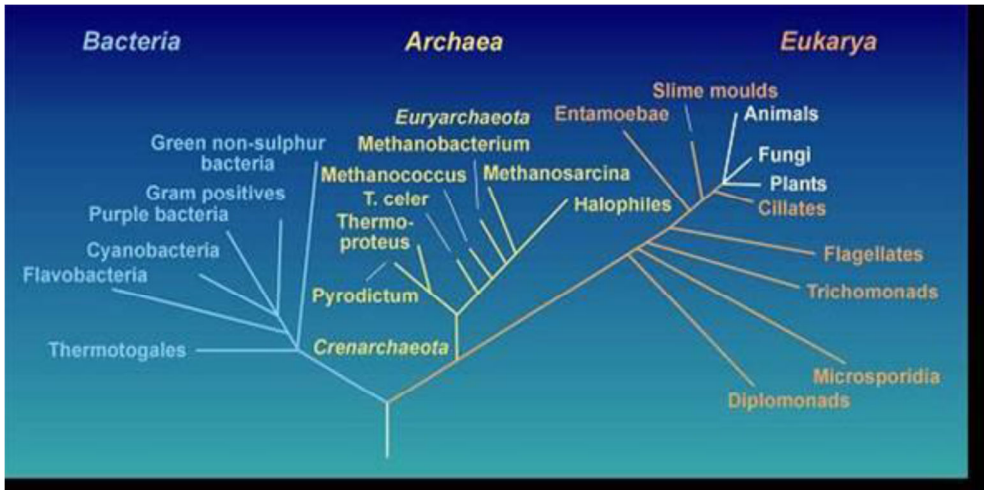


Figure 2.2 : Three domain system (Madigan et al., 2002).

Methanogens are classified into five orders within the kingdom *Archaeobacteria*: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales,* and *Methanopyrales* as shown in Figure 2.3 (Garcia 2000; Talbot et al., 2008; Deublein and Steinhauser, 2008). Organisms from different orders have less than 82% 16S rRNA sequence similarity. Methanogens belonging to different orders also have different cell envelope structure, lipid composition, substrate range, and other biological properties.

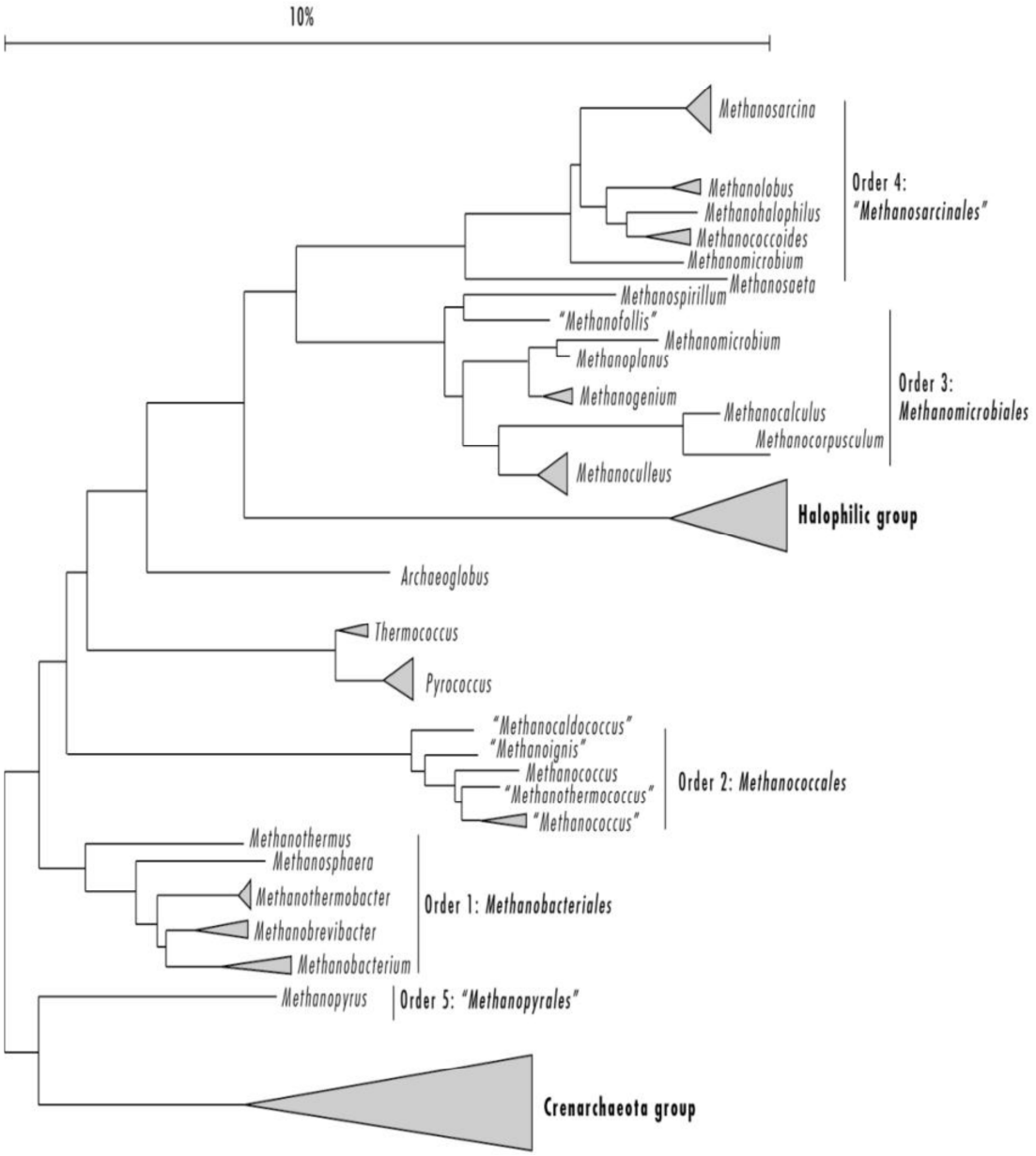


Figure 2.3 : Phylogeny of methanogens, domain *Archaea*. (Non-methanogens are indicated by their group names, large triangles) (Garcia et al., 2000).

Members of the order *Methanobacteriales* generally produce methane using CO_2 as elector acceptor and H_2 as the electron donor. Some species can also use formate, CO, or secondary alcohols as electron donors (Deublein and Steinhauser, 2008).

The order of *Methanobacteriale*s is divided into two families, *Methanobacteriaceae* and *Methanothermaceae*. The family *Methanobacteriaceae* contains three mesophilic genera, *Methanobacterium*, *Methanobrevibacter*, and *Methanosphaera*, and one extremely thermophilic species *Methanothermobacter* (Garcia et al., 2000). The family *Methanothermaceae* is represented by one hyperthermophilic genus,

Methanothermus, which grow optimally above 80^0C (Deublein and Steinhauser, 2008).

Methanococcales produce methane using CO_2 as the electron acceptor and H_2 or formate as the electron donor (Garcia et al., 2000). The order of *Methanococcales* has been divided into two families distinguished by their growth temperatures, *Methanocaldococcaceae* and *Methanococcaceae* (Deublein and Steinhauser, 2008).

Members of the order *Methanomicrobiales*are order of methanogens that use CO_2 as the electron acceptor and H_2 as electron donor. Most species can use formate, and many species also use secondary alcohols as alternative electron donors (Garcia et al., 2000). They are grow only below 60^0C. The order of *Methanomicrobiales*is divided into three families, *Methanomicrobiaceae, Methanospirillaceae*and *Methanocorpusculaceae* (Deublein and Steinhauser, 2008).

Methanosarcinales can produce methane by disproportionating the methyl group containing compounds or by splitting acetate. Some species can reduce CO_2 with H_2, but formate is not used as an electron donor (Garcia et al., 2000). The order of *Methanosarcinales*is divided into two families, *Methanosarcinaceae*and *Methanosaetaceae*.

Methanosaetaspp. are filemantous organisms which are known to grow only on acetae (Deublein and Steinhauser, 2008). *Methanosarcina spp*. usually grow in large aggregates. These aggregates consist of single cells surrounded by a thick wall. *Methanosarcina spp*. use several methanogenic substrates such as acetate, methanol, methylamines and sometimes also H_2/CO_2 (Schmidt et al., 2000). *Methanosaeta* can use acetate at concentrations as low as 5-20 μM, while *Methanosarcina* requires a minimum concentration of about 1 mM (Mladenovska and Ahring,2000; Hori et al., 2006; Smith et al., 2007; Reay, 2010).

The order of *Methanopyrales* is represented by only one species, *Methanopyrus kandleri*. Cells reduce CO_2 with H_2 for methanogenesis. *M. Kandleri* is hyperthermophilic with a growth temperature range of 110°C (Robert, 2007; Deublein and Steinhauser, 2008; Madigan, 2009).

2.3 Environmental and Operational Factors Affecting Anaerobic Treatment System

It has been recognized that the most important factors affecting the anaerobic digestion process are temperature, hydraulic retention time (HRT), organic loading rate (OLR), pH and buffering systems/alkalinity, mixing, the availability of nutrients, the nature of the feedstocks (composition) and the presence of toxic components or inhibitors in the process (Parawira, 2004; Khanal 2010; Clark, 2011).

2.3.1 Temperature

For achieving successful anaerobic digestion several physical and chemical factors must be considered. Temperature is one of the most important physical factors which affect the anaerobic digestion process in many ways such as; ionization equilibrium, solubility of substrates, substrate removal rate and other constants such as specific growth rate, decay biomass yield, and half saturation constant (Vindis et al.,2009).

There are three common temperature ranges for anaerobic digestion:

(1) The lower temperature range, which is referred to as psychrophilic, meant for temperatures lower than 20ºC.
(2) Temperatures within 20-45 ºC, named mesophilic temperatures.
(3) Temperatures in the range of 45-60 ºC, are termed thermophilic temperatures (Monteiro 2011).

Most of the trials mentioned in the literature to enhance biogas production are aimed at achieving the digester temperature to mesophilic range (i.e. optimum temperature). It is noted that systematic studies on biomethanation by psychrophilic microflora are lacking (Yadvika et al., 2004). Although it is known that anaerobic digestion process can take place within a large temperature range, it is optimal at mesophilic (35ºC - 42ºC) and thermophilic (45ºC - 60ºC) conditions (Gerardi 2003; Weiland 2010; Monteiro 2011; Ward et. al.,2008; Khanal,2010).

It is important to maintain a constant temperature during the anaerobic digestion process, (Vindis et al.,2009) as temperature changes or fluctuations significantly affect anaerobic digestion and the quantities of gas produced(Dennis and Burke, 2001). The ultimate yield of biogas depends on the composition and biodegradability of the organic feedstock, but its production rate will depend on the population of

microorganisms, their growth conditions, and fermentation temperature (Lusk, 1998). The structures of the active microbial communities at the two temperature, mesophilic and thermophilic, optima are also quite different (Ward et. al.,2008). Advantages and disadvantages of the mesophilic and thermophilic anaerobic digestion process are mentioned in Table 2.1.

Table 2.1 : Advantages and disadvantages of the mesophilic and thermophilic anaerobic digestion process.

Mesophilic Conditions	**Thermophilic Conditions**
Advantages	**Advantages**
• Mesophilic microflora are able to tolerate temperature fluctuations within ±3ºC • Need less energy input for heating • Less influenced by inhibitory factors of ammonia released during the mineralization of proteins	• Higher metabolic rates • Higher specific growth rates • Process is faster and more efficient • High total biogas production • Low content of volatile solids in the stabilized digestion residues • Ability to fed with higher organic loading rates at lower hydraulic retention times • More efficient killing (%90) of pathogens present in the waste
Disadvantages	**Disadvantages**
• Efficient killing of pathogens cannot be said for mesophilic digestion when used alone. This is a significant criterion for the animal waste treatment since the effluent can be used as a soil fertilizer	• Frequently higher death rates • More vulnerable to temperature fluctuations • Lower microbial diversity • More sensitive to toxicants or inhibitors and temperature fluctuation • Leads to the system imbalanced and susceptible to failure • Additional energy requirements (reduction in the net energy production) • The pH increases through a reduced solubility of carbon dioxide, this presents to a higher proportion of free ammonia

2.3.2 Hydraulic retention time

The hydraulic retention time (HRT) is one of the most important design parameters effecting the economics of digestion operation (Parawira, 2004). It is a defining factor on biogas production and waste stabilization. Thus, it should be long enough to ensure the growth of microorganisms in the system as it is closely related with the growth rates of microorganisms in the system (Gerardi,2003; Demirel and Scherer, 2008). If the retention time is too short, the bacteria in the digester are washed out

faster than they can reproduce, so that fermentation practically comes to a standstill (Hassan, 2003).

The SRT (solids retention time) is the average time that bacteria in another word solid fractions are in the anaerobic digester. The HRT is the time that the wastewater or sludge is in the anaerobic digester (Gerardi,2003). Solids retention time in anaerobic digesters is equal to hydraulic retention time if recycling or supernatant withdrawal is not applied. SRT can be the basis criteria for the reactor volume. The longer retention time is required the higher reactor size needed for a given amount of substrate to be treated (Hassan, 2003). In additon, the digestion process is a function of time needed by microorganisms to degrade the organic matters, so SRT and volume of the digesters should be chosen correctly. The shortest SRT in anaerobic digesters is repoted 10 days at 35°C. Shorter SRTs can result in washout of microorganisms (Medcalf and Eddy, 2003). In addition, complex waste, such as animal manure, must be digested at HRTs of 10 days or more because of the high fraction of recalcitrant organic matter present in cattle manure (Sung and Santha, 2003). For digesters with solids retention time values longer than 15 days at the same temperature, changes in volatile solids reduction are relatively small. In general, SRT in digesters is about 30 days for mesophilic digestion and longer for low-temperature digestion (Medcalf and Eddy, 2003). Typical retention time for thermophilic digesters is also 20 - 30 days (Williams, 2009).

2.3.3 pH and buffering capacity

The anaerobic degradation process is highly pH dependent due to the each of the microbial groups required in the digestion pathways has a certain pH level for optimal growth. The conditions affeted by pH level include utilisation of carbon and energy sources, efficiency of substrate dissimilation, synthesis of proteins and various types of storage material, and the release of metabolic products from the cell (Parawira, 2004).

Methanogenesis occur within a limited pH range of 6.5-8.0, being optimum at pH 7.0-7.5. The process is disrupted severely if pH exceeds 8.5 or decreases below 6.0 (Hassan, 2003). During the both mesophilic and thermophilic conditions the methanogenic activity was inhibited at acid or alkaline pHs, and the highest methane concentration was obtained at pH 7.0 in most cases (Zhang et. al., 2009). The

aceticlastic methanogens are found to be more sensitive to low pH values than the hydrogenotrophic methanogens (Parawira, 2004). The optimum pH level of hydrolysis and acidogenesis has been also reported as being between pH 5.5 and 6.5. This is an important reason why some designers prefer the separation of the hydrolysis/acidification and acetogenesis/methanogenesis steps in two-stage processes (Ward et al.,2008).

The amount of carbon dioxide and volatile fatty acids produced through the anaerobic degradation influence the pH of the digester contents (Yadvika et al.,2004). The pH likely rises with accumulation of ammonia and decreases with VFA accumulation which is produced by the acidogenic bacteria. However, the accumulation of VFA may not always result in a pH drop due to the buffering capacity of the substrate. Buffer capacity is often provided with the alkalinity in anaerobic digestion, which is the equilibrium of carbon dioxide and bicarbonate ions that provides resistance to significant and rapid changes in pH (Medcalf and Eddy, 2003), and the buffering capacity is therefore proportional to the concentration of bicarbonate (Hassan, 2003). Anaerobic digesters are operated in a wide range of alkalinity values depending on the substrate to be degraded. These values differ from 2000 to 18000 mg $CaCO_3$/L (Alvarez et al., 2010). Buffer capacity is a more reliable method of detecting digester imbalance than direct measurements of pH value (Ward et al.,2008).

Animal manure is thought to have an additional alkalinity which can neutrilaze acidification due to the possible VFA accumulation. Under optimal conditions, VFA acidity produced by the acidogenic bacteria is utilized by the bicarbonate produced by the methanogens. Nontheless, if VFA production is exceeded, buffering capacity can fail leading to the collapse of the whole system. Besides, inhibitors for the methanogenesis such as excessive fatty acids, hydrogen sulphide, and ammonia are toxic only in their non-ionised forms. The relative proportion of the ionised and non-ionised forms (and therefore toxicity) is pH-dependent (Parawira, 2004). Ammonia is toxic above pH 7; volatile fatty acids and hydrogen sulphide are toxic below pH 7 (Weiland et al., 2010; Ward et al.,2008; Schön, 2010). So that, pH is a highly important operational parameter for the performance of the anaerobic process.

2.3.4 Mixing

Mixing is a very significant factor which effects the performance of anaerobic digestion, especially operating with particulate substrate like manure. Mixing improves the digestion process by distributing organisms, substrate, and nutrients uniformly throughout the digester as well as getting equal temperature (Gerardi, 2003). In this sence, mixing enhances substrate contact with the microbial community, ensures the uniformity of pH and temperature, prevents stratification and scum accumulation (Ong et al., 2002; El-Mashad and Zhang, 2010), facilitates the removal of biogas from the digestant, aids the reduction in particle size and provides rapid dispersion of any toxic materials coming in the tank as it means toxicity minimizing (Hassan, 2003; Karim et al. 2005; Hoffmann et al., 2008; Kaparaju et al., 2008; Pandey 2011).

Mixing is usually applied through various methods including mechanical mixers, digester contents recirculation and gas recirculation (Hassan, 2003). Mechanical mixers are more effective in terms of power consumed than gas recirculation, (Burke, 2001; Karim et al., 2005; Kaparaju et al., 2008) but they often become clogged or fouled with digester solids (Burke, 2001).

The main factors affecting mixing of the digester content are the mixing strategy, intensity and duration and also the location of the mixer. However, the effect of mixing duration and intensity on the performance of anaerobic digesters are contradictory (Kaparaju et al., 2008).

Mixing does not always take place continuously; it is often intermittent and may be active several times a day or several times an hour. In some studies, digesters were manually shaken once a day (El-Mashad and Zhang, 2010). Mixing in full scale digesters can be performed by intermittent and minimal mixing which refer to mixing for 10 minutes prior to feeding and withholding mixing for 2 h prior to feeding, respectively (Kaparaju et al., 2008).

2.3.5 Organic loading rate (OLR) or solid concentration

The loading rate is the term used to description the daily amount of organic substance fed into the digester in relation to the total volume of digester. If the loading rate is too low, the bacteria will represent a lower metabolic activity and very

small quantities of gas will be produced. If the loading rate is too high, this generated overload situation leads to volatile fatty acids (VFA) build up, gas production drops (Khanal, 2010) and the proportion of carbon dioxide rises (Hassan, 2003). At high loading rates, methanogenic activities might be inhibited by high concentrations of long-chain fatty acids, volatile fatty acids (VFAs) and free ammonia (NH_3) (Wu et al., 2009).

The amount of fermentable material of feed in a unit volume of slurry is defined as solid concentration. The applied solid content in association with the substrate loading rate is critical to the cost, performance and stability of anaerobic digesters. Ordinarily 7–9% solids concentration is best-suited. It was reported that the process was unstable below a total solids level of 7% for manure as substrate while a level of 10% caused an overloading of the fermenter (Yadvika et al.,2004). Average TS content of cattle manure is 10%, sheep manure is 24%, chicken manure is 22%. Burton and Turner (2003) proposed optimal OLR for cattle manure of 2.5-3.5 kg VS/m^3.day. When animal manure and water are mixed in ratio of 1:1, aproximately 8-10% TS containing slurry is prepared. Normally, the amount of 8-10% TS is recommended for easy operation and easy miscibility (Wiese et al., 2007; Ilkılıc et al., 2011).

2.3.6 C/N ratio

The relationship between the amount of carbon and nitrogen present in organic materials is represented by the C/N ratio (Monnet, 2003). It is generally found that during anaerobic digestion, microorganisms utilize carbon 25–30 times faster than nitrogen. Thus, to meet this requirement, microbes need a 20–30:1 ratio of C to N with the largest percentage of the carbon being readily degradable (Hassan, 2003; Yadvika et al., 2004).

A high rate C/N ratio is an indication of rapid consumption of nitrogen by methanogens and results in lower gas production. On the other hand, a low C/N ratio results in ammonia accumulation (Sakar et al., 2009) and pH exceeds 8.5, which is toxic to methanogenic bacteria. Therefore, an optimum C/N ratio in the digester can be achived by suitable mixing materials containing the high and low C/N ratios (Khanal, 2010).

2.3.7 Nutrients

For the growth and survival of the existing groups of microorganisms in anaerobic digesters, certain macro and micro nutrients are essential. Macro nutrients include carbon, nitrogen and phosphorus that are required in relatively large quantities by microorganisms. Whereas, iron, nickel, magnesium, calcium, sodium, barium, tungstate, molybdate, selenium and cobalt can be pronounced as micronutrients that are required in relatively small quantities by microorganisms. They are considered as necessary for various situations of active methanogenesis step and reported to enhance bacterial metabolism and growth (Gerardi, 2003; Khanal, 2010; Schnürer and Jarvis 2010).

Through these essential nutrients, cobalt is required as an activator of enzyme systems in methane forming bacteria. The presence of cobalt into enzyme systems provides to the conversion of acetate to methane more efficiently. Nickel is a unique micronutrient need for methane forming bacteria, while it is generally not essential for the growth of most bacteria. For example, the F430 enzyme in methane forming bacteria contains nickel. The addition of nickel can increase acetate utilization rate of methane forming bacteria (Gerardi, 2003). In addition to cobalt and nickel, selenium and tungsten are also significant in the enzyme systems of acetogenic and methanogenic bacteria. On the other hand, the amount of micronutrients neccesary for the process is very low, changing between 0.05 and 0.06 mg/L. Iron may be an exception required in concentrations between 1 and 10 mg/L.

As an advantages, the use of manure as a substrate, decreases the need for micronutrients however, it has been shown that addition of micronutrients always enhance the performance of an anaerobic digester.

2.3.8 Inhibitors

The complexity of the anaerobic digestion process makes it vulnerable to system shut down caused by the inhibitors. If a substance blocks the metabolism or microbial activity and biogas production it is called an inhibitor. Inhibition of anaerobic digestion processes can easily be monitored by the methane content of the biogas or amount of volatile fatty acids accumulated in the system. In the anaerobic digestion process, methanogenesis is much more sensitive to improper environmental

conditions like inhibitors than other steps. Following headlines are the major inhibitors.

Oxygen

Anaerobic digestion process must be kept in the conditions which is oxygen free. The methanogens are strictly anaerobes so that, oxygen as low as 0.01 mg/l is toxic to methanogens (Kun, 2006; Clark, 2011).

Ammonia inhibition

Ammonia is generated by the degradation of nitrogenous compounds like urea or proteins. Diverse results of ammonia inhibition include; a change in the intracellular pH, increase of maintenance energy requirement, and inhibition of a specific enzyme activity (Chen et al., 2008). Although it is an important buffer, high concentrations of ammonia can lead to failure in digestion systems. Ammonia can be found as ammonium ion (NH_4^+) or dissolved ammonium gas (NH_3) in aqueous solution as forms of inorganic ammonia nitrogen (Chen et al.,2008). The [ammonium]/[ammonia] ratio is pH dependent(Sung and Liu 2003). These compounds are in equilibrium with each other at neutral pH (Clark, 2011). However, higher pH value causes to shift the equilibrium to ammonia gas (Gerardi, 2003; Sakar et al., 2009).

Free ammonia (NH_3) which is non-ionised form, has been suggested to be the major cause of inhibition (Gerardi, 2003; Schön, 2010; Clark, 2011) since it is freely membrane-permeable. The hydrophobic ammonia molecule may diffuse passively into the cell, causing proton imbalance and potassium deficiency (Chen et al.,2008).

Ammonia concentrations less than 1000 mg/L reported to have no adverse effect on methanogens (Deublein and Steinhauser, 2008) whereas up to 3000 mg/L ammonia may have inhibitory effects at higher pHs (Gerardi, 2003; Hassan, 2003; Medcalf and Eddy, 2003; Öztürk, 2005).

Sulfide inhibition

The biological reactions in the anaerobic digestion process may produce sulfides by decomposing the sulfur containing inorganic compounds. These oxidized sulfur compounds can serve as electron acceptors for sulfate reducing bacteria (SRB), which consume organic compounds in the anaerobic reactor and produce hydrogen

sulfide (H_2S) (Medcalf and Eddy, 2003). Sulfate can inhibit metanogenesis due to both the competition for substrate like acetate and hydrogen by SRBs and the production of sulfide from the sulfate reduction by SRBs (Chen et al., 2008). However, low concentrations of sulfide (less than 20 mg/l) are needed for optimal methanogenic activity (Medcalf and Eddy, 2003). Soluble sulfide concentrations less than 100 mg/l can be tolerated with a slight or no acclimation. Soluble sulfide concentrations between 100 and 200 mg/l do not show inhibitory effect after an acclimation period. Sulfate concentrations higher than 200 mg/l had an inhibitory effect on anaerobic systems directly (Hassan, 2003; Burton and Turner 2003; Schön, 2010).

Volatile fatty acids inhibition

The accumulation of VFAs is one of the most important criteria in the monitoring of the anaerobic digestion system. It is commonly agreed that VFA build up is the result of unbalanced digestion conditions (Ahring et al., 1995). The decrease in pH accompanying accumulation of VFAs is the main cause of toxicity and reactor failure in the anaerobic digestion process (Parawira, 2004). The VFA accumulation may be resulted by unsuitable environment conditions such as over loading, nutrient depletion or infiltration of inhibitory materials.

Much attention has been directed to the relationship between VFA concentration and the performance of an anaerobic fermenter (Pullammanapallil et al., 2001). It has been shown that VFAs are important intermediary products in the metabolic pathway of methane production (Sasaki et al., 2011) and cause microbial stress if present in elevated levels, decrease pH, and lead to failure of the digester (Gerardi et al., 2003). Therefore, the concentration of VFAs is an major consideration for good performance of a digester (Ward et al., 2008).

The most common VFAs found in an anaerobic digester are; acetic acid, propionic acid, butyric acid and isovaleric acid. In most of the unsuccessful digesters, commonly acetic and propionic acids accumulate in the system as causing the reduction of methane production (Gerardi, 2003; Hori et al., 2006). Acetic acid is usually present in higher concentrations than other fatty acids during anaerobic digestion however, propionic and butyric acids have more inhibitory effect to the methanogens (Montero et al.,2010; Weiland et al., 2010).

Metals inhibition

They can be distinguished into light and heavy metals. Light metals are present in the form of cations in solution and in the case of anaerobic digesters; they usually include sodium, potassium, calcium and magnesium. They are usually added in the form of chemicals for pH control, but they can also arise from the breakdown of biomass. They are required for microbial growth at moderate concentrations, but they can cause severe inhibition or even toxicity at high levels (Clark, 2011).

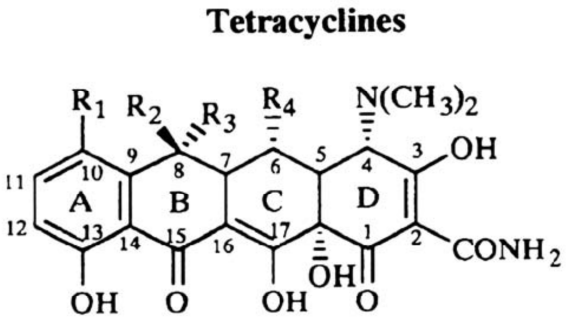
Heavy metals such as chromium, iron, cobalt, copper, zinc, cadmium, and nickel may cause toxic effect on anaerobic processes. Whether heavy metals would be stimulatory or inhibitory to anaerobic microorganisms is determined by the total metal concentration, chemical forms of the metals, and process related factors such as pH and redox potential. Heavy metals are not biodegradable and can accumulate to potentially toxic concentrations (Chen et al., 2008). In this exceeding amount, heavy metal ions inhibit activity of microorganisms and inactivate their certain enzymes, however, trace amounts of heavy metals are essential for the microorganism activity (Clark, 2011).

Inhibition of veterinary antibiotics

Veterinary antibiotics (VAs) are widely used in many countries worldwide to treat disease and protect the health of animals. They are also incorporated into animal feed to improve growth rate and feed efficiency (Thile-Bruhn and Beck, 2005; Arıkan et al., 2006; Alvarez et al., 2010; Jeong et al., 2010). In the USA, approximately 70% of the estimated 16 million kg of antimicrobial compounds consumed has been used for non-therapeutic purposes in 2000 (Sarmah et al., 2006). According to data collected from a surwey of members of the Animal Health Institute (AHI), the sales of antibiotics used to treat, prevent and control disease and maintain the health of animals rose 7.5 % in the USA from 2003 to 2004 (Kümmerer, 2008).

Among veterinary antibiotics such as sulfonamides, macrolides, fluroquinolones; tetracyclines are very much favoured in veterinary medicine. Tetracyclines are protein synthesis inhibitors (Bowman, 2009) and binding to ribosome resulting in the inhibition of protein synthesis. It is primarily active against gram-negative bacteria, but also will kill some gram-positive bacteria (Madigan, 2009; Tylova,

2010). These chemicals are characterized by a partially conjugated four-ring structure with a carboxyamide functional group as shown in Figure 2.4.



	R_1	R_2	R_3	R_4
Tetracycline (TC)	H	CH_3	OH	H
Chlortetracycline (CTC)	Cl	CH_3	OH	H
Oxytetracyline (OTC)	H	CH_3	OH	OH

Figure 2.4 : Molecular Structure of Tetracyclines (Glazer, 1995; Fogarty, 1990; Sarmah et al., 2006).

They are relatively hydrophilic because of the presence of several hydroxly groups, an amide moiety and a tertiary amine substituent. Thus, they can cross the outer membrane of gram negative bacteria efficiently (Glazer, 1995). Besides, under a broad range of environmental conditions, tetracyclines (tetracycline, chlortetracycline and oxytetracycline) can adsorb strongly to solid matter like clays, soil and sediments (Halling-Sorensen 2003; Sanford et al., 2009).

Oxytetracycline (OTC) is a common antibiotic with a broad range of activity and low cost. OTC is administered to livestock animals (including cattle, swine, poultry and fish) to promote growth and for prophylactic and therapeutic treatment (Alvarez et al., 2010). OTC was first isolated from *Streptomyces rimosus* in 1940s (Glazer, 1995; Madigan, 2009).

In the digestive system of animals, OTC is degraded into its metabolites such as 4-epi-Oxytetracycline (EOTC), α-apo-oxytetracycline (α-Apo-OTC), and β-apo-oxytetracycline (β-Apo-OTC) (Arikan et al., 2006). These metabolites can also be additional inhibitors for the microbial communities working in the anaerobic digestion. Bacterial activity may be inhibited by antibiotic metabolites produced in the gastrointestinal tract rather than by the original molecule (Masse et al., 2000). These compounds are strongly adsorbed in manure samples because they create

complexes with metal ions, especially with divalent ones, humic acids, proteins, particles and organic matter in the manure matrix (Alvarez et al., 2010). Thus, the presence of antibiotics or antibiotic metabolites in manure can inhibit the digestion performance of anaerobic bacteria as resulting of the reducing use of these substrate by microorganisms (Arikan et al., 2006; Alvarez et al., 2010).

As antibiotics are poorly adsorbed in the gut of the animals, the majority is excreted unchanged in faeces and urine (Kemper, 2008; Jeong et al., 2010). Between 17% and 76% of antibiotics administered to animals are excreted via urine and faeces in an unaltered form or as metabolites of parent compounds (Alvarez et al., 2010). About 23% of oxytetracycline was excreted in the manure without any change in a study by Arıkan et al., (2006).The results given by Martinez-Carballo et al. (2007) reveal that in liquid manure up to 46 mg/kg CTC, 29 mg/kg OTC, and 23 mg/kg TC could be detected. Metabolisms rate of the main antibiotic groups are shown in Table 2.2.

Table 2.2 : Metabolisms rate of the main antibiotic groups (Kümmerer, 2008).

Antibiotics group	**Metabolism**
Tetracyclines	Minimal (<20%)
Sulfonamides	High (>80%)
Macrolides	Minimal (<20%)
Fluoroquinolones	Moderate high (20-80 %)
Beta-lactams	High (>80%)

When the animal waste containing antibiotics is applied to land as a soil conditioning it should be considered the potential impact of antibiotic residues on the environment. Frequent use of antibiotics has also raised concerns about increased antibiotic resistance of microorganisms (Sarmah et al., 2006; Bowman,2009;Alvarez et al., 2010). Besides to these environmental effects, antibiotics in the animal manure are also inhibitors of the biogas production from anaerobic digestion of these animal wastes. Different forms of tetracycline resulted in a decrease in methane production in the order of 20 to 40%. Differences in results may be explained by changing study design. These differences include concentration of the antibiotics used, whether antibiotics were added directly to the manure and how the digester was set up and run such as temperature (Bowman, 2009).

Fate and inhibitory effect of oxytetracycline in anaerobic digestion processes have been studied for quite sometimes. Despite some contrary results, it can be said that

oxytetracycline reduces biogas and methane yields in biogas digesters, in most cases without causing complete system failure. Some conclusions which can be drived from these works are; toxicity of OTC is increased by the presence of metabolites and that OTC alone is not as much competent to cause significant inhibition on the anaerobic digestion process (Masse et al., 2000; Lallai 2002; Arıkan et al., 2006; Alvarez et al, 2010).

Studies in general only target physical aspects of oxytetracycline inhibition and microbiological side of the phenomena is generally overlooked. Therefore, the key point to enlighten the unknown behind oxytetracycline inhibiton would be identifiying the microbial diversty which are most immediately affected by oxytetracycline toxicity (Shi et al., 2011).

2.4 Feedstocks for Biogas Production

The term feedstock is defined to include any substrate that can be converted to methane by anaerobic bacteria (Steffen, 1998). Any type of organic waste can be used as a substrate for the anaerobic digestion process as long as it contains carbohydrates, fats and lipids (Alvarez et al., 2010).

Through the organic compounds present in substrates, degredation of fats result with the highest biogas yield and it is required the longest retention time due to its poor bioavailability. Carbohydrates and proteins are easier to degrate but result in lower biogas yields as shown in Table 2.3 (Steffen, 1998; Weiland, 2010).

Table 2.3 : Maximal Gas Yields and Theoretical Methane Contents of Different Substrates (Baserga, U., 1998).

Substrate	Biogas (Nm^3/t TS)	CH_4 (%)	CO_2 (%)
Carbohydrates	790–800	50	50
Raw Proteins	700	70-71	29-30
Raw Fats	1,200-1,250	67-68	32-33
Lignin	0	0	0

The biogas yield of the individual substrates varies considerably dependent on their source, content of organic matter, and substrate composition as shown in Figure 2.5.

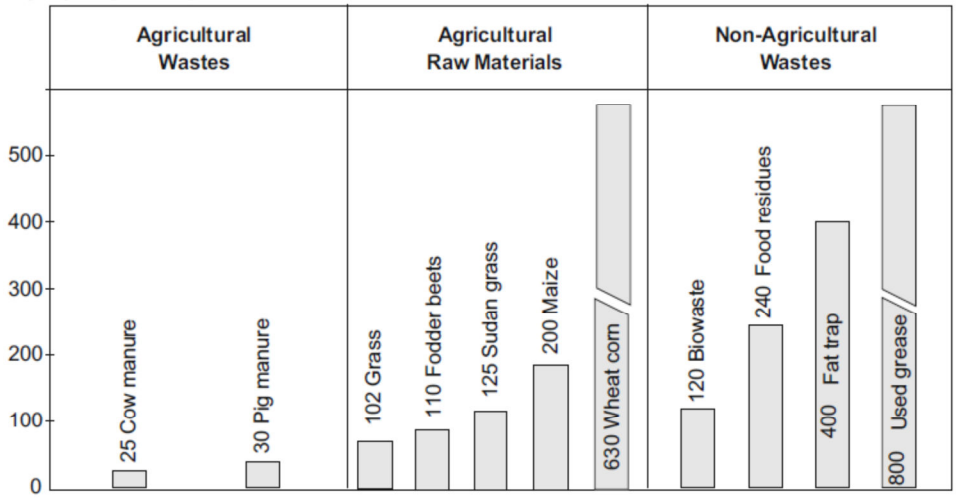


Figure 2.5: Mean Biogas yields of different substrates (m^3/t Fresh Material) (Weiland, 2010).

2.4.1 Livestock manure for anaerobic digestion

In history, anaerobic digestion has been mostly associated with animal manure and sewage sludge. Manure from ruminants, particularly bovines, is very useful to start the fermentation process, since it already contains the essential methanogenic populations. On the other hand, cattle draw a higher percentage of nutrients out of the fodder and the leftover lignin complexes from high-fibber fodder are very resistant to anaerobic fermentation. Therefore, recently, most of the agricultural plants digest manure from pigs, cows and chicken with the addition co-substrates to increase the biogas production and methane yield. (Steffen, 1998; Li et al., 2010; Monteiro et al.,2011). These co-substrates are generally harvest residues and food waste from households and energy crops (Weiland, 2010). Several studies have been made on the co-digestion of various organic wastes with manure (Demirbaş, 2006; Liu et al., 2009; Comino et al., 2009; Li et al., 2010; El-Mashad and Zhang, 2010; Goberna et al., 2010; Cavinato et al., 2010). Beside to that, some researches has been conducted on digestion of multi-component substrates (Misi and Froster, 2001; Alvarez and Liden, 2008) for improved methane production.

Depending upon the livestock species and the age of the animals, the proportion of volatile solids in the manure can range from under 45 to almost 85% of the total solids content (ASAE 2002b). The volatile solids provide an approximation of the organic matter content of the manure (ASAE 2002a). Manure contains both organic and inorganic nutrients that may be dissolved into or suspended within the liquid

phase. The inorganic nutrients present in livestock manure include the macro or primary nutrients nitrogen (N), phosphorous (P), potassium (K), and sulphur (S), the secondary nutrients calcium (Ca) and magnesium (Mg), and micro nutrients such as boron (B), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mb), and zinc (Zn) (ASAE 2002b; Lague et al., 2005).

Livestock manure also contains many microorganisms that help to work the anaerobic digestion process efficiently. On the other hand, it contains microorganisms including bacteria, fungi, protozoa and viruses. Some of these microorganisms may be pathogenic to humans. Manure treatment techniques, such as anaerobic digestion which can be very effective in reducing the bacterial load of livestock manure prior to land application and this provides to environmental protection (Lague et al., 2005).

2.5 Biogas from Anaerobic Digestion Systems

Biogas is a mixture of gas which is highly flammable and results from the decomposition of organic wastes in the absence of oxygen. Composition of the gas depends on the waste type, digestion time and other operational parameters (Ilkılıc et al., 2011). The most important parameters for the biogas generation rates were the digestion time, temperature, species of feeding substrate, pH, and the TS concentration in the slurry. Other factors that affect the rate and amount of biogas output include the water/solids ratio, carbon/nitrogen ratio, mixing of the digesting material, and the particle size of the material being digested (Demirbaş, 2006). In general, biogas contains 40-70% of methane (CH_4), 30-60% of carbon dioxide (CO_2), moisture and small amounts of hydrogen sulfide (H_2S) (Ilkılıc et al., 2011).

Methane has 25 times the warming effect of CO_2 and has a relatively short atmospheric lifetime (approximately 12 years) when compared to the atmospheric lifetime for carbon dioxide, which has a half-life of roughly 100 years (Monteiro et al.,2011; Bracmort, 2010). When its atmospheric concentration and half life are considered, CH_4 is thought to contribute 4–9% of the global greenhouse gas (GHG) effect (Attwood et al., 2011). Therefore, some asserts that efforts to overcome methane from anthropogenic sources may ensure near-term climate change abatement(Bracmort, 2010).

2.6 Process Technology of Biogas Plants

Various process types are applied for biogas production which can be classified as wet and dry fermentation (Ward et al., 2008). In wet fermentation, digesters are operated below 10% TS that allows the application of completely stirred tank digesters. The digested material can be used for the purpose of the fertilization on fields (Nelson and Lamp, 2002). Solid matters like energy crops are mixed with liquid manure in order to achieve appropriate TS concentration. Dry digestion processes are run with TS concentrations ranging from 15% to 35% and operated both continuously and batch reactors while the wet digestion processes are operated only continuously. In general, wet digestion processes are more frequently used than dry digestion processes in the agricultural sector (Weiland, 2010).

The most common wet fermentation reactor is the vertical continuously stirred tank fermenter. It is applied nearly 90% of modern biogas plants in Germany. Often, the fermenter is covered with a gas tight single or double membrane roof to store the gas in the fermenter top before utilization. Mechanical, hydraulic or pneumatic mixing are applied for the effective stirring in order to contact microorganisms with the substrate, to enhance the upflow of gas bubbles and to get the constant temperature in the whole digester. Almost 90% of biogas plants use mechanical mixing equipment. Horizontal digesters are also applied in anaerobic digestion for biogas. These are plug-flow systems equipped with a low rotating horizontal paddle mixer (Nelson and Lamp, 2002).

Morever, the anaerobic digestion process can be applied in a single or multi step process. In order to improve the stability and rate of degredation hydrolysis and acidogenesis steps are seperated from the methanogenesis step, thus this is called two phase digesting systems (Ward et al., 2008). In this way, hydrolysis and methanogenesis stages can be maximally optimised (Hassan, 2003). For instance, in a comparison of one- and two-stage thermophilic reactors treating cattle manure, it was found that the two-stage digester had a 6–8% higher specific methane yield and a 9% more effective volatile solids removal than the conventional single stage reactor (Nielsen et al.,2004; Ward et al., 2008).

2.7 Utilization of the Digestion Products

The biogas is utilized in gas engines for Combined Heat and Power (CHP) generation (Monnet, 2003; Ahring, 2003-II;Aoki et al., 2006). Electric efficiencies can be achieved up to 43% (Weiland, 2010). Heating value of biogas is ranged from 17000 to 25000 kJ/Nm^3 as depending on the methane content. Table 2.4 shows that fuel value of biogas is depend on the methane gas content in the mixture (Ilkılıc et al., 2011).

Table 2.4 : Maximal Gas Yields, Theoretical Methane Contentsand heat efficiency of Different Substrates (Baserga, U., 1998).

Organic matter	Biogas (Nm^3/t TS)	CH_4 (%)	CO_2 (%)	Heat efficiency (KJ/Nm^2)
Carbohydrates	800	50	50	17782
Raw Proteins	700	70	30	24894
Raw Fats	1200	67	30	23639

Through the biogas composition methane which has an energy potential, must be dissocited from other energy diluent gases (CO_2, H_2S ve H_2O) (Ilkılıc and Deviren 2011).The upgraded gas must have a methane content of more than 95% in order to fulfill the quality requirements of the different gas appliances. In addition, biomethane should not contain bacteria and molds that could create unacceptable risks for human health and equipment (Weiland, 2010).

The residue coming from the anaerobic digestion process, which are called the digestate, can be spread on farmlands as fertilizer (Holm-Nielsen et al., 2009; Rico et al., 2011). The digestate isrich in mineralized nitrogen and the C/N ratio is lowered which increases the short-term N fertilization effect (Monnet, 2003; Weiland, 2010; Masse et al., 2011).60% of the nitrogen in cow slurry is regarded as effective nitrogen on the long run. After digestion this is considered to be 78%. This advance in effective nitrogen will effect the fertilization capacity of the product. This has been taken into account. While, the total effective nitrogen required is the same in both situations, less mineral fertilizer will be used since more nitrogen is available from the digestate (de Vries et al., 2010). In addition, the digestate penetrates into soil more easily thus, the loss of nitrogen to ammonia is lowered. The digestate is also less odorous and purified from pathogens effectively (Salter, 2006). This treatment

leads to a reduction up to 80% of the odour (Monnet, 2003). These improved characteristics of the digestate make it highly suitable for utilization as fertilizer (Weiland, 2010).

2.8 Molecular Methods Used in Microbial Ecology of Anaerobic Treatment

Classical microbiology techniques used in identification of environmental microorganisms are mostly based on cultivation and required the methods on selective growth media. These methods have certain limits which prevent an efficient identification of the community and it is also time consuming method (Sanz et al., 2007). Since, there are many groups of microorganisms difficult to grow, this technique is not able to detect whole microorganisms (Schmidt et al., 2000; Zhou et al., 2011). Thus, non-culture based analysis of microbial populations via phylogenetic analysis is becoming an increasingly important tool in the determination of microbial communities. Molecular biology tools increased understanding of composition, dynamics and interactions within microbial ecosystems (Zhou et al., 2011). Molecular phylogeny has provided a new basis for the direct identification and quantification of microorganisms (Olsen and Woese, 1993). Nucleic acids are biomarkers and hereditary molecules most probably because of their important role in protein synthesis, making them one of the earliest evolutionary functions in all cellular life-forms (Woose, 1987).

Particularly, 16S rRNA, and its encoding genes are ideal biomarkers (Dahllöf, 2002; McArthur, 2006; Sanz et al., 2007; Narihiro and Sekiguchi, 2007). The prokaryotic small subunit (SSU) rRNA gene, or 16S rRNA gene, is highly conserved but contains variable sequence regions. These variations allow classification of microorganisms into two domains (*Archaea* and bacteria) and more discrete taxonomic levels (Talbot et al.,2008). It is possible to design general and specific primers and probes for the study of evolution to species level. The rRNA is highly conserved in nucleotide sequence as well as in secondary structure since its function remains same through years of evolution. Random changes in the variable regions occur time to time and reflect to evolutionary relationship of organisms (Amann et al., 1995). There are several molecular biology approaches in the studies on microbial ecology of the anaerobic reactors, a summary is given in Figure 2.6 and Table 2.5.

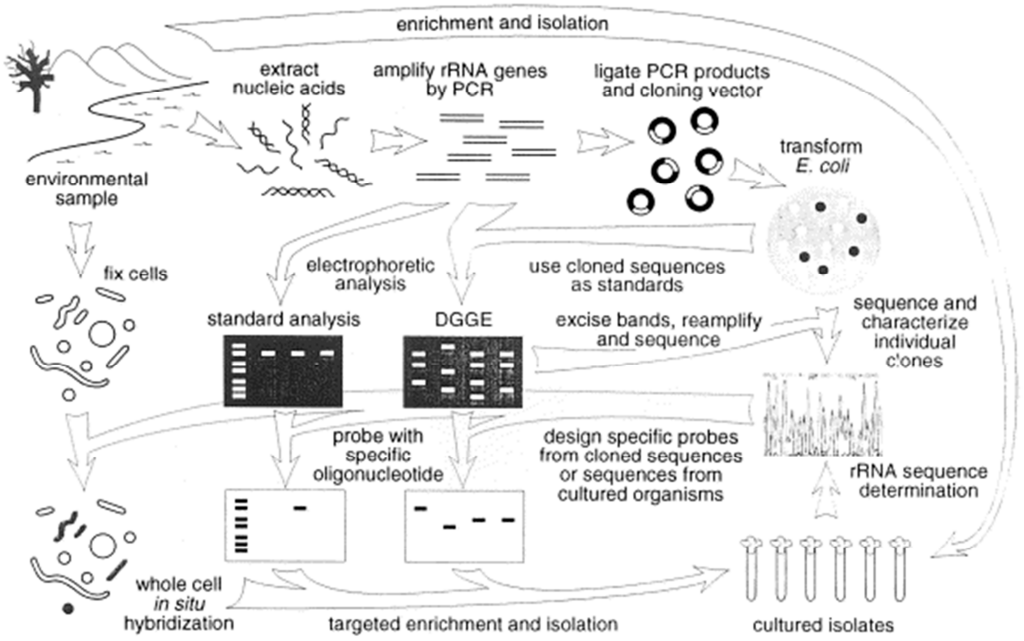


Figure 2.6 : Summary of phylogenetic methodologies used in microbial ecology (Scow et al, 2004).

Table 2.5 : Summary of common molecular methods used in microbial ecology

Approach	Description	Remarks
Cultivation	Study micro-organisms in defined circumstances.	Only a minor fraction of the micro-organisms can be cultivated.
PCR	Specific and sensitive amplification of genetic material (DNA/RNA).	Primers developed from known sequences and can cause bias.
Real-time PCR	Sensitive quantitative amplification suitable for high-throughput over a wide dynamic range.	Sensitive quantitative amplification suitable for high-throughput over a wide dynamic range.
Fingerprinting (DGGE/SSCP/TRFLP)	Rapid overview of diversity. Ideal for comparisons of ecosystems in time or between different samples.	Bias in nucleic acids extraction and PCR. Only dominant populations can be visualised.
Sequencing	Gold standard for sequence retrieval.	Nucleic acids extraction, PCR and cloning can be biased.
FISH *In situ* isotope tracking	Enumeration of micro-organisms *in situ*. Allows localization and quantification.	Laborious without automatisation and requires sequence information for probe development. Cell permeabilization and fixation can cause bias.

Analytical techniques targeting 16S rDNA or functional genes were widely used for microbial quantification (Schmidt et al., 2000). These methods include hybridization based techniques, such as membrane hybridization and fluorescence in situ

hybridization (FISH) as well as polymerase chain reaction (PCR) based techniques, such as denaturing gradient gel electrophoresis (DGGE) and cloning sequencing (Dahllöf, 2002; Zhang and Fang, 2006; Sanz et al., 2007; Talbot et al.,2008; Cetecioglu et al., 2009). In the literature, there are many studies in which these molecular tools are used for investigation the methanogenic populations quantitatively (Schmidt et al., 2000; Leung et al., 2001; Keyser et al., 2006; Akarsubasi et al., 2006; Sawayama et al., 2006; Kobayashi et al., 2009; Montero et al., 2009; Ince et al., 2007, 2009; Kim et al., 2010; Song et al., 2010; Bialek et al., 2011; Shin et al., 2011; Kolukirik et al., 2011). These studies provide valuable qualitative information, including the syntrophic and competitive interactions between microbial structure and process performance (Dahllöf, 2002). It is also useful to understand the microbial diversity of an engineered anaerobic process (Amann et al., 1995).

2.8.1 Polymerase chain reaction (PCR)

PCR is a tecnique used to amplify the amount of target DNA up to 10^6-fold or more and has revolutionized molecular biology. PCR is a relatively simple enzymatic reaction used to generate copies of a target DNA sequence through a series of temperature cycles. During each PCR cycles,denaturation–annealing–extension cycles, the amount of target DNA is theoretically doubled, resulting in an exponential increase in the amount of DNA (Zhang and Fang, 2006). PCR products are typically visualized by agarose gel electrophoresis and the size is estimated by comparison with DNA standards of known size run in the same gel (Maier, 2009).

PCR process mainly based on three steps: denaturation, annealing, and extension. In denaturation step double stranded DNA templates melted and separated by high temperature. In annealing step the reaction temperature is lowered so that the primers can attach to the single-stranded DNA template. Then temperature is increased again to a level (72 °C mostly) in which Taq polymerase can elongate the chain by adding nucleotides (dNTPs). This cycle of binding of primer and elongation and then disassociation repeated 30-40 times to recover enough DNA segment of interest. The addressed sequence amplified in order of 2 (2n where n is the cycle number). The resulted product will be run on an agarose gel to monitor efficiency of the PCR.

Mostly Ethidium Bromide (EtBr) is used to stain DNA which renders DNA visible under UV light.

2.8.2 Real time quantitative PCR (Q-PCR)

Although PCR amplification is a rapid method to detect methanogens, it is not quantitative. In contrast, Real-time PCR is an approach developed to provide quantitative measurement of a target from the early phase of PCR amplification (Zhou et al., 2011). Real-time PCR (RT-PCR) or Quantitative PCR(Q-PCR) technology is highly flexible and many alternative instruments and fluorescent probe systems have been developed recently. The decreased hands-on time, increased reliability and improved quantitative accuracy of RT-PCR methods have contributed to the adoption of RT-PCR for a wide range of new applications (Logan, 2009).

In the real time PCR fluorescent molecules are present in the reaction mix and bind to amplicons as they are made during the PCR process. Thermocyclers with fluorescence readers are used to quantify amplicon accumulation once at the end of each PCR cycle. So that,real-time PCR allows more sensitive detection of amplicons as well as quantification of the target molecule present in the original sample (Zhang and Fang, 2006; Talbot et al., 2008).

The bias often observed in the PCR template-to-product ratios can be largely avoided. This is most commonly achieved through the use of fluorescence-based technologies. There are some approaches to generate fluorescence in real-time PCR. The first is the use of a general fluorescent dye, SYBR Green I, that intercalates into dsDNA. The second is the use of some type of fluorescent probes that specifically binds to the target sequence, essentially combining PCR and gene probe technologies into one step. One example of real-time fluorescent probes are 5'-exonuclease or TaqMan probes, which have enzymatically released fluors (Maier, 2009). In addition to hydrolysis probes (TaqMan), hybridization probes (LightCycler) are also used for generating fluorescence in real-time PCR. Another agent that fluoresces is fluorescent hairpins (Zhang and Fang, 2006; Talbot et al., 2008).

For absolute quantification, a RNA standard curve of the gene of interest is prepared in order to calculate the number of copies. In this case, a serial dilution of a known amount (number of copies) of pure RNA is made and subjected to amplification. The

unknown signal is compared with the standard curves so as to calculate the starting concentration.

2.8.3 Fluorescence in situ hybridization (FISH)

Probes with fluorescent labels can be used to investigate cells *in situ* (i.e., in a culture or in an environmental sample) in a technique called fluorescent *in situ* hybridization (FISH). In this case, reagents are added to facilitate penetration of the probe through the cell membrane, and this probe ultimately hybridizes to its target sequence. Cells containing the target sequence are visualized under a fluorescent or confocal microscope (Pernthaler et al., 2001). Combination with a confocal laser-scanning microscope allows the visualization of three-dimensional microbial structures (granules, biofilms) (Sanz et al., 2007). This tecnique has the advantage of allowing visualization of spatial relatinships between populations within a community to be elucidated (Schmidt et al., 2000; Pernthaler et al., 2001; Maier, 2009).

This molecular tool consists of four main steps:

- The fixation and permeabilization of the cells.
- Hybridization with fluorescently labeled oligonucleotide probes.
- Washing to remove the unbound probes.
- Detection the hybridized cells by epifluorescent microscopy or flow cytometry (Amann, 1995).

First step is the fixation of microbial cells with appropriate chemical fixatives and then cells are hybridised under optimal conditions on a glass slide or in solution with oligonucleotide probes. These probes are generally 15–25 nucleotides in length and are labelled covalently at the 5'end with a fluorescent dye (Sanz et al., 2007). After the washing step is applied, specifically stained cells are detected by epifluorescence microscopy or flow cytometry (Pernthaler et al., 2001). The determination of composition and number of microbial community can be achieved mostly by rRNA-targeted oligonucleotide probes without cultivation, directly in their natural environment. Because, its product (16S rRNA) is in ribosomes, the targeted sequence occurs in multiple copies in metabolically active cells in all prokaryotes (Seviour and Nielsen, 2010). This allows a quantification of rRNA concentrations both in single cells and in the environment (Pernthaler et al., 2001).

Despite the advantages, FISH technique has its own limitations. The most significant one is that not all bacterial and archaeal cells can be permeabilisied by oligonucleotide probes using standart fixation protocols (Amann et al., 1995). It is not always possible to design a specific probe for a certain group of microorganism. The design and assessing optimum conditions for hybridization for a new probe is a difficult dedication (Sanz et al., 2007).

2.8.4 Denaturing gradient gel electrophoresis (DGGE)

Pattern analysis or fingerprinting is often carried out by evaluating banding patterns of PCR products on gels (Dahllöf, 2002) to estimate the level of diversity in environmental samples, to follow changes in community structure, to compare diversity and community characteristics in various samples and simply to identify differences between communities (Dahllöf, 2002; Hofman-Bang et al., 2003; Talbot et al., 2008). DGGE is a gel electrophoresis method that separates genes/ DNA fragments of the same size (obtained after PCR of DNA extracted from an environmental sample) that differ in base sequence, at least by one nucleotide into distinct bands on a chemical denaturing gradient polyacrylamide gel.The number of bands corresponds to the number of dominant species. Coupled with sequencing and phylogenetic analysis of the bands, this method can give a good overview of the composition of a given microbial community (Dahllöf, 2002; Kan et al., 2006; Sanz et al., 2007).

2.8.5 Molecular cloning, sequencing and phylogenetic analysis

The process of creating identical copies of a gene, has enabled scientists to find new or closely related genes, as well as characterize and identify unculturable or unknown isolates (Maier, 2009). Using molecular cloning, large quantities of genes or chromosomal fragments can be isolated in pure form (Madigan et al., 2002). Also, cloning can be used to identify the organisms in an environmental sample. The DNA fragments can be produced after digestion with restriction enzymes of the DNA extracted from a sample (i.e., shotgun cloning), or after PCR or RT-PCR (if RNA is the template) (Hofman-Bang et al., 2003).

Cloning consists of 5 steps:

1. Isolation and fragmentation of source DNA which can come from PCR amplification.
2. Joining or ligation of the DNA fragments into a cloning vector such as plasmid.
3. Insertion of the resulted recombinant DNA molecule into a clone host such as E.coli through transformation.
4. Propagation, selection and screening for clones that contain the recombinant DNA molecules.
5. Analysis of the source DNA fragment (Maier, 2009).

2.5 Aim of the Study

It has been pointed out that energy production from biomass has gained interest all over the world including Turkey. Many type of biomass can be used in the anaerobic digestion process. Manure is among the most popular substrates used for the production of biogas in which inhibitory substances like veterinary antibiotics are commonly detected. Although effect of these substances on biogas production has been studied by engineering perspective, studies regarding their possible-effects on microbial populations are rather scarce. Therefore, this study aims to evaluate the effect of OTC, a commonly used veterinary antibiotic, on active microbial community structures and biogas production in thermophilic manure digesters. In this study, inhibition effect of OTC was investigated under changing operational parameters such as different solid content and mixing rates to understand response of microbial structuresand performance ofthe thermophilic batch digesters.

3. MATERIALS AND METHODS

3.1 Digesters Experiments

3.1.1 Manure sampling and animal medication

A female, Holstein race, 3.5 years old, 440 kg body mass dairy cow was kept in a pen at the Istanbul University Veterinary Faculty Barn. The manure in rectum was collected and stored at 4°C until later use as the "blank manure". This animal was then medicated with veterinary antibiotic oxytetracycline (OTC) with the commercial name Teknomycin LA 200(TEKNOVET, Turkey). The dairy cow was medicated once with 50 ml Oxytetracyline injection solution (20 mg/kg). This is a standard dosage in veterinary practice. Equal doses were injected to right and left body between *musculus semitendinosus* and *musculus semimembranosus* muscles. Manure was collected from rectum every 24 hours for 5 days and mixed together until homogenous state has been established. This mixed manure was then lableled as "medicated manure" and used throughout the experiments. Collected manure samples are stored in volume of 1 L containers and brought to laboratory in cold chain. Until the experiments, samples are kept in $+4^0$C.

3.1.2 Lab-scale digesters setups

Digesters were prepared in 1 L glass reactors in which active volume was 600 ml. For each reactor, 1 literslurry was prepared which contains 1/10seedsludge. While 600 ml slurry was put in the reactor, 100 ml slurry was analyzed as the sample of 0th time. The remaining 300 ml slurry was stored at 4°C and usedto replacethe lostvolume which was taken from the digester for sampling. The pH of eachdigesterwasset to bepH 7.0. Digesters were flushed with nitrogen gas for 3 minutes to provide anaerobic conditions. After the controlling of the air-tightness, digesters were placed on the temperature controlledincubatorshaker as seenFigure 3.1.

Figure 3.1 : Digesters placed on the temperature controlledincubatorshaker.

The amount of biogas produced was measured with the miligas counters. Biogas composition was also measured with the HP 6850 GC. At the each sampling time approximately 100 ml slurry was taken for physicochemical, analytical and molecular analysis.

In the Set1, temperature was 55 ± 1.0 °C and mixing rate was 90 rpm. In this set, digesters which contain low solid content of 5-5.5% and high solid content of 7.5-8% TS were designed for the investigation of the effect of different solid contents. For this set, hydraulic retention time was set to 20 days and samples were collected in every 5 days. Temperature was as follows: 37 °C for 1.day, 40 °C for 2.day, 45 °C for 3. day, 50 °C for 4. day and 55 °C for 5. day for acclimization of microorganisms to increasing temperature.

In the Set2,temperature is 55 ± 1.0 °C and mixing speed is 120 rpm. In this set, digesters which contain low solid content of 4.5-5% % and high solid content of 5.5-6.5% TS were designed for the investigation of the effect of different solid contents.Set2 was clone of Set1 except mixing rate. Initial conditions of all digesters for both two sets are given in Table 3.1.

Table 3.1 : Initial conditions of the digesters of Set1 and Set2.

Sets	Digesters	Temperature (^{0}C)	Mixing Rate(rpm)	TS (%)
Set1	D1	55	90	Low TS content (5±0.5% TS)
	D3			
	D4			High TS content (7 ±1 % TS)
	D5			
Set2	D1	55	120	Low TS content (5±0.5% TS)
	D3			
	D4			High TS content (7 ±1 % TS)
	D5			

3.2 Physical-Chemical Analytical Analyses

During the operating of digesters some parameters were monitored such as temperature, pH, compositon of VFA, biogas production, methane composition and yield. VFA, TS and TVS concentrations were determined in the samples according to American Public Health association APHA, 2005.

Gas pressures were measured with a manometer (HACH PM-9107) for every 5 days. Gas compositions were determined using Gas Chromatograph HP Agilent 6850 with a thermal conductivity detector and HP Plot Q Column (30 m, 530 μm). Methane and biogas productions were calculated and given as the volume in ambient conditions (1 atm, 20 °C). VFA measurements were carried out in a Perkin Elmer Gas Chromatograph (Clarus 600) with an FID detector and Elite-FFAP column (30 m, 0.32 mm).

OTC measurement with the high-performance liquid chromatography (HPLC)

The amounts of antibiotics in the manure sample which contains OTC was measured with the HPLC analysis of the collected manure.

Chemicals and Reagents:

Acetic acid glacial (BDH-GPR), Oxalic acid dihydrate (Merck),Methanol and Acetonitril (LiChrosolv) were commercially supplied. Oxytetracycline was purchased from Agros Chemicals. Methanol and Acetonitril were HPLC grade. The other chemicals were of analytical grade. Double distilled water was used throughout the analysis.

Apparatus and Chromotographic Conditions:

HPLC instrument was a Shimadzu, (Schimadzu LC-10 AD) HPLC equipped with an UV detector; (UV VIS Detector, SPD 10-A) operating at 357 nm. The analytical column used in this study was InertsilODS-3 HPLC column, 25 cm x 4.6 mm ID, 5 μM.An autosampler, SIL-10 AD was used for injection. The injection volume was 20 μl. Degassing of the solvents was achieved by sonication, in a Transonic ultrasonic bath, ELMA D-78224 Singen/Htw prior to use. All of the results were analysed by the system software; Class VP (Schimadzu Scientific Instruments Inc.)

The Inertsil ODS-3 analytical HPLC column was used at ambient temperature. The mobile phase consisted of 75% 0.1M oxalic acid buffer and %25 Methanol: Acetonitril (1:1,5) solution which was delivered isocratically at a flow rate of 1 ml/min. The mobile phase was degassed prior to use. The total run time was 30 min. Wavelenght for the detection of oxytetracycline was 357 nm.

Before every analysis, analytical column was conditioned with the mobile phase, until a clean baseline was observed. After an acceptable baseline was achieved, standards and then the samples were analyzed.

Extraction of OTC from Manure

Extraction was done according to a method modified from Yuan et al. (2010). 5 g wet manure was put into 50 mL polycarbonate centrifuge tubes with 0.5 g Oxalic acid ($C_2O_4H_2 \cdot 2H_2O$), 4 mL acetic acid and 7.5 mL of 90% methanol and shaked at 100 rpms for 30 minutes. The tubes were further centrifuged at 11000 rpm for 10 minutes. This procedure was repeated for 3 times and the supernatants were collected in 50 mL volumetric flasks. Flasks were diluted to 50 mL with double distilled water and centrifuged again at 14000 rpm for 3 minutes and filtrated through 0.2 µm Millipore filters. The extracts were kept in 2 mL amber vials at -20 ºC until the day of HPLC analysis.

3.3 Molecular Analyses

3.3.1 Fluorescence in situ hybridization (FISH)

After the gas composition and gas pressure values were obtained, 5 mL of the samples were transferred to Falcon tubes and diluted 1:1 with absolute ethanol and stored at -20^{0}C and fixed with Paraformaldehyte (PFA) within 3 days.

For the standard PFA fixation, 1 mL ethanol-sample mixture was transferred to 1.5 mL Eppendorf tubes and washed with 0.5 mL 3X Phosphate Buffer Saline (PBS) for two times and resuspended in 0.25 mL 3x PBS and 0.75 mL freshly prepared 4% PFA and incubated for 3 hours at +4^{0}C. After incubation, cells were washed once with 3x PBS and resuspended in 1 mL 1:1 ethanol: 1x PBS mixture and stored at -20^{0}C until hybridization.

For the hybridization, oligonucleotid probes targeting 16S ribosomal RNAs (rRNAs) listed in Table 3.2 were used.

Table 3.2 : 16S rRNA-targeted oligonucleotide probes used in this study.

Probe	Target Group	Probe Sequence (5'-3')	Labelling (5')	Reference
UNIV1393	Virtually all known organisms	ACGGGCGGTGTGTAC	CY3	Raskin et al., 1994a
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	CY3	Stahl et al., 1988
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	CY3	Amman et al., 1990a
MB310	*Methanobacteriales*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin et al., 1994a
MG1200	*Methanomicrobiales* relatives	CGGATAATTCGGGGCATGCTG	CY3	Raskin et al., 1994a
MS1414	*Methanosarcina* + relatives	CTCACCCATACCTCACTCGGG	CY3	Raskin et al., 1994a
MSMX	*Complete acetoclastic methanogens*	GGC TCG CTT CAC GGC TTC CCT	CY3	Raskin et al., 1994a
NON338	Non sense probe	ACTCCTACGGCAGGCAGC	CY3	Raskin et al., 1994a

The methanogen targeted probe sequences and classification of the methanogens are given in Figure 3.2. Their optimum hybridization conditions are given in Table 3.3.

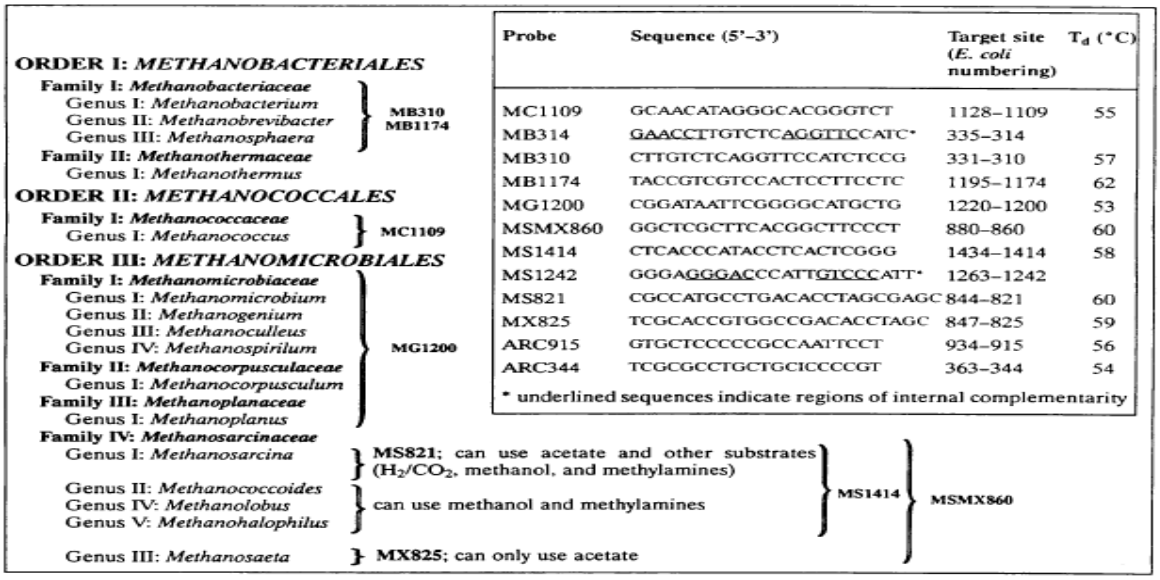


Figure 3.2 : Classification of methanogens in relationship to the oligonucleotide probes characterized (Raskin et al., 1994).

Table 3.3 : Optimum hybridization conditions for oligonucleotide probes (Kolukırık, 2004).

Probe	Formamide concentration	Hybridization temperature (^{0}C)	Washing temperature (^{0}C)	NaCl concentration
UNIV1393	10%	37	37	450 mM
ARC915	35%	46	48	84 mM
EUB338	10%	46	46	450 mM
MB310	20%	46	48	225 mM
MG1200	30%	46	48	112 mM
MS1414	35%	46	48	84 mM
MSMX	35%	46	48	84mM

For each hybridization, two negative controls were prepared; one for assessing non-specific bindings (with Non338 probe), and the other (lacking a probe) monitoring autofluorescence. In addition to negative controls, one positive control was prepared to assess success of cell permeabilization and rRNA content of the cells (with universal probe UNIV1392).

20-25 µl of the fixed samples were transferred to new microfuge tubes. The amount was determined by the microorganism density in the sample. The samples were than washed 2 times with 1 ml 3X PBS and once with ddH_2O. After washing, the pellet was resuspended in 0.5 mL ddH_2O. The slides were dehydrated through ethanol series (50%, 80%, 96%) for 3 minutes. 17 µl hybridization buffer (2 mg/ml Ficoll, 2 mg/ml Bovine serum albumen, 2 mg/ml polyvinyl pyrolidone, 5 mM EDTA, Tris HCl, pH 7.2, 25 mM NaH_2PO_4, NaCl, pH 7.0, 0.1% SDS) and 3µl targeted probes were added and incubated at the optimal hybridization temperature for the given probe for 4 hours. Following hybridization, the cells were washed twice in a wash buffer containing 20 mM Tris-HCl (pH 7.2), 0.01% SDS, 4.5 M NaCl before a final wash in MilliQ water. The cells were resuspended in 200 µl of MilliQ water, and then dried. 10 µl of DABCO (1,4-diazabicyclo[2.2.2]octane) [Sigma D-2522]: 0.233g DABCO 800 µl ddH2O200 µl TRIS-HCl (pH=7.2) was added to the cells, and a coverslip was applied and sealed with nail polish before epifluorescence microscopy**.**

In DAPI staining, the total cells present in the samples were previously determined by counting 4,6-diamine phenylindol (DAPI) stained cells. Hybridization procedure of a regular sample was followed except the hybridization time in incubator. Hybridization time needed for DAPI is 15-20 minutes at 46^0C. Slides were examined under Olympus BX 50 epifluorescence microscope equipped with a 100 W high-

pressure mercury lamp, U-MWIB and U-MWG filter cubes. Images were captured using a Spot RT charged coupled device (CCD) camera having special software supplied by the camera manufacturer (Diagnostic Instruments Ltd., UK).The dilution percent needed is determined by counting DAPI added cells. For all times, counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated. Average of the counts gave the representative number of total microorganisms in each sample. Images were processed and analyzed using Image-Pro Plus version 6.3 image analysis software (Media Cybernetics, USA).

Different fluorochromes are excited and emitted at different wavelengths. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used in this study are given in Table 3.4.

Table 3.4 : Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used.

Fluorochrome	Color of Fluorescence	Maximum Excitation Wavelength (nm)	Maximum Emission Wavelength (nm)	Filter Cube Used
CY3	Red	552	565	U-MWG
DAPI	Blue	365	397	U-MWG

3.3.2 Real time PCR (Q-PCR)

The Q-PCR assays were performed by using Roche LightCycler DNA Master SYBR Green I kit and Roche Light Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). The primers used in this study are given in Table 3.5.

Table 3.5 : Information regarding the 16S rDNAspesific primers used in this study

Primer	Target Organism	Annealing Temperature	Reference
Bac519f	Bacteria	53C°	Lane, 1991
Bac907r			
MSaeta170f	*M.Saeta* sp.	59C°	Hwang et. al, 2005
MSaeta390r			
MBac857f	*M.Bacteriales*	60C°	Hwang et. al,. 2005
MBac1996r			
MMic282f	*M.Microbiales*	59C°	Hwang et. al, 2005
MMic832r			
MSarles812f	*M. Sarcinales*	55C°	Hwang et. al, 2005
MSarles1159r			

Light Cycler Software 4.05 program provided by Roche was used analyze Q-PCR data. The program consisted of 4 sections; denaturation (95^0C), amplification (95^0C, 53-60^0C (annealing temperature in Table 3.5), 72^0C), melting (95^0C, 53^0C, 95^0C) and cooling (40^0C).

3.3.2.1 RNA extraction

RNA was extracted using Purelink RNA Mini Kit (Life Technologies California, US) with slight modifications. Approximately 500 μL sample taken from the digesters was added up to lysing matrix tubes. The tube contains mixture of ceramic and silica particles to lyse all microorganisms in sample and was supplied by MP Biomedicals. 600 ul LB ((594 ul * n) Lysing buffer + (6 ul * n) 2-mercaptoethanol mixture) was added and vortexed for 10 seconds. Then lysing matrix tubes were spinned in Ribolyser (Fast Prep TM FP120 Bio 101 Thermo Electron Corporation) for 45 seconds at speed of 6.5. The tubes were then centrifuged to precipitate the pelletat 14000 rpm for 5 minutes at 4^0C. After centrifugation supernatants were transferred to clean 1.5 ml appendorf tubes and added 500 ul 70% ethanol ((350 ul * n) absolute ethanol + (150 ul * n) RNAase free water mixture). To mix the composition tubes were vortexed for 10 seconds. The 600 ul mixture is taken in spin cartridge and centrifuged at 12000 rpm for 15 seconds at room temperature. After that the collection tube was emptied and put the filter in collection tube again. These last two steps were repeated until end up the sample mixture. 700 ul Wash Buffer I was added into spin cartridge. Tubes were centrifuged at 12000 rpm for 15 seconds at room temperature and the collection tubes was removed and put the spin cartridge in a new collection tube. 500 ul Wash Buffer II (contains ethanol) was added into spin cartridge. Tubes were centrifuged at 12000 rpm for 15 seconds at room temperature and the collection tubes was emptied and put the filter in collection tubes again. 500 ul Wash Buffer II was added into spin cartridge again. Tubes were centrifuged at 12000 rpm for 15 seconds at room temperature and the collection tubes were emptied and put the filters in collection tubes again. After that tubes were centrifuged at 12000 rpm for 15 seconds at room temperature. The collection tubes were removed, the filters were placed to recovery tubes. Covers of tubes were cut and these covers were hold in sterile environment. 50 ul RNAse free water was added and mixed 1-2 times with pipet than closed with spin cartridge covers. Finally, tubes were incubated for 1 minute at room temperature. Than, tubes were centrifuged

at 12000 rpm for 2 minutes. After centrifugation, spin cartridges were removed in sterile environment. Tubes were closed with covers of recovery tubes which is cut before. Application-ready RNA was obtained in the tube. RNA quantity was determined using Nanodrop 2000 (Thermo Scientific, Australia).

3.3.2.2 cDNAsynthesis from extrated RNA

cDNA was synthesized using SuperScript® VILO™ cDNA Synthesis Kit according to procedure. The following components were taken in a tube on ice: 5X VILO™ Reaction Mix (4 µl), 10X SuperScriptR Enzyme Mix (2 µl), RNA (up to 2.5 µg) x µl, DEPC-treated water (20 µl). Tube contents was mixed gently and incubated as following at 25°C for 10 minutes, at 42°C for 60 minutes and the reaction was terminated at 85°C at 5 minutes.

The cDNA generated from total RNA quantities above 100 ng will need to dilute. In this study 20 times diluted cDNA was used for Q-PCR experiment.

4. RESULTS AND DISCUSSION

4.1 Performance of Batch Digesters

In this study, for thermophilic anaerobic manure digesters,HRTs were set to 20 days in both Set1 and Set 2 and samples were taken for analytical, molecular and physicochemical analysisfor every 5 days. Digesters were monitored for biogas production, total solid (TS) reduction, biogas and volatile fatty acid concentrations. In *situ* Hybridization (FISH) and RNA based Q-PCR were used to monitor of active microbial populationsdynamics.

OTC measurements

Precision and accuracy

The analytical conditions maintained were mentioned earlier. Retention time of OTC was found to be 7.3±0.1 min. In order to confirm the correctness of the method, duplicate analysis of five working standard solutions covering the range from 1 to 100 mg/L were made. 20 µL of these standards were injected into the HPLC system and its concentrations were calculated by the software. A calibration curve was plotted with concentration against area. Results of the assay are presented in Figure 4.1.

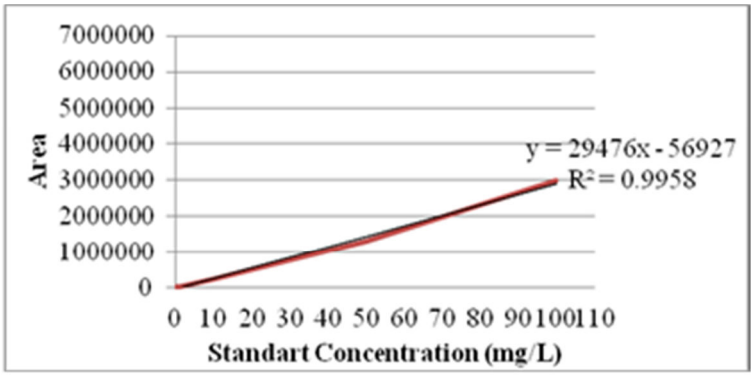


Figure 4.1: Calibration curve for standard solutions.

Determination of the extraction efficiency

The accuracy of extraction was verified by extracting a known amount of OTC spiked into non medicated manure, and analyzing with HPLC. The spiking concentrations were 5, 20, 200 and 1000 mg/g manure. After each extraction OTC was collected in 50 mL of extract. The extract was injected into HPLC. All of the analyses were conducted triplicate. The extraction efficiencies of OTC from manure are given in Table 4.1.

Table 4.1: Extraction efficiencies of OTC from manure.

Amount Collected in 50 mL extract (mg/L)	Concentration in Manure (mg/kg)	Recovery Rate (%)
100	1000	99±0.02
20	200	92±0.1
2	20	85±0.1
0.5	5	77.6±0.04

Sample analysis

After plotting the calibration curve and calculating the extraction efficiency samples were extracted and analyzed. Following extraction, samples were stored at -20ºC until the day of HPLC analysis.

OTC concentration of the sample taken from digesters was collected during the operation on 0., 5., 10. and 20. days and measured with high-performance liquid chromatography (HPLC). OTC concentration and removal efficiency of OTC were shown in Table 4.2.

Table 4.2 : The change of the OTC concentrations with the operating time.

Digesters	OTC concentration (mg/l) in the operating days				OTC removal %	Half life of the OTC (day)
	0. day	5. day	10. day	20. day		
1. Set D1	0	0	0	0		
1. Set D3	2.46	1.29	1.06	0.59	76.0	13
1. Set D4	0	0	0	0		
1. Set D5	4.74	2.88	1.13	0.82	82.7	12
2. Set D1	0	0	0	0		
2. Set D3	1.51	0.82	0.58	0.54	64.2	15
2. Set D4	0	0	0	0		
2. Set D5	2.67	2.08	1.25	1.03	61.4	16

In initial condition, OTC was measured in concentration between 1.51-4.74 mg/l in the OTC containing digesters. Maximum removal rate of OTC during the operation, in 20 days, was 82.7% in digeter D5 of the Set1 (Table 4.2). Figure 4.2 and Figure 4.3 show the change of the OTC concentration/removal of OTC in digesters during 20 days.

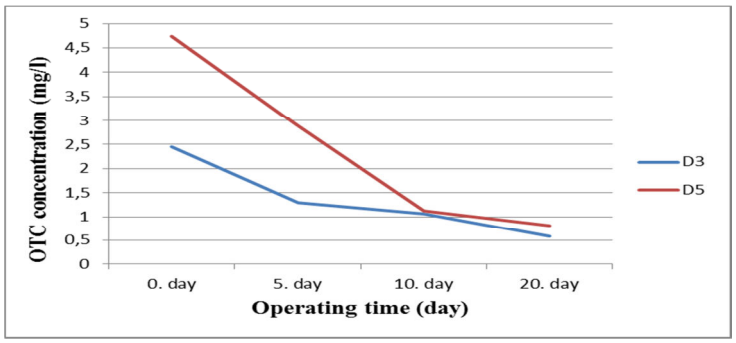


Figure 4.2 : OTC concentrations in digesters of the Set1 during operating time.

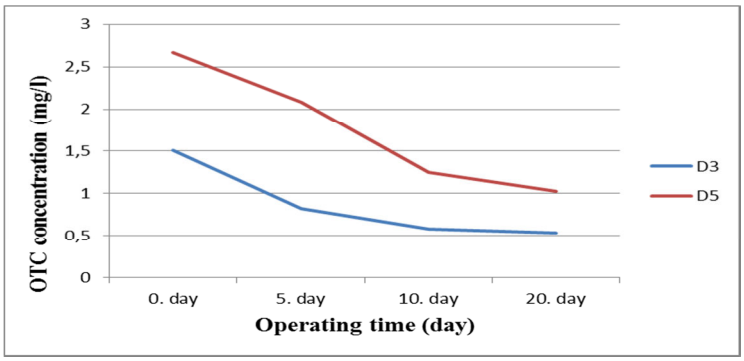


Figure 4.3 : OTC concentrations in digesters of the Set2 during operating time.

In this study, half life of OTC was found 14 days as shown in Table 4.9 in thermophilic cattle manure digesters. Arıkan et al. (2006) reported that a 59% removal of OTC was achieved during 64 days of mesophilic anaerobic digestion of manure, yielding a calculated OTC half-life value of 56 days. The rapid degradation of OTC at high temperatures was shown in literature studies and might be a useful clue to manure treatment designs (Wang and Yates, 2008).

Reduction in TS content

TS reduction given in Table 4.3 and Table 4.4 was calculated in the range of 19-30% for the Set1 and 10-20% for the Set2. The maximum TS reduction of 29.1% was achieved in D1 digester in Set1 in which TS content decreased to 3.9% from 5.5%.

Table 4.3 : TS, TVS ranges of the manure slurry and reduction of TS and TVS inSet1.

Digesters	Operating time	TS content %	TVS content %	TVS/TS	Reduction in TS (%)	Reduction in TVS (%)
1. Set D1 (control)	0	5.5	4.4	80	29.1	29.5
	10	4.1	3.3	82		
	15	3.9	3.1	79		
1. Set D3 (OTC)	0	5.1	4.1	80	19.6	19.5
	10	4.3	3.6	82		
	15	4.1	3.3	81		
1. Set D4 (control)	0	7.8	6.3	81	21.8	22.2
	10	6.5	5.3	82		
	15	6.1	4.9	81		
1. Set D5 (OTC)	0	7.7	6.2	81	20.8	20.9
	10	6.5	5.3	82		
	15	6.1	4.9	80		

In Set1 and Set2, different results in TS reduction can be related with using different manure samplings which have various compositions in terms of physical, chemical and biological properties.

Table 4.4 : TS, TVS ranges of the manure slurry andreductionof TS andTVS in Set2.

Digesters	Operating time	TS content %	TVS content %	TVS/TS	Reduction in TS (%)	Reduction in TVS (%)
2. Set D1 (control)	0	4.7	3.8	82	19.1	18.4
	10	4.1	3.3	81		
	15	3.8	3.1	80		
2. Set D3 (OTC)	0	4.8	3.9	82	10.4	10.3
	10	4.6	3.6	79		
	15	4.3	3.5	81		
2. Set D4 (control)	0	6.3	4.8	81	19.0	14.6
	10	5.7	4.6	81		
	15	5.1	4.1	80		
2. Set D5 (OTC)	0	5.7	4.6	81	17.5	17.4
	10	5	4	80		
	15	4.7	3.8	81		

Reduction in TS content (%) in the both Set1 and Set2 during operating time are given in Figure 4.4 and 4.5. In both sets, higher TS reduction was seen in control digesters D1and D4. In D3 digesters of both sets,lowestreduction in TS content (19.6% in Set1 and 10.4% in Set2) were observed.

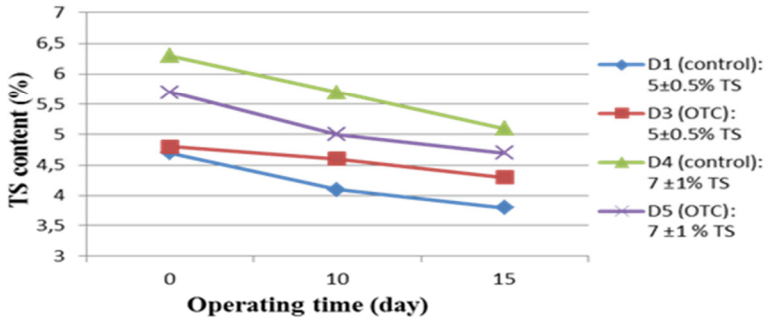


Figure 4.4 : Reduction of TS content in the Set1 during operating time.

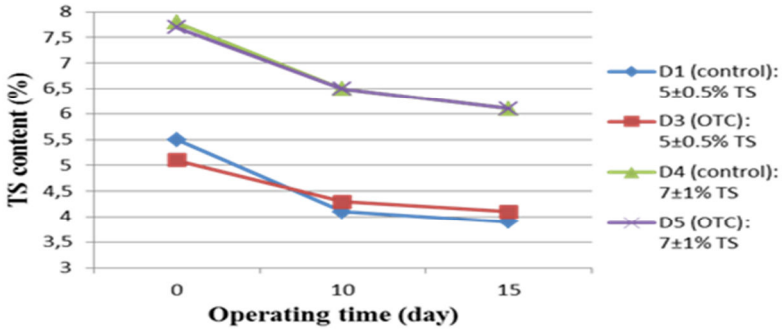


Figure 4.5 : Reduction of TS content in the Set2 during operating time.

Nielsen et al. (2004) was found that TS reduction in the thermophilic anaerobic digester with treatment of cattle manure was achieved to %40 for HRT 15 days and in operational days between 189-211. In our study, %30 TSreduction obtained only within 20 days. Similarly, in the study by Ahring et al. (2001), 10 days after start-up at 55^0C, reduction in volatile solids was determined to be around 28% with HRT 15 days.

Biogas and methane yields

In the Table 4.5, biogas yields obtained from the digesters in Set1 and Set2 during operational 20 days are shown. Biogas yield in terms of volume of the biogas per amount of TVS increased in all digesters in both sets day by day due to the increasing biogas production during operating time.

Table 4.5 : Biogas Yields of the digesters in Set1 and Set2 during 20 days.

Digesters	Cumulative Biogas Yield (L/kgTVS) during operating time (days)																		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1.Set D1	6	12	27	36	41	46	53	61	67	70	73	80	87	93	95	95	108	115	119	124
1.Set D3	4	6	22	30	34	44	52	56	57	63	71	76	80	87	93	94	105	109	110	111
1.Set D4	4	11	24	38	41	49	57	60	63	68	71	78	83	89	97	105	120	124	130	134
1.Set D5	4	9	23	31	40	54	67	67	71	74	76	82	87	93	93	93	106	108	109	110
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
2.Set D1	3	1	16	27	35	49	58	66	72	73	83	89	91	99	102	104	116	121	127	132
2.Set D3	4	8	17	26	35	45	56	65	70	72	80	88	92	95	97	106	110	113	115	118
2.Set D4	4	9	20	30	46	59	68	71	74	76	87	92	97	100	102	103	114	119	122	123
2.Set D5	4	8	19	29	36	49	59	61	64	66	78	86	90	93	94	95	106	110	111	111

Increasing of biogas yields during 20 days can be clearly seen for both Set1 and Set2 in the Figure 4.6 and Figure 4.7 respectively. In the both sets, biogas yield was more in control digesters (D1 and D4) than in OTC containing digesters (D3 and D5).

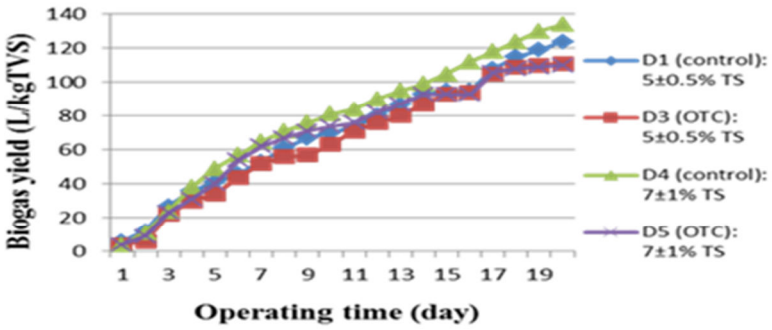


Figure 4.6 : Biogas yield of the Set1 during operating time.

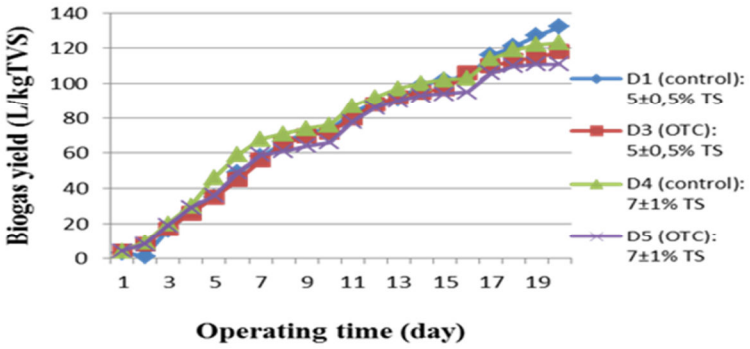


Figure 4.7 : Biogas yield of the Set2 during operating time.

In all digesters, biogas and methane yields were achieved in 20 days between the range of 110-134 L/kgTVS and 77-94 L/kgTVS respectively. According to Table

4.6, in the Set1, maximum biogas yield, 134 L/kgTVS was produced in 20 days in the control digester (D4). In the Set2, maximum biogas, 132 L/kgTVS was produced in the control digester (D1).

Table 4.6 : The relationship between the OTC concentration (mg/l) and operating conditions with biogas and methane yield (Digestion for 20 days).

Digesters	Initial OTC Concentration (mg/l)	Biogas yield (L/kgTVS)	Methane yield (L/kgTVS)	OTC inhibition on Biogas %
1. Set D1	0	124	87	11
1. Set D3	2.46	111	78	
1. Set D4	0	134	94	18
1. Set D5	4.74	110	77	
2. Set D1	0	132	92	18
2. Set D3	1.51	118	83	
2. Set D4	0	123	84	10
2. Set D5	2.67	111	79	

In this study, when Set1 and Set2 were compared with each other, 90 and 120 rpm mixing rate did not effect on the biogas production performance significantly. Substrates containing high solid contents like animal manure are difficult to mix, therefore type of mixer used may be more important than the mixing rate in order to provide an efficient mixing also digester performance.

In theory, when solid content is increased or mixing is applied biogas production will also increase. In contrast, our study did not show significant change on biogas yield when mixing rate was increased from 90 to 120 rpm. This may be due to the using different manure samplings which have various compositions, when the slurrries for the both Set1 and Set2 were prepared.On the other hand, mixing may not work efficiently with high total solid content (TS) thus the expected increase in biogas yield was not detected. This study also showed the decrease in biogas yield when solid content is increased in Set2. The reason for this may be due to some experimental errors. Besides all, it can be assumed that presence of higher OTC concentration with increasing solid content may cause inhibition effect on biogas production or VFA accumulation may be generated in this case and thus the expected increase in biogas production rate was not detected. Similar problem was seen in the previous study carried out by Karim et al. (2005), there was no effect of mixing on digesters performance when fed with 5% TS manure slurry. Furthermore, digesters fed with 10% TS manure slurry were mixed by slurry recirculation, impeller, and

biogas recirculation producing approximately 29%, 22% and 15% more biogas than unmixed digester, respectively. Thus, it was pointed out by Karim et al. (2005) that different mode of mixing provides changing effects on digestion performance. In addition, according to studies from literature under high loading rate, intensive mixing can cause the acidification and failure of the system. Hence, it can be argued that the absence of mixing can be more beneficial than vigorous mixing. On the other hand, the slowly mixed digesters exhibited an overall better startup than the non-mixed digester (Stroot et al., 2001; McMahon et al., 2001; Vavilin and Angelidaki, 2005; Gomez et al., 2006; Kaparaju et al., 2008; Conklin et al., 2008; Ghanimeh et al., 2012).

Table 4.6 also showed that in all digesters, inhibition in biogas production at the end of 20 days were between %10-18 for the digesters containing OTC between the range of 1.5-4.7 mg/L. The inhibitory effect of tetracyclines in manure and on the anaerobic digestion processes has been studied earlier. In most of these works, the drug was administrated orally by the animal. In an early study, Masse et al. (2000) reported that the presence of oxytetracycline in manure slurries reduced methane production by 25%.Arıkan et al. (2006) reported 27% reduction in cumulative biogas production during the anaerobic digestion of cow manure, in which the OTC concentration was 3.1 mg/L in the slurry. Likewise, in the work of Stone et al. (2009), it was found out that 28 mg/L CTC in manure slurry of a swine fed with CTC inhibited methane production 28.4%. On the other hand, according to Loftin et al. (2005), tetracycline and chlorotetracycline added externally at the concentration of 10 mg/l also showed an inhibition of the methane evolution by 7–27% and 10–43%, respectively. In the previous study, inhibition in biogas production at the end of 30 days were 41%, 57% and 61% for the microcosms containing 50 mg/L, 100 mg/L and 200 mg/L OTC, respectively (Coban, 2011). In contrast, Lallai et al. (2002) did not observe any reduction in methane production in batch reactors operating with pig waste slurry containing 125 and 250 mg/L OTC which added externally in reactors.

In manure digesters, not only OTC itself, but mostly its metabolites were reported to be inhibitors (Fedler and Day, 1985). These metabolites are produced in the digestiontrack of the animal. Results of this and previous studies showed that smaller antibiotic concentrations in medicated manure can result in higher biogas inhibitions

comparing to the external addition of antibiotic to the manure. This supports the idea that metabolites of OTC produced in the animal are the main inhibitors.

The results of the studies show similar results among each medication but it has been found that it may be varying time to time due to the slight differences in operational parameters such as the source of inoculum or source of substrate, composition of substrate, inoculum/ manure ratio, reactor size and type of the operation.In previous studies, methane yields were reported at a wide range of value changing from 140 to 250 L CH_4/gVS.day from the thermophilic anaerobic digesters of cattle manure (Ahring et al., 1995; Nielsen et al., 2004; Liu et al., 2009; Kaparaju et al., 2009). It is also important to note that biogas yield and also methane productivity will differ with the type of animal, type of fodder used and thus will vary with the manure collected from different farms (Hansen et al., 1998; Moller et al., 2004).

In this study, methane content of the biogas was 70% in all digesters of the both sets. Table 4.7 showed the methane contents of biogas produced in digesters. This rate is considerable when it is compared with other studies in literature. Methane content higher than 65% is shown the stability of the system and 70% methane content is pointed out the well operated system performance (Sakar et al., 2009).

Table 4.7 : Biogas composition in Set1 and Set2 according to GC analysis.

Digesters	Operating time (day)	N_2 (%)	CH_4 (%)	CO_2 (%)
1. Set D1	10	6	64	30
	20		70	30
1. Set D3	10	9	62	29
	20		70	30
1. Set D4	10		65	35
	20		70	30
1. Set D5	10		71	29
	20		70	30
2. Set D1	10		62	38
	20		70	30
2. Set D3	10		63	37
	20		70	30
2. Set D4	10	5	65	30
	20		68	32
2. Set D5	10		72	28
	20		71	29

Arıkan et al. (2006) reported that methane productivity was 60% of total biogas in mesophilic condition.Pandey and Soupir (2011) reported that methane content in biogas from the digestion of dairy manure operating at 52.5°C varied between 44 and 70% with mean of 56 ± 18%.The methane content of the biogas produced from temperature-phased anaerobic digestion (TPAD) system in the stabilization of dairy cattle wastes was between the range of 58-62% (Sung and Santha, 2003). Ahring et al. (2001) repoted that methane content was between the range of 65-71% of total biogas from the thermophilic digestion of cattle manure. Rico et al. (2011) studied with digestion of dairy manure in CSTR was repoted that between 121-123 operating days, methane content was reached to 72% while in our study, this rate was obtained only within 20 days. According to literature studies, methane content of biogas produced in thermophilic digesters is generally found more than in mesophilic digesters (Goberna et al., 2010; Cavinato et al., 2010). This is one of advantages of thermophilic digestion systems.

VFA measurements

Acetic and propionic acid were found as the major VFAs while isobutyric, isovaleric, butyric and valeric acids were in minor concentrations in all digesters.VFA concentrations of the samples taken from digesters at different sampling time are given in Table 4.8 and Table 4.9.

Table 4.8 :VFA concentrations of the samples taken from digesters in Set1 at different sampling time.

Digesters	Operating time (days)	Volatile Fatty Acids (VFA)								
		Acetic Acid (mg/l)	Propionic Acid (mg/l)	Isobutyric Acid (mg/l)	Butyric Acid (mg/l)	Isovaleric Acid (mg/l)	Valeric Acid (mg/l)	Isocaproic Acid (mg/l)	Caproic Acid (mg/l)	Heptanoic Acid (mg/l)
1.Set D1	0	47	0	0	0	0	0	0	0	0
	5	55	164	0	0	11	0	0	0	13
	10	48	91	0	0	0	0	0	0	0
	15	76	12	0	0	0	0	0	0	0
	20	49	0	0	0	0	0	0	0	0
1.Set D3	0	370	88	12	28	13	11	0	0	0
	5	97	291	0	0	35	6	0	0	0
	10	56	0	0	0	0	0	0	0	0
	15	58	0	0	0	0	0	0	0	0
	20	58	0	0	0	0	0	0	0	0
1.Set D4	0	147	25	0	4	0	0	0	0	0
	5	87	281	0	0	15	0	63	0	0
	10	56	0	0	0	0	0	0	0	0
	15	49	0	0	0	0	0	0	0	0
	20	50	0	0	0	0	0	0	0	0
1.Set D5	0	931	263	33	103	33	29	0	0	0
	5	240	732	26	8	103	42	43	0	0
	10	101	0	0	0	0	0	0	0	0
	15	89	0	0	0	0	0	0	0	0
	20	65	0	0	0	0	0	0	0	0

Table 4.9 :VFA concentrations of the samples taken from digesters im Set2 at different sampling time.

Digesters	Operating time (days)	Volatile Fatty Acids (VFA)								
		Acetic Acid (mg/l)	Propionic Acid (mg/l)	Isobutyric Acid (mg/l)	Butyric Acid (mg/l)	Isovaleric Acid (mg/l)	Valeric Acid (mg/l)	Isocaproic Acid (mg/l)	Caproic Acid (mg/l)	Heptanoic Acid (mg/l)
2.Set D1	0	36	0	0	0	0	0	0	159	0
	5	37	179	30	15	25	7	0	7	0
	10	29	201	0	0	0	0	0	0	0
	15	34	110	0	0	0	0	0	140	0
	20	70	60	0	0	0	0	0	0	0
2 Set D3	0	170	42	0	13	3	0	0	0	0
	5	82	228	44	11	51	15	18	8	12
	10	39	239	0	0	0	0	0	0	0
	15	45	62	0	0	0	0	0	0	0
	20	50	9	0	0	0	0	0	0	0
2.Set D4	0	61	0	0	0	0	0	0	0	0
	5	65	245	0	0	20	0	43	0	15
	10	38	24	0	0	0	0	0	0	15
	15	44	35	0	0	0	0	0	0	0
	20	40	0	0	0	0	0	0	0	0
2.Set D5	0	486	138	18	51	17	15	0	0	0
	5	101	410	56	0	94	25	21	6	0
	10	61	0	0	0	0	0	0	0	0
	15	45	0	0	0	0	0	0	0	0
	20	42	0	0	0	0	0	0	0	0

Acetic acid concentrations in all digesters of the both sets were between 29 and 931 mg/L. Propionic acid concentrations were found between the range of 0-732 mg/L in all digesters. The highest acetic (931 mg/L) and propionic acid (732 mg/L) were detected in the digester D5 of the Set1 on 0. and 5. day respectively.

VFA accumulation connected to the presence of OTC was not detected in this study. In another words, inhibition caused by volatile fatty acid accumulation can not be pronounced due to the continuation of methanogenesis and stability of the methane percentages in biogas. Similarly, Arıkan et al. (2006) repoted that the presence of OTC in manure from medicated calves showed no significant effect on biogas composition or on reductions in volatile solids. Also, concentrations of the VFAs detected were below reported toxic concentations (Angelidaki et al., 1995). This probably means that the hydrogen pressure in the system was kept low enough to prevent VFA accumulation due to the consumption of it by hydrogenotrophic methanogens (Cuetos et al., 2010).

In this study, VFA accumulation can not be pronounced. It is fact that, among increasing of the total solids microbial activity also increases. Therefore, it could be said that VFAs were consumed throughout the operation by increasing active microbial population. In addition to that, the alkalinity existing in the digesting substrate, cow manure, may neutralize the excess volatile acids to maintain the pH in the optimum range.According to VFA results, particularly propionic acid, it can be said that Set1 worked better than Set2. In almost all of digesters in Set1, propionic acid was consumed efficiently and also was not detected in 10 days (Table 4.8 and Table 4.9). Similar with our results, the results of a previous study demonstrated that a stable digester operation at thermophilic conditions (50 and 60^0C) was well possible on cattle manure, as manifested in a constant methane production accompanied with constant low VFA concentration in the effluent (ElMashed et al., 2004). Similarly, theonly VFAs, detected in effluents from digester fed with dairy manure, were acetic and propionic acids and the VFA accumulation was not critical (Rico et al., 2011).

Figure 4.8 and 4.9 shows the total VFA concentrations as equivalent of acetic acid in all digesters of Set1 and Set2 during 20 days. First 5 days increasing in total VFA concentrations was observed and after 5 days, total VFA concentrations

decreased substantially in all digesters of both sets. It can be clearly seen that VFAswere consumed efficiently and VFAs accumulation was not observed.

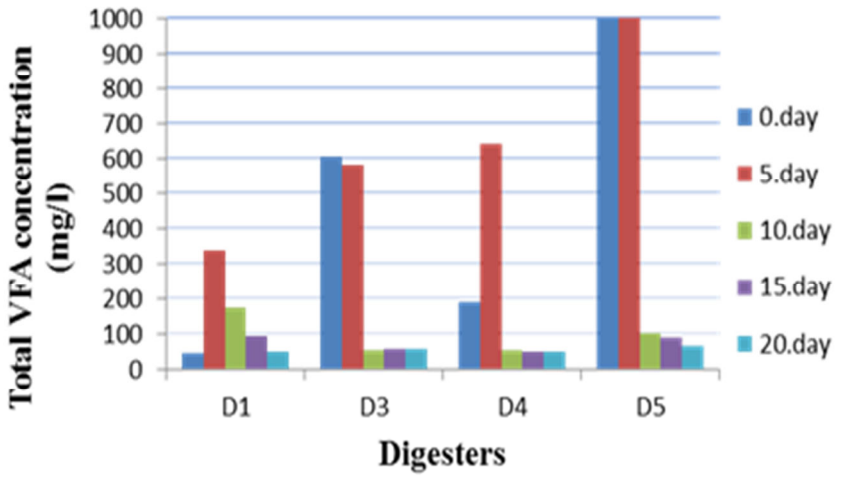


Figure 4.8: Total VFA concentrations (mg/l) as equivalent of acetic acid in digesters of Set1 during operational time.

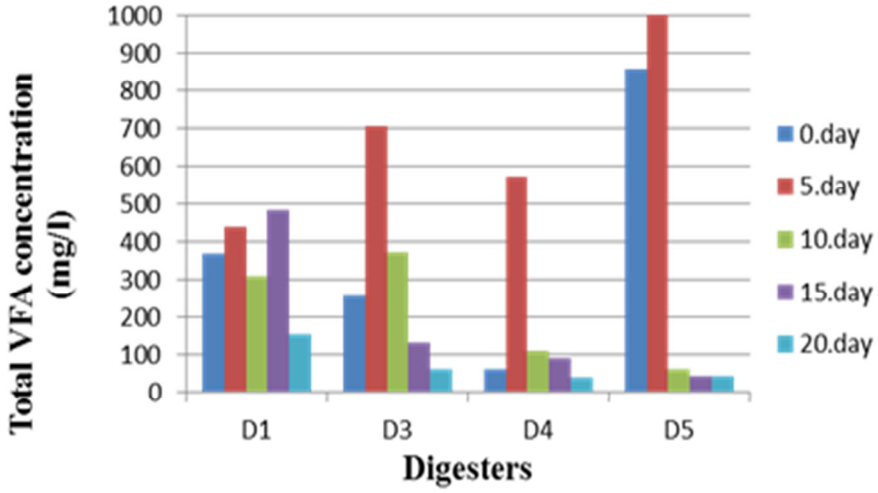


Figure 4.9 : Total VFA concentrations as equivalent of acetic acid in digesters of Set2 during operational time.

4.2Results of MolecularAnalyses

Changes in microbial population dynamics were determined using Fluorescence *in situ* hybridization (FISH) and quantitative real time PCR (Q-PCR). FISH was used to determine the number of active cells of bacteria, *Archaea* and different phylogenetic groups of methanogens in control and OTC containing digesters.

4.2.1Results of FISH analysis

Changes in active cell numbers of bacteria, *Archaea*andmethanogens at the operational days 0. and 20. are given in Table 4.10 and Table 4.11. 16S rRNA-targeted oligonucleotide probes given in Table 3.2, Eubmix, Arc915, MB310, MG1200, MSMS1414, MSMX were used to detect active cell numbers of *Archaea,* Bacteria*, Methanobacteriales, Methanomicrobiales*, *Methanosarcina*and complete acetoclastic methanogens.So that, differences between the MSMX and MSMS1414 probes show the active cell number of *Methanosaeta spp.*. According to results given in Table 4.10 and Table 4.11,*Methanobacteriales*was most abundant methanogen as reaching percentage of 48%. *Methanosaeta spp.* were detected in manure digesters showing the quite low cell number.

Table4.10 : Percentages of the active bacteria, *Archaea*and methanogens in Set1.

S1 Probe	Total cell number /DAPI (%)	Operational day	S3 Probe	Total cell number /DAPI (%)	Operational day
Eubmix	10	0	Eubmix	15	0
Arc915	52	0	Arc915	41	0
MB310	24	0	MB310	23	0
MG1200	14	0	MG1200	13	0
MSMS1414	20	0	MSMS1414	12	0
MSMX	21	0	MSMX	13	0
Eubmix	6	20	Eubmix	7	20
Arc915	46	20	Arc915	38	20
MB310	23	20	MB310	13	20
MG1200	15	20	MG1200	11	20
MSMS1414	11	20	MSMS1414	16	20
MSMX	12	20	MSMX	17	20
Eubmix	23	0	Eubmix	30	0
Arc915	37	0	Arc915	59	0
MB310	15	0	MB310	43	0
MG1200	8	0	MG1200	7	0
MSMS1414	13	0	MSMS1414	17	0
MSMX	14	0	MSMX	18	0
Eubmix	6	20	Eubmix	13	20
Arc915	43	20	Arc915	37	20
MB310	16	20	MB310	18	20
MG1200	10	20	MG1200	14	20
MSMS1414	16	20	MSMS1414	4	20
MSMX	17	20	MSMX	5	20

Table 4.11 : Percentages of the active bacteria, *Archaea*and methanogens in Set2.

S1 Probe	Total cell number /DAPI (%)	Operational day	S3 Probe	Total cell number /DAPI (%)	Operational day
Eubmix	21	0	Eubmix	20	0
Arc915	41	0	Arc915	22	0
MB310	29	0	MB310	13	0
MG1200	7	0	MG1200	1	0
MSMS1414	3	0	MSMS1414	6	0
MSMX	5	0	MSMX	8	0
Eubmix	2	20	Eubmix	16	20
Arc915	38	20	Arc915	75	20
MB310	16	20	MB310	48	20
MG1200	3	20	MG1200	14	20
MSMS1414	4	20	MSMS1414	10	20
MSMX	18	20	MSMX	12	20
Eubmix	7	0	Eubmix	37	0
Arc915	60	0	Arc915	44	0
MB310	30	0	MB310	25	0
MG1200	12	0	MG1200	7	0
MSMS1414	14	0	MSMS1414	11	0
MSMX	15	0	MSMX	12	0
Eubmix	28	20	Eubmix	9	20
Arc915	65	20	Arc915	38	20
MB310	24	20	MB310	9	20
MG1200	20	20	MG1200	11	20
MSMS1414	19	20	MSMS1414	7	20
MSMX	20	20	MSMX	18	20

Change of the active bacteria, *Archaea*and methanogens range with operational day in all digestersof both sets are given in Figure 4.10-4.17. It can be seen from the Figure 4.10-4.13, in the Set1, range of active bacterial population decreased in all digesters at the end of batch operation. *Methanobacteriales* did not change significantly in control digesters D1 and D4 whereas, in OTC containing digesters D3 and D5, activity of *Methanobacteriales*decreased in 20th day.Active*Methanomicrobialesspp*. showed increasing trendin all digesters at the end of operating day. *Methanosarcina* decreased in digesters D1 and D5 while increased in D3 and D4. Thus, it can be said that active *Methanosarcina* population oscillated in digesters showing no spesific trend. Besides, *Methanosaeta spp.* were detected in low cell numbers in all digesters.

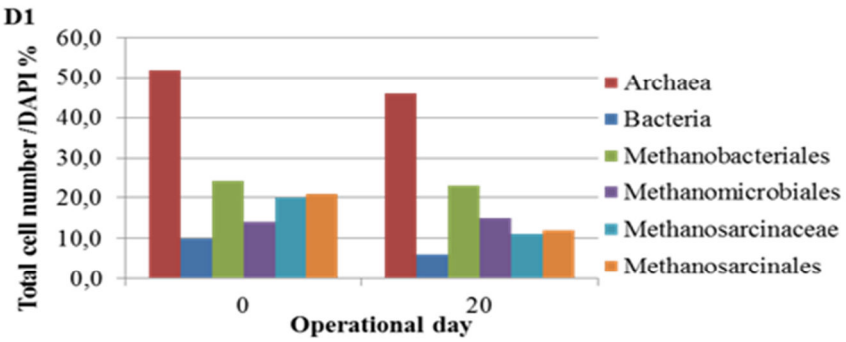


Figure 4.10 : Change of the active bacteria, *Archaea* and methanogens range with operational day in D1 digester of Set1.

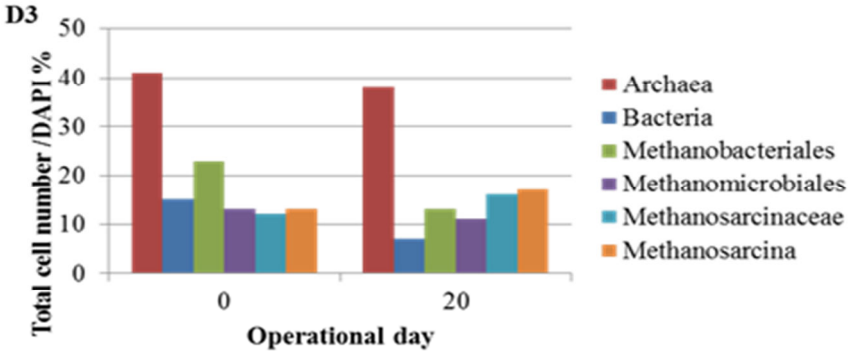


Figure 4.11 : Change of the active bacteria, *Archaea* and methanogens range with operational day in D3 digester of Set1.

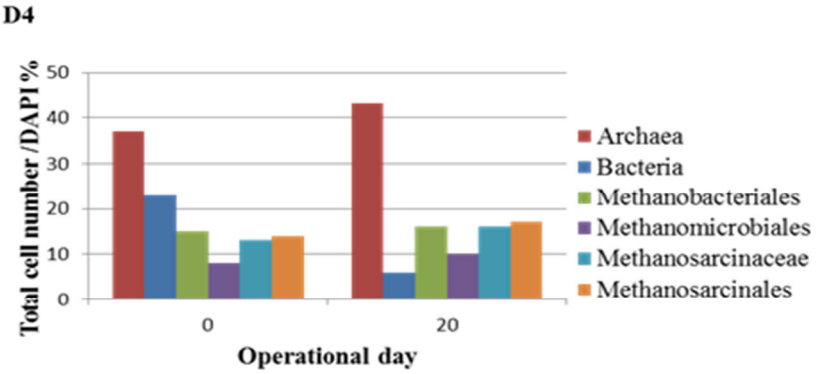


Figure 4.12: Change of the active bacteria, *Archaea* and methanogens range with operational day in D4 digester of Set1.

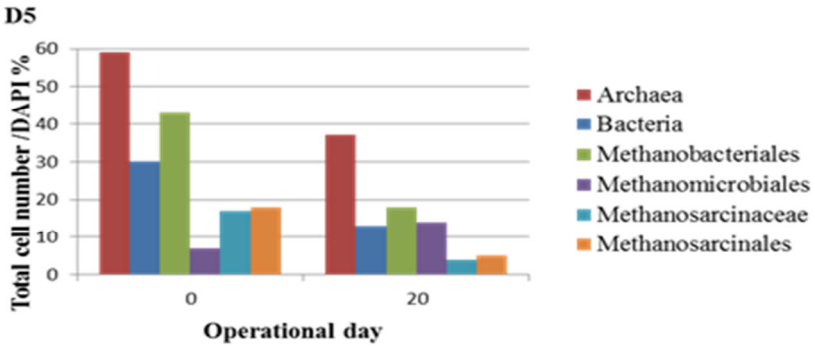


Figure 4.13 : Change of the active bacteria, *Archaea* and methanogens range with operational day in D5 digester of Set1.

In the Set2,according to Figure 4.14-4.17, at the end of operation (20th days), bacterial cellnumber decreased in all digesters except D4. Active *Methanobacterialesspp*. decreased in all digesters however, their cell number increased significantly in OTC containing D3digester. *Methanomicrobiales*cell numberincreased in all digester and only decreased in D1. While *Methanosarcina* population oscillated in digesters in Set1 showing no spesific trend,in Set2,*Methanosarcina*showed the remarkable increase in all digesters except digester D5. Besides, *Methanosaeta spp.* were detected in low cell numbers in all digesters.

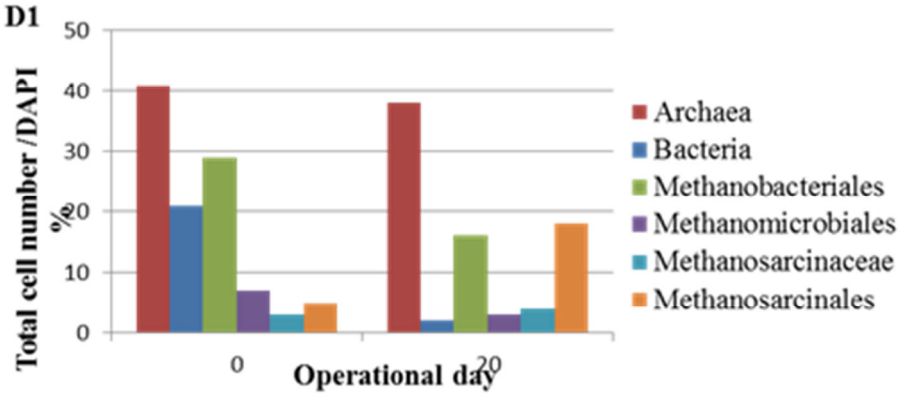


Figure 4.14 : Change of the active bacteria, *Archaea* and methanogens range with operational day in D1 digester of Set2.

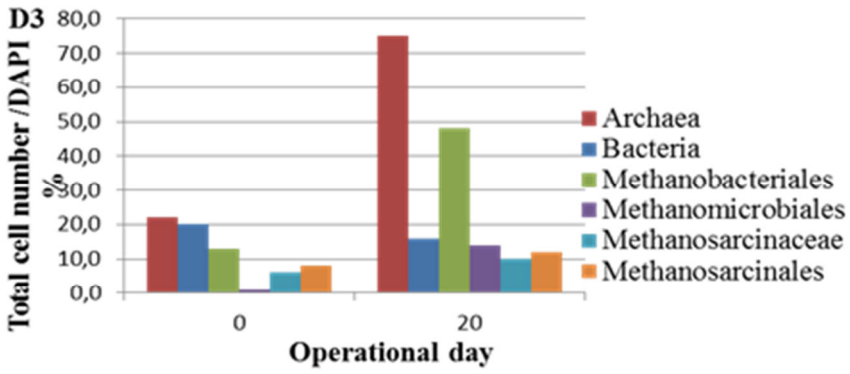


Figure 4.15 : Change of the active bacteria, *Archaea* and methanogens range with operational day in D3 digester of Set2.

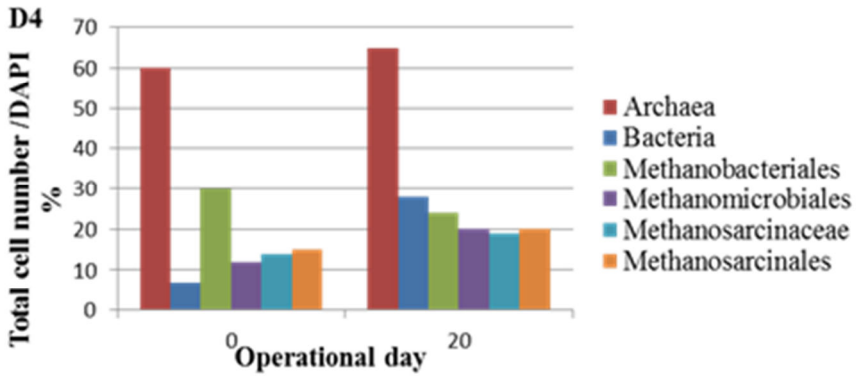


Figure 4.16 : Change of the active bacteria, *Archaea* and methanogens range with operational day in D4 digester of Set2.

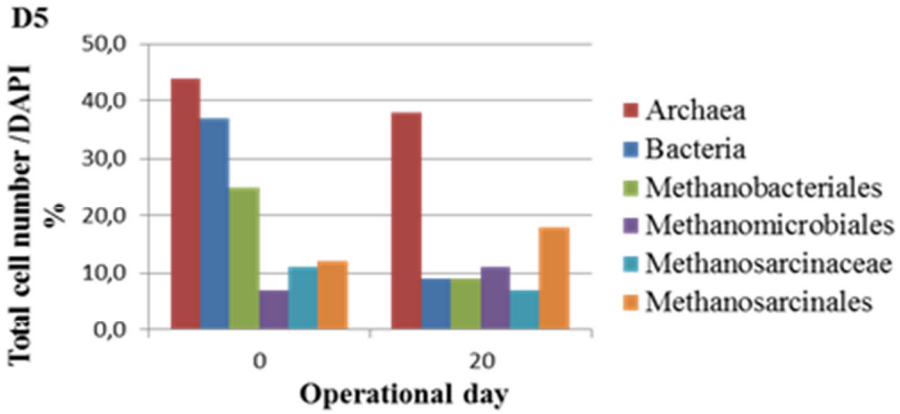


Figure 4.17 : Change of the active bacteria, *Archaea* and methanogens range with operational day in D5 digester of Set2.

According to overallFISH results of this study, *Methanobacteriales*was most abundant methanogen in all digesters as reaching percentage of 48%. In literature, this result was supported by Hattori et al.(2000).*Methanosaeta spp.* were detected in low cell numbers in all digesters. In contrast to the literature, in this

study,*Methanosaeta* was found in manure digesters as addition to *Methanosarcina*. However, acccording to literature, *Methanosarcinaspp*. seems to be the sole acetoclastic methanogen present in full-scale thermophilic biogas plants treating animal manures while *Methanosaetaspp*. were not commonly found in the samples (Mladenovska and Ahring, 2000). In a previous study that support it, Zinder et al. (1984) mentioned that *Methanosarcinaspp*. were a predominant acetoclastic thermophilic methanogens with optimum temperature value of 55–58^{0}C.

FISH technique was also used previously for the definition of microorganisms in manure digesters (Karakashev et al., 2005; Karakashev et al., 2006). However, these studies investigated the structure of general population rather than effects generated by inhibitors like OTC. *Methanosaeta* and *Methanosarcina* representtoacetoclastic methanogens in anaerobic digesters. According to Karakashev's study (2005), *Methanosaetaceae* was the dominant acetotrophic methanogen in digesters fed with sewage sludge however, in reactors fed with swine manure *Methanosarcinaceae* was dominant.

According to Schmidt et al. (2000), in biogas reactors treating solid wastes like manure, only *Methanosarcina spp*. were identified. At high levels of NH_3 and VFA, like the case in manure digesters, the dominance of *Methanosarcinaceae* was observed, while in sewage sludge digesters with low levels of NH_3 and VFA, *Methanosaetaceae* dominated. Acetate-utilizing methanogens having thin filaments (*Methanosaetaceae)* with high surface seemed to be more sensitive to ammonia concentrations than hydrogenotrophic methanogens which grow as rods or *Methanosarcinaceae* whichconsist of thick clumps. Therefore, *Methanosaeta* is not reported to be dominating, particularly in swine manure biogas reactors (Schmidt et al., 2000).Similarly, most other studies repoted that reactors treating animal manure contain *Methanosarcina spp*. dominantly (Chachkhiani et al., 2004; Demirel and Scherer, 2008; Steinberg and Regan, 2009; Kaparaju et al., 2009).In addition, *Methanosarcina* and *Methanosaeta* were found less abuntant in CTC containing manure than in manure without CTC (Stone et al., 2009). Sanz et al. (1996) reported also that methanogens are found more sensitive to most of antibiotics such as tetracylines, than acetogens.

4.2.2Results of Q-PCR analysis

For the Q-PCR assays, 16S rRNA sequence specific primers were used to quantify total copy numbers of active bacterial, *Archaeal* and methanogenic 16S rRNA gene. Results can be seen from Table 4.12 and Table 4.13.

Table 4.12 : Q-PCR analysis results of Set1 digesters.

SET1		Bacteria	M.bacteriales	M.microbiales	M.saeta	M.sarcinales
Day	**Digester**	**Gene copy number**	**Gene copy number**	**Gene copy number**	**Gene copy number**	**Gene copy number**
D0	D1	3.39E+10	2.52E+09	1.11E+08	3.10E+06	3.32E+08
D0	D3	2.96E+10	1.02E+09	1.65E+08	6.76E+06	6.02E+08
D0	D4	7.77E+09	2.41E+09	8.69E+07	4.60E+06	1.02E+08
D0	D5	1.64E+10	4.74E+09	3.38E+07	3.56E+05	1.74E+08
D5	D1	2.02E+09	1.18E+09	8.95E+06	5.56E+05	1.81E+09
D5	D3	3.24E+09	1.36E+09	1.25E+07	4.42E+06	1.62E+09
D5	D4	1.87E+09	1.45E+09	2.22E+07	3.56E+06	1.41E+09
D5	D5	2.11E+09	2.35E+09	7.32E+06	1.70E+06	2.02E+09
D10	D1	1.40E+09	1.60E+09	4.69E+06	2.04E+07	9.84E+08
D10	D3	2.96E+08	7.45E+08	3.97E+06	4.76E+05	5.62E+08
D10	D4	7.13E+08	1.38E+09	1.01E+07	1.03E+06	7.58E+08
D10	D5	3.23E+08	1.67E+09	1.09E+07	1.32E+05	5.10E+08
D15	D1	2.10E+08	1.06E+09	5.20E+06	1.34E+06	6.18E+08
D15	D3	1.37E+08	7.84E+08	7.20E+06	5.22E+05	3.82E+08
D15	D4	7.91E+06	4.49E+08	2.77E+05	2.46E+07	4.10E+06
D15	D5	3.96E+07	3.63E+08	1.68E+06	1.02E+06	4.18E+07
D20	D1	5.99E+07	3.22E+08	3.60E+06	8.38E+05	5.22E+07
D20	D3	9.68E+06	6.30E+08	2.03E+05	9.04E+05	1.40E+07
D20	D4	4.91E+06	1.25E+08	3.90E+05	2.40E+05	1.36E+06
D20	D5	4.24E+06	1.46E+08	3.34E+05	1.53E+05	6.70E+06

Tablo 4.13 : Q-PCR analysis results of Set2 digesters.

SET2		Bacteria	M.bacteriales	M.microbiales	M.saeta	M.sarcinales
Day	**Digester**	**Gene copy number**	**Gene copy number**	**Gene copy number**	**Gene copy number**	**Gene copy number**
D0	D1	1.53E+09	6.08E+09	2.86E+07	5.24E+05	6.16E+07
D0	D3	1.13E+09	3.90E+09	1.00E+07	4.78E+05	1.31E+08
D0	D4	2.35E+09	2.88E+09	1.38E+06	2.06E+05	3.64E+06
D0	D5	2.30E+09	1.38E+10	1.84E+06	2.94E+05	1.52E+07
D5	D1	1.38E+08	2.66E+09	1.51E+05	2.56E+05	1.46E+08
D5	D3	6.65E+08	8.66E+09	3.75E+06	3.78E+05	3.50E+08
D5	D4	2.05E+08	6.04E+09	1.78E+06	5.00E+05	1.36E+09
D5	D5	3.36E+08	1.25E+10	1.67E+06	7.44E+06	3.48E+08
D10	D1	1.63E+08	1.18E+10	7.25E+05	7.40E+05	7.20E+07
D10	D3	5.90E+07	4.62E+09	1.26E+05	3.88E+05	5.86E+07
D10	D4	2.13E+08	1.19E+10	4.27E+06	3.62E+05	7.56E+07
D10	D5	3.83E+08	1.47E+10	4.62E+06	1.49E+06	1.75E+08
D15	D1	2.13E+08	1.98E+10	9.75E+05	1.03E+05	4.84E+08
D15	D3	2.17E+08	2.18E+10	3.13E+05	912E+05	2.18E+08
D15	D4	7.98E+07	2.04E+10	9.91E+05	9.04E+05	6.86E+06
D15	D5	1.05E+08	2.30E+10	7.40E+06	5.76E+05	5.34E+07
D20	D1	3.11E+08	3.05E+10	2.53E+06	1.27E+06	1.04E+09
D20	D3	3.79E+08	2.04E+10	2.24E+06	2.96E+05	3.10E+08
D20	D4	8.36E+07	3.06E+10	3.91E+05	4.48E+04	4.20E+06
D20	D5	4.96E+06	1.12E+10	3.17E+04	3.64E+05	2.82E+05

Figure 4.18-4.27show time-dependent changes of bacterial, *Methanobacteriales, Methanomicrobiales, Methanosaeta ve Methanosarcinales* gene copy number in both sets. In Set1 digesters, bacterial gene copy number showed decreasing trendduring operational time. *Methanobacteriales*did not show a significant change but also showed a decreasing trend only in D5 during the operational time. *Methanomicrobiales* tended to decrease in all digesters within operational days and *Methanosaeta*gene copy number decreased and also showed oscillates in digesters showing no spesific trend. In addition, gene copy number of *Methanosaeta spp.* was low in number in all digesters. *Methanosarcinales,* at the beginning, showed increasing trend until 5. day but after day 10th tend to decrease especially in digester D4 significantly.

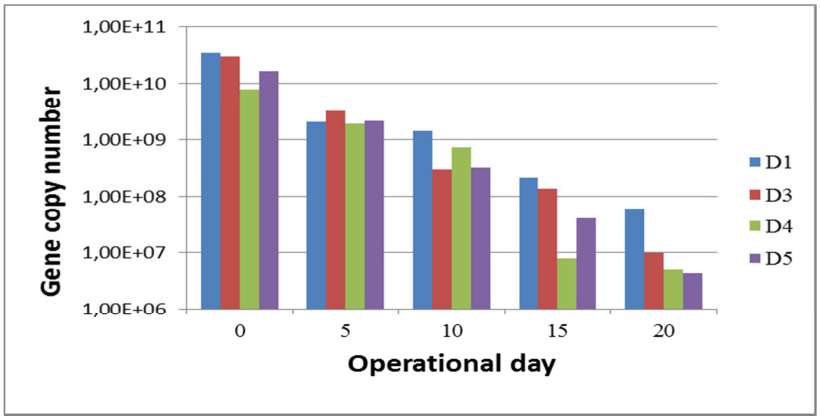


Figure 4.18 : Time-dependent changes of active Bacteria in Set1.

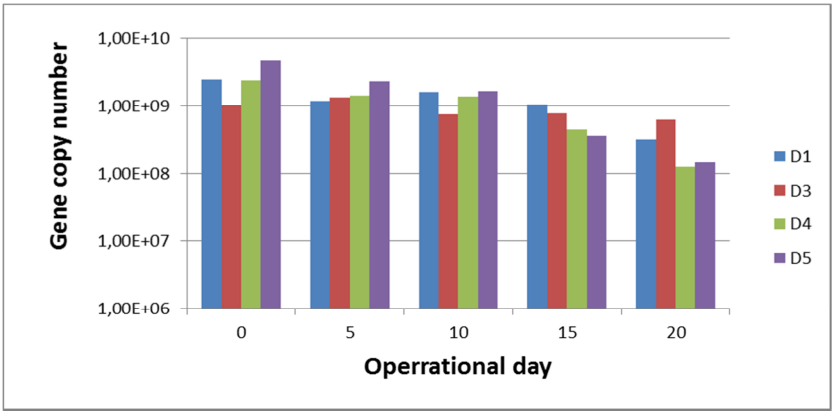


Figure 4.19 : Time-dependent changes of active *Methanobacterialesspp*. in Set1.

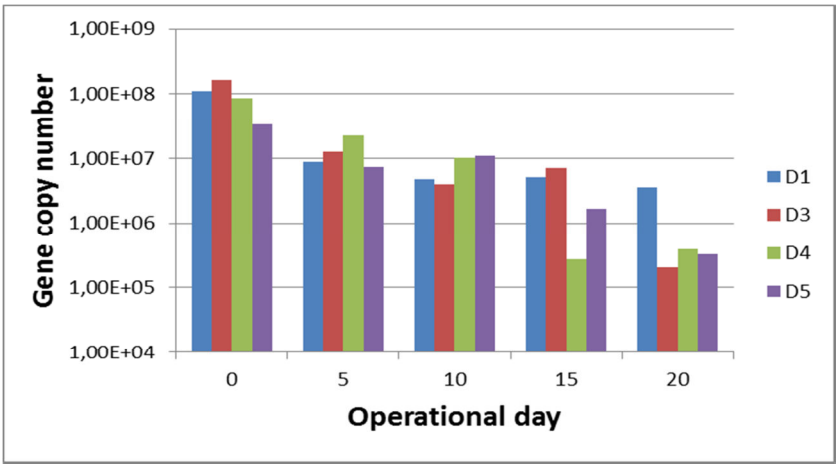


Figure 4.20 : Time-dependent changes of active *Methanomicrobialesspp*. in Set1.

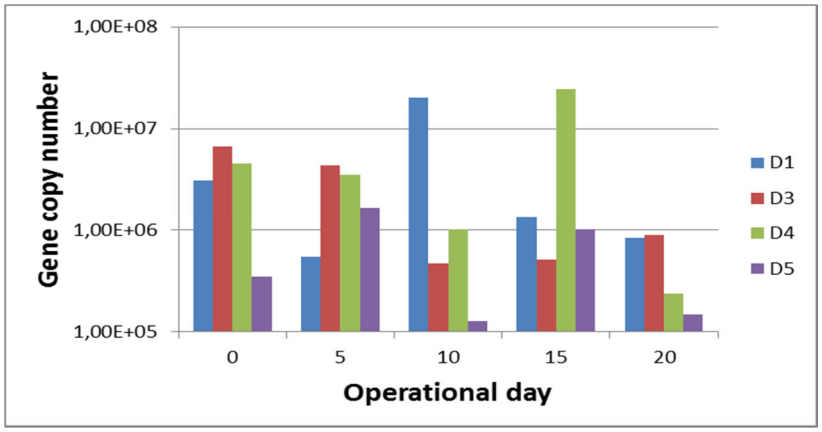


Figure 4.21 : Time-dependent changes of active *Methanosaetaspp*. in Set1.

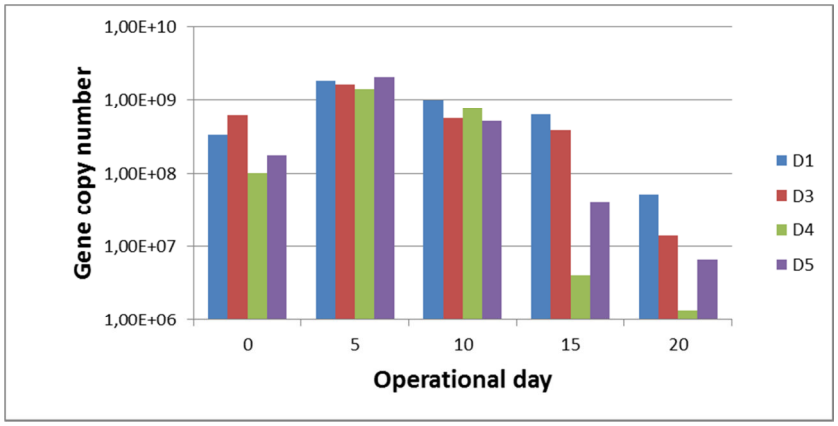


Figure 4.22 : Time-dependent changes of active *Methanosarcinalesspp*. in Set1.

In Set2 digesters, during operation, bacterial and *Methanomicrobiales* gene copy number decreased while *Methanobacteriales*and *Methanosarcinales* gene copy number increased. *Methanosaeta* gene copy number was found slightly lower than other species and did not show significant change within operational time. It can be seen that *Methanobacteriales ve Methanosarcinales* gene number showed an increasing trend in digesters with high mixing rate, but showed a decreasing trend in digesters with low mixing rate.

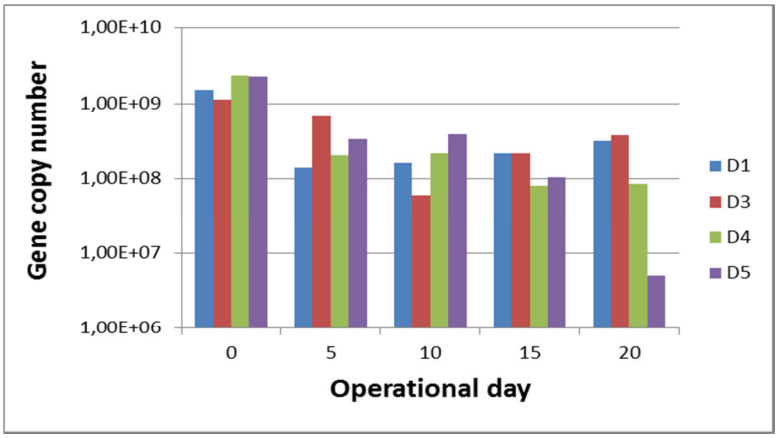


Figure 4.23 : Time-dependent changes of active Bacteria in Set2.

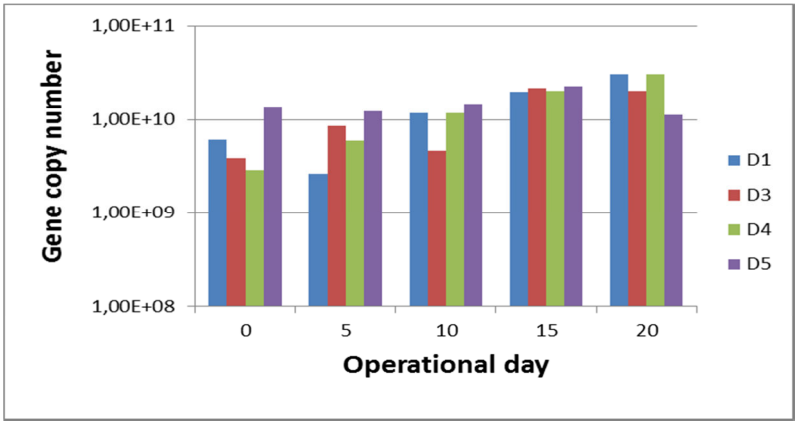


Figure 4.24 : Time-dependent changes of active *Methanobacterialesspp*. in Set2.

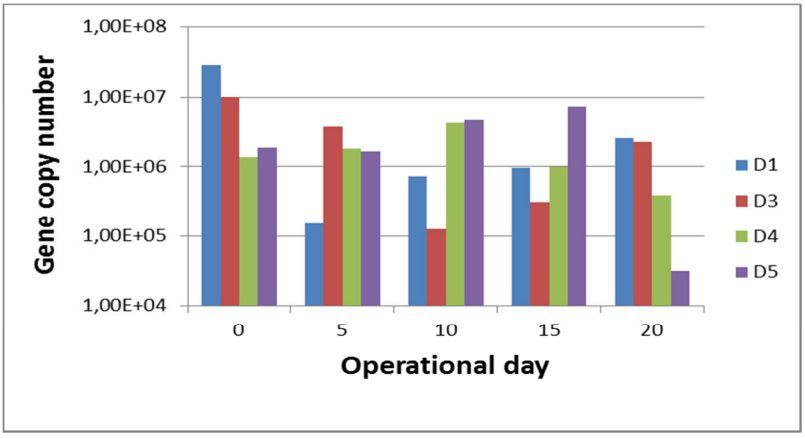


Figure 4.25 : Time-dependent changes of active *Methanomicrobialesspp*. in Set2.

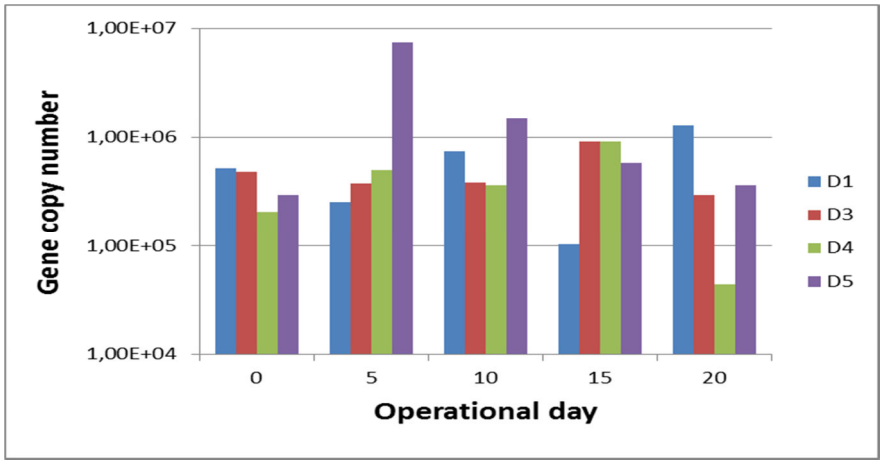


Figure 4.26 : Time-dependent changes of active *Methanosaetaspp*. in Set2.

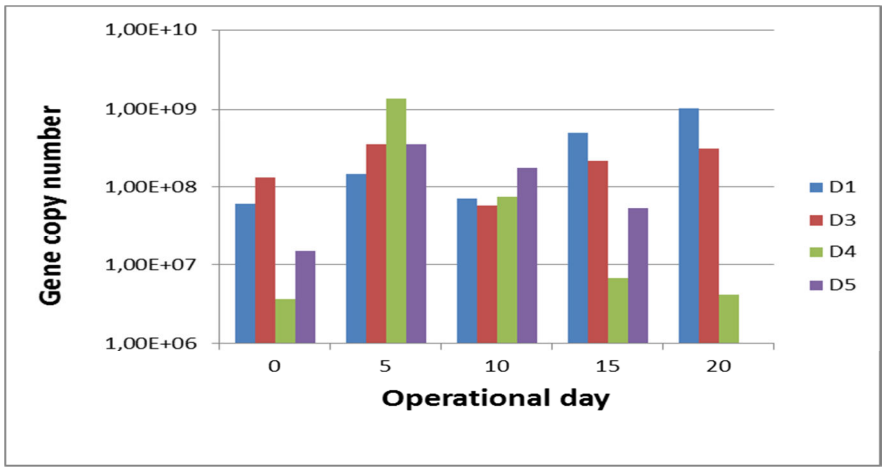


Figure 4.27 : Time-dependent changes of active *Methanosarcinalesspp*. in Set2.

5. CONCLUSIONS AND RECOMMENDATIONS

The main objective of this study was to determine inhibitory effect of OTC on biogas production, biogas quality and microbial communities in the thermophilic biogas digesters using cattle manure medicated by intramuscular injection as substrate under changing operational conditions such as different solid content and mixing rate.

In this study, maximum biogas yield132-134 L/kg TVS was found in control digesters in Set1 and Set2. Also, biogas yields results show that 90 and 120 rpm mixing rate did not effect biogas production performance significantly. Inhibition in biogas production at the end of 20 days were between 10-18% for the digesters containing OTC between the range of 1.5-4.7 mg/L.TS reduction was achieved to highest value of %30 during 20 days. Acetic and propionic acids were dominantly detected in slurries where VFA accumulation was not caused an inhibition. OTC concentration showed a decreasing trend during operational time and half life of OTC was calculated as 14 days for thermophilic anaerobic digestion.

Dynamics of active populations were investigated by molecular methods such as FISH and RNA based Q-PCR. According to FISH results, active bacterial population decreased in all digesters at the end of batch operation whereas active *Methanomicrobiales* population increased. *Methanobacterialesspp*. decreased in OTC containing digesters.Active *Methanosarcina* population oscillated in digesters showing no spesific trend. According to overall FISH results of this study, *Methanobacteriales*was most abundant methanogen in all digesters as reaching percentage of 48%. *Methanosaeta spp.* were detected in low cell numbers in all digesters. According to Q-PCR results, bacterial, *Methanobacteriales, Methanomicrobiales, Methanosaeta ve Methanosarcinales* gene copy numbers decreased within20 days in Set1 digesters. *Methanobacteriales* and *Methanosarcinales* gene copy numbers decreased in Set 1 digesters where mixing rate was 90 rpm whereas gene copy numbers increased in Set 2 digesters where 120

rpm were maintained. Bacterial, *Methanomicrobiales*, *Methanosaeta* gene copy numbers were decreased in all digesters during operational time. *Methanosaeta* gene copy number was low and did not show significant decreasing trend within operational time. Both activity and gene copy number of *Methanosaeta spp.* was low in number in digesters suggesting methanogenesis was performed mainly by *Methanosarcinalesspp*. and hydrogenotrophic archaea like *Methanobacteriales and Methanomicrobiales.*

In this study, lab-scale batch digesters were set to determine the acute effects of OTC on anaerobic digestion performance. Continuous operation may be applied to determine the chronic effects of OTC on anaerobic digestion performance. Besides, different type of animal manure and also co-digestion should be applied to determine the change of the biogas production yield and OTC inhibition.

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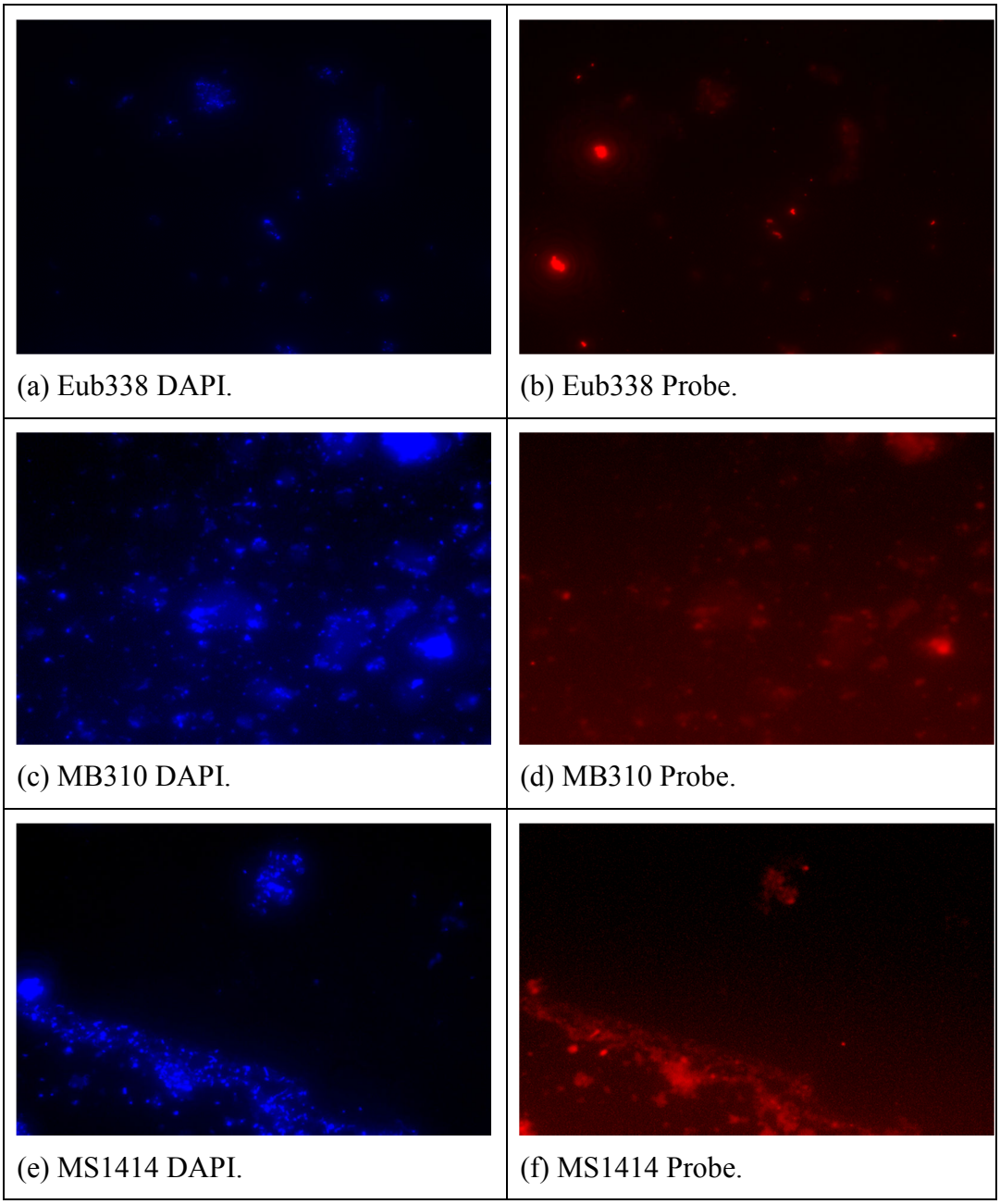
APPENDICES



(a) Eub338 DAPI. (b) Eub338 Probe.

(c) MB310 DAPI. (d) MB310 Probe.

(e) MS1414 DAPI. (f) MS1414 Probe.

Figure A.1: Epifluorescence Micrographs of (a,b)bacteria, (c,d)*Methanobacteriales*and (e,f)*Methanosarcinales.*

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