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**INVESTIGATING THE MICROBIOLOGY AND
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STRENGTH INDUSTRIAL WASTEWATERS**

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ABSTRACT

Anaerobic degradation of industrial wastewaters has proven a viable alternative for waste treatment with a reduced carbon footprint. However certain challenges remain in terms of achieving robust process performance which is directly underpinned by microbial communities governing the anaerobic process. Understanding the role of these communities poses a challenge mainly due to following factors: a) Limited findings which correlate process performance with community dynamics of both acidogens and methanogens, b) Differing responses of microbes to different substrates and process parameters. Therefore the major objective of this project was to observe microbial community dynamics and its impact on process performance by using molecular microbial techniques to track community development and changes.

The first part of the study focused on industrial wastewater feedstock selection protocol. Three different wastewaters INDWW, WW1 and WW2 were selected on basis of total COD levels. This was followed by a study of the methanogenesis potential of INDWW at mesophilic (35°C) temperature, with investigation into the microbial community responses. The results obtained in this experiment suggested that the presence/absence of certain specific microflora affect process performance, especially that of acidogenic bacteria and acidogenesis. A study was also undertaken to observe the impact of one step high temperature thermal shock on mesophilic seed and its subsequent effect on microbial community dynamics while treating INDWW in a thermophilic anaerobic process. The process of mesophilic seed acclimation to thermophilic conditions affected the bioprocess via a combination of factors including a direct effect of temperature on the selection of microbial communities.

A single stage batch anaerobic degradation process was performed to treat INDWW over three batches of substrate loading and 145 days of operation.

There was substantial methane production and COD reduction in Batches I and II. Microbial community analysis revealed that *Methanosarcinaceae*; a group of acetoclastic (acetate using) methanogens, increased its copy numbers initially concurrent with accumulation of acetate. Substantial propionate accumulation was observed even in the presence of propionate oxidisers, leading to a hypothesis that excessive acetate levels may have hindered propionate degradation along with a combination of other factors. Significant extracellular protein production in EPS (extracellular polymeric substances) as well as increase of total lipid was observed, implying wastewater substrate might have exerted stress on the microbial communities leading to production of compatible solutes in the form of EPS and/or increase in membrane lipid content to increase rigidity of cellular structure.

Developing a mixing ratio for WW1 and WW2 for improved methane production was an important project activity. The results obtained were used for subsequent laboratory scale studies for achieving improved COD removal and increased methane production. A batch study was performed to compare the microbial community dynamics of three different seed biomass when degrading the optimized WW1:WW2 wastewater mixture. The results imply that diversity of biomass plays a key role in acidogenic and methanogenic reactions, especially the diversity of *Methanosarcinaceae*. The study also illustrated the production of biological metal chelators, "siderophores" and glycolipid biosurfactants while degrading the wastewater mixture, indicating a form of stress mitigation but also slowing down of the process.

Due to limited feedstock availability, a study was performed to monitor the microbial dynamics during start-up of a two stage lab scale CSTR treating the optimized WW1:WW2 wastewater mix. Results elucidated that it was possible to achieve near complete phase separation. Diverse acidogenic flora in the acid reactor caused increased VFA production and a combination of hydrogenotrophic and acetoclastic methanogenesis was responsible for increased

methane production in methane reactor. Production of siderophores and biosurfactants imply that biochemical responses would be highly substrate dependent and not process dependent.

Major conclusions derived from this project are 1) Validation of earlier studies regarding acidogenic reactions and its importance as the rate limiting step in anaerobic degradation, 2) Methanogens less affected by dynamics and development of acidogenic communities, 3) Microbial communities markedly vary in response to differing substrates and not so much with respect to process changes, 4) Microbial responses significantly affect outcome of the process viz. methane production efficiency and COD reduction. Understanding the community dynamics and biochemical responses of microflora within anaerobic systems would definitely help in better process control and prevent process failure, especially during start-up of anaerobic process.

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LIST OF ABBREVIATIONS

APHA	American Public Health Association
CO ₂	Carbon dioxide
CH ₄	Methane
H ₂	Hydrogen
VFA	Volatile Fatty Acids
TS	Total Solids
VS	Volatile solids
TSS	Total suspended solids
VSS	Volatile suspended solids
TDS	Total Dissolved solids
COD	Chemical oxygen demand
TCOD	Total chemical oxygen demand
SCOD	Soluble chemical oxygen demand
HRT	Hydraulic retention time
GC	Gas Chromatography
FID	Flame Ionisation Detector
TCD	Thermal conductivity detector
Kg	Kilogram
gm	Gram
L	Litre
mL	Millilitre
μL	Microlitre

M	Molar
mM	Millimolar
BMP	Biochemical methane potential
PCR	Polymerase Chain Reaction
DNA	De-oxyribose nucleic acid
RNA	Ribose nucleic acid
r-RNA	Ribosomal RNA
ΔG^0	Standard Gibbs free energy
STP	Standard temperature and Pressure
T-RFLP	Terminal-restriction fragment length polymorphism
DGGE	Denaturing gradient gel electrophoresis
AMPTS	Automated methane potential testing system
ECP	Extracellular protein
ATP	Adenosine tri-phosphate
NMDS	Non metric multidimensional scaling

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND

Public concern has been on the rise regarding waste pollution impacting human health and environmental quality. This concern has not been addressed in a proper manner due to a concomitant increase in human population itself, thereby leading to a direct increase in industrial activity. However with the onset of strict regulations regarding waste discharge into the environment, there has been a shift to adopt greener technologies which leave a lesser footprint in the environment. There is also an effort to recover maximum energy in some form from the wastes before its disposal. One such technology is an anaerobic degradation system that treats organic waste and wastewater for resource recovery and reuse. The anaerobic degradation system relies on complex consortia of anaerobic microorganisms to degrade and decompose organic matter in the wastes to methane and carbon dioxide in an oxygen-free environment. The process is also naturally occurring in many environments like waterlogged soils, swamps, sediments, animal and human digestive guts (Ward *et al.*, 2008). Anaerobic degradation possesses the inherent natural ability to generate renewable biofuels like methane, hydrogen and alcohols which have the potential in the long run to serve as an alternative for non-renewable fossil fuels like petroleum hydrocarbons and coal (Ghosh, 1997). It also conserves the fertiliser value in stabilised wastes and destroys pathogens at thermophilic conditions (Bendixen, 1994). These promising qualities of anaerobic degradation have spurred research into developing different types of systems to capture these beneficial features for sustainable use of resources. Engineered anaerobic degradation systems have been in use for many years to treat low- to high-strength organic waste effluents from a variety of sources such as food and beverage industries, paper pulp mills, palm and olive oil mill effluents, agricultural and dairy industries, petrochemical and animal waste effluents; domestic sewage sludge and recently, the organic fraction of municipal solid

wastes (Angelidaki *et al.*, 2003; Demirel and Yenigun, 2002; Chynoweth *et al.*, 1998).

The ever increasing human activity has led to a plethora of waste streams being generated, each having varying degrees of negative environmental impact. Waste streams consisting of a high proportion of organic matter such as livestock manure, food processing wastes, or waste activated sludge (WAS) from waste water treatment plants (WWTPs) are detrimental when discharged into aquatic environments such as streams or lakes due to nutrient overloading which can lead to the eutrophication of waterways. Additionally, these wastes may harbor potentially pathogenic microorganisms such as *Escherichia coli* or *Cryptosporidium* spp. that must be removed prior to disposal (Ahring *et al.*, 2002). Anaerobic degradation is one such process which has been in use for many years to achieve the aforementioned standards. During the anaerobic degradation process, complex consortium of microbial communities simultaneously acts upon a specific substrate and product to produce the terminal end products of methane and carbon dioxide (Harper and Pohland, 1986). In a single-stage anaerobic system, all microorganisms co-exist in a delicate relationship participating in four important biological processes viz. hydrolysis, acidogenesis, acetogenesis, and methanogenesis (de Lemos Chernicharo, 2007). Complex and insoluble long chain polymers of carbohydrates, proteins and lipids are first converted into soluble monomers of sugars, amino acids and fatty acids by extracellular enzymes which are secreted by hydrolytic bacteria during the process of hydrolysis. In the acidogenesis stage, the monomers are converted into simple compounds such as volatile fatty acids (VFA), alcohols, hydrogen, carbon dioxide and ammonia by the acidogenic fermentative bacteria. These intermediate products are degraded by a syntrophic consortium of acetogenic bacteria into acetic acid and methanogenic microorganisms convert it to methane and carbon dioxide end products (Harper and Pohland, 1986, Pavlostathis and Giraldo-Gomez, 1991). A stable anaerobic

degradation operation requires the acidogenic, acetogenic and methanogenic bacterial groups to be in balance. However, due to differences in growth kinetics, nutritional requirements and sensitivity to environmental conditions between these organisms, there most often occurs an imbalance between the phases of acid fermentation and methane production. This commonly results in accumulation of VFA and other products which potentially inhibit the methanogenic microorganisms in a single-stage anaerobic degradation system.

To overcome the process deficiency of a single-stage anaerobic system, a two-phase or two-stage anaerobic digestion system consisting of a first-stage acidogenic reactor and a second-stage methanogenic reactor was developed (Pohland and Ghosh, 1971). Through tweaking of the reactors' operating conditions, optimum conditions were achieved to favour the fast-growing acid-forming fermentative bacteria and the slow-growing syntrophic VFA-consuming acetogenic bacteria in the first reactor and methane-forming *Archaea* in the second reactor. There have been numerous studies since, which highlight the efficacies of a two-stage anaerobic system in enhancing process stability particularly at high organic loading rates and increasing volatile solids destruction when compared to a single stage system (Demirer and Chen, 2005). In the aforementioned case study, anaerobic systems operating at thermophilic conditions were found to tolerate higher organic loading rates, possessed capacity for greater volatile solids removal and greater pathogens destruction than at mesophilic conditions. However on the downside, there are also contradictory reports citing that thermophilic systems produced poor supernatant quality and had lower process stability, especially in the face of high propionate concentrations (Kugelman and Guida, 1989). There exist other applications of acid-phase anaerobic digestion, such as production of hydrogen rich biogas as an alternative sustainable biofuel (Jan *et al.*, 2007), removal of pathogenic microbes which pose health hazards by causing opportunistic infections (Kunte *et al.*, 2004), and enhancing the solubilisation of particulate

organics (Elefsiniotis *et al.*, 1996). These findings have seeded renewed research efforts targeting the acidogenic reactor potentials for these applications on a large commercial scale.

Anaerobic degradation is a microbial mediated process with the microbial communities influencing the overall stability of the system and in turn, the economic viability of anaerobic degradation systems. Whilst studies have been undertaken to examine the microbial communities in an anaerobic system, the microbial diversity and their activity in such systems are still pretty much undefined (Rivière *et al.*, 2009). Certain aspects of microbial diversity have been elucidated over the years with the advent of various molecular techniques; however these datasets were often small, comprising fewer than 500 sequences. Rough estimates of total species richness in anaerobic degradation systems which treat municipal WAS suggest that approximately 10-13,000 microbial species are involved in WAS anaerobic degradation and 75% of them have been witnessed (Rivière *et al.*, 2009). Estimates for other systems treating different feedstock are less available or inconclusive with the comparisons of diversity with previous results often turning out to be contradictory.

Anaerobic degradation of organic wastes has been in use as a waste treatment method for many years now. However, it has only been in recent years that this process has been viewed seriously as a potential source of energy through the production, capture and use of methane biogas. Unfortunately, the conventional feedstock of sewage sludge does not generate enough methane in order for it to replace conventional fuel. Hence there is a shift in focus from conventional feedstock to industrial wastes which are rich in organic content and can act as potential energy reserves if treated properly. Many of the current industrial wastewater streams are rich in organic substances which hold the potential for resource recovery. However, inhibition from the substances not amenable to biological transformation may lead to an accumulation of recalcitrant organic

compounds. Nevertheless, the whole premise of using industrial wastewater as a potential feedstock for anaerobic degradation and subsequent methane resource recovery holds tremendous potential for commercialization. To optimize the process, it is imperative to observe the microbial communities which govern the process and also identify the biochemical process which occur in the system. With a clearer picture of the microbial communities and their prospective function in the degradation process, it would be possible to fine-tune existing anaerobic degradation processes or theoretically engineer a process containing suitable microflora which achieve optimum degradation rates under controlled conditions.

1.2 OBJECTIVES AND SCOPE OF RESEARCH

1) Identifying potential industrial wastewater feedstock optimum for methane generation, main wastewater properties being high chemical oxygen demand (COD) content; imperative for generation of high quantity and quality of methane end product. Three potential feedstocks were identified (INDWW, WW1 and WW2) out of which INDWW was utilized for studies in Chapters 4, 5 and 6. Due to shutting down of source industry of INDWW, WW1 and WW2 were used to achieve an optimized mixture (high TCOD) and subsequently used in studies described in Chapters 7, 8 and 9

2) To evaluate the methanogenesis potential of selected feedstock at mesophilic (35°C) temperature while concomitantly observing the microbial community response during the degradation process. This is described in Chapter 4

- 3) To highlight the effect of thermal shock on a mesophilic seed biomass and its subsequent performance during thermophilic (55°C) anaerobic degradation of a high strength industrial wastewater. This is discussed in Chapter 5.
- 4) To monitor the temporal microbial community responses and the microbiological and biochemical processes involved in a single stage batch reactor treating one particular high strength industrial wastewater. The outcome of this study is presented in Chapter 6.
- 5) To investigate an optimal mixing ratio for two different industrial wastewaters for achieving maximum methane production whilst observing microbial community responses. This is discussed in Chapter 7.
- 6) Comparative study of microbial community dynamics and biochemical responses during batch anaerobic degradation of optimized wastewater mix seeded with biomass obtained from three different sources. The results of this study are discussed in Chapter 8.
- 7) To examine the microbial community dynamics and responses during start-up of a two phase CSTR process. The results of this study are presented in Chapter 9.

CHAPTER 2 REVIEW OF LITERATURE

2.1 INTRODUCTION

This chapter reviews certain information to provide background knowledge for the design of experiments and most importantly for interpretation of experimental results presented in this report: 1) Brief overview of the microbiology and biochemistry of anaerobic degradation process; 2) Anaerobic reactor types; 3) Factors affecting anaerobic degradation, and 4) Nucleic acid-based molecular methods for microbial analysis, with particular emphasis on real time PCR and DGGE techniques

2.2 ANAEROBIC DEGRADATION – A BRIEF OVERVIEW

Several groups of microorganisms, in the absence of oxygen, undertake a biological conversion process. During this process, complex organic matter is converted to terminal end products of methane, carbon dioxide, hydrogen sulphide and ammonia (de Lemos Chernicharo, 2007). These microorganisms are prokaryotes and are grouped under *Bacteria* and *Archaea* domains in the universal phylogenetic or evolutionary tree of life proposed by Woese and depicted in Figure 2.1. (Canfield *et al.*, 2005).

Figure 2.1 Universal tree of life according to Carl R. Woese (Source: Canfield *et al.*, 2005)

The domain *Bacteria* is more diverse and contains greater number of microorganisms in comparison to the *Archaea* domain. It has been reported that there are 871 different genera and 5,007 species belonging to the *Bacteria* domain while 69 different genera and 217 species were found within the *Archaea* domain (Barton., 2005). However, these numbers are purely estimation and are expected to increase over time with increased research activities in this area. Pathogenic microorganisms harmful to plants, animals and humans such as *E. coli*, *Salmonella*, *Clostridium perfringens*, *Enterococcus faecalis*,

Campylobacter etc. are found in the *Bacteria* domain and there exist no known pathogens to both humans and animals in the domain *Archaea*.

The anaerobic bioconversion process consists of five distinct steps; hydrolysis, acidogenesis, acetogenesis, methanogenesis and sulfidogenesis (de Lemos Chernicharo, 2007). A specific community of prokaryotic microorganisms, linked to their specific substrates, mediates each step of the anaerobic degradation process. The microbial communities governing the steps of hydrolysis, acidogenesis, acetogenesis and sulfidogenesis fall in the *Bacteria* domain while the *Archaea* domain contains within it those communities responsible for methanogenesis (methane generation). However there are certain exceptions to this trend. A common example is the *Archaeoglobus* genera in the *Archaea* domain which are also sulphate-reducers (Barton, 2005).

Figure 2.2 - Schematic outline of the microbial mediated anaerobic degradation process (adapted from de Lemos Chernicharo, 2007)

2.2.1. Hydrolysis

The first step in any anaerobic degradation process is hydrolysis. In this process, the insoluble organic particulates and polymers such as carbohydrates, proteins and fats are broken down to simpler soluble materials. This is achieved by the microbial communities secreting different exo-enzymes (eg, cellulase, amylase and xylanase for carbohydrates; proteases for proteins and lipases for fats). The communities which achieve this function are commonly referred to as

the hydrolytic fermentative bacteria. However hydrolysis is not necessarily an enzyme-catalysed biological process. It can also be mediated by certain physicochemical factors and hence is considered as a less defined process which can be affected by extrinsic factors at any given point of time. The factors which can affect hydrolysis are reactor temperature, hydraulic retention time, substrate composition (types of carbohydrates, proteins, fats), substrate particle size, pH of the medium, enzymes production, enzyme diffusion and subsequent adsorption to substrate particles (de Lemos Chernicharo, 2007). In the anaerobic degradation of complex particulate substrates such as piggery waste, cattle manure and sewage, hydrolysis is considered to be the rate limiting step (Eastman and Ferguson, 1981). Hydrolysis also determines the maximum substrate concentration available for the acidogenesis at any given retention time and thereby affecting the overall kinetics of the anaerobic process. Bacteria, belonging to the genera *Acetivibrio*, *Bacteroides*, *Clostridium*, and *Coprothermobacter* for example (Etchebehere *et al.*, 1998; O'Sullivan *et al.*, 2005; Yang *et al.*, 1990) have been isolated from the hydrolysis phase of anaerobic systems. Hydrolysis occurs extracellularly and therefore does not directly contribute to energy harvesting metabolism of an involved microbial community. This commonly leads the bacterial species active in the hydrolysis phase, to be active during the acidogenic phase as well, in order to sustain energy for cell growth and replication. This has direct implications on the microbial community profile of an anaerobic system.

2.2.2. Acidogenesis or Fermentation

In this phase, the monomeric or oligomeric compounds produced during hydrolysis are metabolized intra-cellularly into a variety of fermentation products viz. primarily short chain fatty acids (SCFAs) with minor amounts of lactate and alcohols (IWA Task Group for Mathematical Modelling of Anaerobic Digestion Processes, 2002). Out of the organic carbon processed

during the acidogenic phase, ~50% is converted into acetate while ~20% is released as CO₂ (Ahring, 2003). Butyrate, propionate, and valerate along with a few non volatile SCFAs, form up the remaining balance. Hydrolysis and acidogenic fermentation can be governed by the same trophic group of facultative anaerobes (can survive in the presence of oxygen but grow better in minimal conditions) and obligate (survive without oxygen) anaerobic microorganisms. Acidogenic fermentative bacteria are the fastest growers amongst the anaerobes with a doubling time of as low as 30 minutes (Novaes, 1986). Some fermentative microorganisms that have been identified till date predominantly belong to species of *Streptococcus*, *Bacteroids*, *Clostridium*, *Propionibacterium*, *Butyrivibrio*, *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus*, *Peptococcus*, *Selenomonas*, *Lactobacillus* and *Enterobacteriaceae* (Banerjee *et al.*, 1998).

Optimum growth rate for acidogenic bacteria is thought to occur in the pH range between 5 and 6 and the bacteria are tolerant to lower pH values. Past research have identified a diverse group of microbes involved in the acidogenesis phase of anaerobic degradation. The phyla *Actinomycetes*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, and *Proteobacteria* are all known to contain species involved in acidogenesis. Scientists have also identified genera of bacteria which are found exclusively in the acidogenesis phase. These include the family *Anaerolineaceae* in the phylum *Chloroflexi*, the genera *Bifidobacterium* and *Paludibacter* in the phylum *Bacteroidetes*, and the thermophilic bacteria in phylum *Thermotogae* which are found in most thermophilic anaerobic degradation systems as well as some mesophilic systems (Ueki *et al.*, 2006; Yamada *et al.*, 2006).

Table 2.1 Examples of carbohydrate and protein fermentation products

Substrate	Reaction	ΔG° (kJ/mol)
Glucose	$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2$	-206.3
Glucose	$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2$	-254.8
Glucose	$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2$	-254.8
Amino acid	Glutamate + $3H_2O \rightarrow 2CH_3COO^- + HCO_3^- + H^+ + NH_4^+ + H_2$	-33.9
Amino acid	2Glycine + $4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 2H^+ + 2NH_4^+ + 2H_2$	-51.5
Amino acid	Alanine + $3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + NH_4^+ + 2H_2$	+7.5
	Glycine + $2H_2 \rightarrow 2CH_3COO^- + 2NH_4^+$	-38.9

ΔG° (kJ/mol) at pH 7, 1 atm, 25°C (Sources: Gallert *et al.*, 1998; McInerney, 1988; Thauer *et al.*, 1977)

The fermentative bacteria undertake different pathways to produce different types of metabolic products in response to differing environmental factors viz. substrate concentration, pH and dissolved hydrogen concentration (Gallert and Winter, 2005). The production of acetate, hydrogen and carbon dioxide is thermodynamically favoured at hydrogen partial pressures of $<10^{-4}$ atm. This occurs due to the reaction providing microbial communities with a natural greatest energy yield which can subsequently be utilized by the microbes for its growth. However at higher hydrogen partial pressures of $>10^{-4}$ atm, which occurs in the case of increased organic loading where hydrogen tends to accumulate, ethanol, propionic and butyric acid are produced via pyruvic acid pathway in addition to acetate, hydrogen and carbon dioxide (Harper and Pohland, 1987). Hydrolysis of Amino acids (obtained from hydrolysis of proteins) leads to formation of volatile fatty acids, ammonia, hydrogen, etc through either anaerobic oxidation linked to hydrogen production (also known as de-amination) or the Stickland reaction (**Table 2.1**).

Hydrogen is not produced during the Stickland reaction as the reducing equivalents (hydrogen) produced during the oxidative decarboxylation of amino acids are used up in the system as electron donors. This occurs in order to

convert other amino acids via a reduction reaction. A simple example, wherein the conversion of glycine to acetate and ammonia is undertaken by certain proteolytic bacteria of *Clostridium* species, elucidates this phenomenon. Thus, the production of hydrogen either as biofuel or methanogenic substrate from protein-rich substrates prove difficult when Stickland fermentation reaction is the predominant pathway for degradation. The reductive deamination of long-chain amino acids such as valine, leucine and iso-leucine lead to the formation of isomeric forms of VFAs like iso-butyrate and iso-valerate. Unlike a drop in pH due to the formation of VFA during carbohydrate fermentation, fermentation of amino acids does not cause a pH drop due to the release of ammonia as a by-product. To counteract the VFAs produced during anaerobic degradation, ammonium ions and carbon dioxide-bicarbonate-carbonate ions provide enough alkalinity to stabilise wastewater pH (Gallert and Winter, 2005). Lipids or triglycerides hydrolysis produce long chain fatty acids (LCFAs), glycerol and galactose end products. The soluble glycerol and galactose moieties which are also released during the lipid hydrolysis are anaerobically oxidized by fermentative acidogenic bacteria to VFAs, carbon dioxide and hydrogen (McInerney, 1988). However, acidogenic bacteria cannot oxidise long-chain fatty acids (LCFAs) of carbon length C12 to C18 and medium-chain fatty acids of carbon length C7 to C11. These intermediates are utilized by the acetogenic bacteria via the β -oxidation pathway to produce acetate, hydrogen and carbon dioxide end products (Gurr and James, 1980).

2.2.3. Acetogenesis

Acetogens are those microbes which generate acetate and carbon dioxide as the by-products of bio conversion of organic compounds. These organisms can be subdivided into two groups – obligate acetate-forming hydrogen-producing (proton-reducing) acetogens and acetate-forming hydrogen utilising acetogens. The acetate-forming hydrogen producing acetogenic bacteria have doubling

times as low as 1.5 to 4 days (Novaes, 1986). They include *Syntrophobacter wolinii* (propionate degraders) and other newer species like *Syntrophomonas wolfei* (butyrate and higher fatty acid degraders), *Syntrophus buswellii* (aromatic benzoate degraders) and sulphate-reducing *Desulfovibrio spp.* (Archer and Kirsop, 1991). Past research has shown that *Syntrophobacter wolinii* (propionate degraders) have been reported to take up to 7 days to regenerate (Boone and Bryant, 1980). The acetate-forming hydrogen-utilising homoacetogens are fast growers like the fermentative bacteria and have a minimum doubling time as low as 1.75 to 29 hours. *Acetobacterium*, *Clostridium*, *Acetoanaerobium*, *Acetogenium*, *Eubacterium* and *Pelobacter* are some genera which are included under homoacetogens. The acetogenic bacteria are found to grow in an optimal pH of around 7 (Angelidaki *et al.*, 2003). While acetate is the most abundant VFA produced during acidogenesis phase, propionate and butyrate are also produced as by products. The acetate can be directly used as a substrate in the methanogenesis pathway of certain methanogens. However the other VFAs cannot be used directly for methanogenesis by any methanogen. These non-acetate VFAs have to be converted to acetate or $\text{CO}_2 + \text{H}_2$ in order to attain maximum methane production. When the non-acetate VFAs are allowed to build up in concentration, they cause an overall decrease in the pH of the system. This prevents the growth and/or metabolism of certain microbes, especially the methanogens (Garcia *et al.*, 2000). Propionate concentrations as low as 30 mM have been shown to have inhibitory effects on methanogenesis (Barredo and Evison, 1991).

It has been exhibited that obligate hydrogen-producing acetogenic bacteria can only survive when low concentrations of hydrogen are present in the environment (Stams *et al.*, 2003). Because of this obligate requirement, they only grow in a symbiotic relationship with hydrogenotrophic microorganisms. The hydrogenotrophic microbes utilise the hydrogen produced from anaerobic oxidation reactions of VFAs, alcohols and long- and medium-

chain fatty acids by the hydrogen-producing acetogenic bacteria. **Table 2.2** elucidates some of the anaerobic oxidation reactions of VFAs and ethanol which are commonly observed in an anaerobic degradation system which contain mixed anaerobic microorganisms. Acetate, hydrogen and carbon dioxide end products can directly be consumed by the methanogenic *Archaea* to form methane in the absence of sulphate-reducing bacteria or homoacetogens (Stams *et al.*, 2005).

Table 2.2 - Degradation pathways of important non acetate fermentation products

Substrate	Reaction	ΔG° (kJ/mol)
Propionate	$\text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + 3\text{H}_2 + \text{CO}_2$	+76.1
n-Butyrate and iso-Butyrate	$\text{CH}_3(\text{CH}_2)_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{H}_2$	+48.1
Iso-valerate	$\text{CH}_3(\text{CH}_2)_3\text{COOH} + 2\text{H}_2\text{O} + \text{CO}_2 \rightarrow 3\text{CH}_3\text{COOH} + \text{H}_2$	+20.2
n-valerate	$\text{CH}_3(\text{CH}_2)_3\text{COOH} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + \text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2$	
Caproate	$\text{CH}_3(\text{CH}_2)_4\text{COOH} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + \text{CH}_3(\text{CH}_2)_2\text{COOH} + 2\text{H}_2$	+48.4
Caproate	$\text{CH}_3(\text{CH}_2)_4\text{COOH} + 6\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 8\text{H}_2 + 2\text{CO}_2$	+48.3
Ethanol	$\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2$	+9.6
Hydrogen	$4\text{H}_2 + 2\text{CO}_2 \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2\text{O}$ (homoacetogenic)	-95.0

ΔG° (kJ/mol) at pH 7, 1 atm, 25°C (Sources: Pind *et al.*, 2003; Pohland and Kim, 2000; Thauer *et al.*, 1977)

The values of Gibbs free energy change for these reactions (with the exception of homoacetogenic reaction) are positive under standard conditions (1 atm, pH 7 and 25°C). This indicates that the reactions are thermodynamically unfavourable unless the H_2 partial pressure is kept $<10^{-4}$ atm. However, oxidation reactions are thermodynamically favourable only when inter-species hydrogen transfer occurs, a process whereby hydrogen released by the syntrophic acetogenic bacteria is captured by hydrogen-utilising methanogens or hydrogen-utilising sulfate-reducing bacteria (SRB) residing in close proximity. *Syntrophomonas* oxidising butyrate and higher carbon fatty acids to

acetate, H₂ and CO₂; species of *Syntrophobacter* which undertake propionate oxidation to acetate, H₂ and CO₂ in association with H₂-utilising sulfate-reducing bacteria such as *Desulfovibrio* (Boone and Bryant, 1980) or hydrogen-utilising methanogens such as *Methanobacterium* or *Methanospirillum* or homoacetogens such as *Acetobacterium*, *Clostridium*, *Acetoanaerobium*, *Acetogenium*, *Eubacterium* and *Pelobacter* are some examples of syntrophic acetogenic and methanogenic microorganisms communities (Archer and Kirsop, 1991).

2.2.4. Methanogenesis

Methanogenesis is the final step in the anaerobic degradation of organic substrates. In this step, methane and carbon dioxide, the most reduced and oxidised forms of carbon respectively, are formed as end products. A highly specialised group of obligate anaerobes which utilise a limited number of substrates comprising of acetate, hydrogen + carbon dioxide and other one-carbon compounds such as, methanol (CH₃OH), methylamines (CH₃NH₂Cl), methyl mercaptan (CH₃SH), carbon monoxide (CO), and belonging to the *Archaea* mediate the methanogenesis step (Gerardi, 2006). These compounds serve as direct precursors for methane formation in the methanogenesis step. There are three groups of methanogenic organisms which mediate degradation of compounds from the acetogenesis step into methane. These groups are classified into Class I hydrogenotrophic methanogens (methanogens utilizing H₂ and CO₂), Class II methyltrophic methanogens (methanogens utilizing methylated compounds) and Class III acetoclastic methanogens (methanogens utilizing acetate). Methanogens exhibit optimal growth around the neutral range of pH 6.8 – 7.2, although different species tend to exhibit different characteristics when faced with different growth conditions (Novaes, 1986). **Table 2.3** exhibits some reactions through which methane is produced in an anaerobic degradation system.

Table 2.3 - Reactions depicting the conversion of intermediates to methane

Substrate	Reaction	ΔG^0 (kJ/mol)
Carbon dioxide/Hydrogen	$4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$	-131
Carbon monoxide	$4\text{CO} + 2\text{H}_2\text{O} \rightarrow \text{CH}_4 + 3\text{CO}_2$	-210
Formate	$4\text{HCOOH} \rightarrow \text{CH}_4 + 3\text{CO}_2 + 2\text{H}_2\text{O}$	-304
Methanol	$4\text{CH}_3\text{OH} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 2\text{H}_2\text{O}$	-319
	$\text{CH}_3\text{OH} + \text{H}_2 \rightarrow \text{CH}_4 + \text{H}_2\text{O}$	-113
Methylamine	$4\text{CH}_3\text{NH}_3\text{Cl} + 2\text{H}_2\text{O} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 4\text{NH}_4\text{Cl}$	-230
Acetate	$\text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2$	-31

ΔG^0 (kJ/mol) at pH 7, 1 atm, 25°C (Table Source: Brock *et al.*, 1994)

Methanobacteriales, *Methanococcales*, and *Methanopyrales* all fall under the Class I group while the second and third groups, Class II and Class III methanogens only includes the order *Methanomicrobiales* and *Methanosarcinales* respectively (Anderson *et al.*, 2009). The Class I and II methanogens are both considered as hydrogenotrophic, in the sense that they use formate, and/or $\text{CO}_2 + \text{H}_2$ as their only precursors for methane generation. The acetoclastic or acetotrophic Class III methanogens, are characterized as being able to use acetate and with certain genus, methanol, methylamines or other one carbon compounds are also used (Anderson *et al.*, 2009). Class I methanogens play an important role in maintaining the reactor equilibrium by consumption of free hydrogen produced in the reactor. This is critical as the propionate to acetate conversion by syntrophic means is thermodynamically unfavorable except under low hydrogen partial pressures. Past studies have shown that syntrophic acetogens are located in close physical proximity to hydrogenotrophic methanogens thereby facilitating in easier interspecies hydrogen transfer (Ariesyady *et al.*, 2007). *Methanobacterium*, *Methanoculleus*, and *Methanospirillum* are three important hydrogenotrophic methanogens

isolated from methanogenic phase of anaerobic system (Hori *et al.*, 2006; Leclerc *et al.*, 2004).

Hydrogenotrophic methanogens while nevertheless important in the maintenance of low hydrogen partial pressures in the reactor vessel still do not contribute to the majority of methane produced. This is because maximum methane production occurs from conversion of acetate to methane due to acetate being the major intermediate product of aceto/acidogenesis (Mackie and Bryant, 1981). The Class III acetoclastic (acetotrophic) methanogens are responsible for this conversion of acetate to methane with the two primary families being *Methanosarcinaceae* and *Methanosaetaceae*. Members of the *Methanosarcinaceae* family possess the ability to use methylated compounds as well as formate, carbon dioxide and hydrogen apart from acetate. In comparison, all known species of *Methanosaetaceae* obligately use acetate as their substrate for methanogenesis. *Methanosaeta* spp. have a very low minimum acetate concentration ($K_s = \sim 5\text{-}70\ \mu\text{M}$) for growth and conversion of acetate to methane (Jetten *et al.*, 1992). *Methanosarcina* spp., have a much higher acetate threshold ($K_s = \sim 1\ \text{mM}$), and a higher growth rate when compared to *Methanosaeta*. Studies conducted, examining population dynamics of acetoclastic methanogens, evince that members of *Methanosaetaceae* predominate under conditions of low acetate concentration ($< 1\text{mM}$) and *Methanosarcinaceae* predominate under conditions of higher acetate concentration (Hori *et al.*, 2006). Methanogenesis is inhibited and/or altered in the presence of certain electron acceptors such as metal oxides [$\text{Fe}(\text{OH})_3$, MnO_2], nitrogen oxides (NO_3^- , NO_2^-), or oxidized sulfur compounds (SO_4^{2-} , SO_3^{2-}) (Schink, 2006). However methanogenesis usually has the potential to recover after these alternative electron acceptors are depleted. The rate of methanogenesis is highly dependent on the amounts of electron acceptor (e.g. acetate versus sulphate) and donor (e.g. hydrogen) present and even the slightest imbalance could render the anaerobic system ineffective due to improper bioconversion of organics to methane (Zeikus, 1979).

2.3 ANAEROBIC REACTOR CONFIGURATIONS

Selecting a particular reactor configuration for anaerobically treating complex wastewaters is highly dependent on a number of factors such as a) physicochemical characteristics of waste, b) wastewater strength and c) operating cost involved. The most common anaerobic reactors can be grouped into three broad categories namely dispersed or suspended-growth, attached-growth and hybrid processes (Jordening and Buchholz, 2005; Hall, 1992; Speece et al, 1997).

In a suspended-growth process, as the name suggests, the microorganisms are in suspension. The most common example of this process is the completely stirred mixed tank reactor (CSTR) with and without biomass recycle (contact process). This process is well suited for the treatment of sludge, animal manure slurry and concentrated wastewaters which contain a high proportion of particulate biodegradable organic materials (Angelidaki et al, 2005; Hall, 1992). However, without biomass recycling, the lack of retention devices to concentrate the wastewater biomass leads to the suspended biomass being continuously discharged along with the treated effluent since the hydraulic retention times (HRT) and solid retention times (SRT) are the same (Hall, 1992). Thus, the effluent quality is highly dependent on the SRT (HRT) of the reactor. This type of setup is most commonly used for pre-acidification of wastewater with typical HRT of 6 to 24 hours (Lettinga and Hulshoff Pol, 1986). It also serves as a holding tank to equalise variations in the flow or in the pollution strength of the wastewater. The inherent disadvantage of this system (apart from biomass loss) is the large size of reactors involved.

In the contact CSTR process, an external settling tank is included to separate and recycle the biomass discharged from the system. This effectively decouples the SRT from HRT. However, due to biogas production, the gravity settling of

biomass can cause problems with settleability. Flocculant addition or vacuum degassing would be necessary to achieve optimal biomass settleability.

In fixed-film or biofilm processes, the microorganisms are concentrated onto an inert medium such as sand, granular activated carbon, gravel and even plastic material. Examples of these processes are fixed bed (anaerobic filters), rotating bed and fluidised bed. Due to the process' inherent ability to retain increased volumes of biomass, the overall SRT is increased even with the application of low hydraulic retention times (<24hr). Hence they are commonly referred to as high rate wastewater treatment systems. One major limitation of this process is the susceptibility to clogging and hence, not particularly suitable for high particulate wastewater. They are most commonly used to treat dilute and soluble organic wastewater (Buitron et al., 2006; Hall, 1992).

Hybrid processes combine advantages of suspended-growth and attached-growth processes in a single system. Examples are high-rate upflow anaerobic sludge blanket (UASB), high-rate expanded granular sludge bed (EGSB), high-rate baffled reactor, low-rate anaerobic lagoon, high-rate two-stage or two-phase anaerobic systems comprising of the dispersed- and/or attached-growth reactors in series whereby the first reactor serves as the acidogenic phase with short HRT and the second reactor as the methanogenic phase with long HRT. Recent developments in the two phase anaerobic system resulted in the temperature-phased thermophilic and mesophilic anaerobic system (TPAD), a patented process developed at Iowa State University to meet the US faecal coliform requirement for Class A biosolid (Harikishan and Sung, 2003). However these processes perform the best when soluble wastewater with little to no particulate content is used.

Apart from the configurations, anaerobic reactors can also be tweaked to operate in different modes. They include batch, semi-continuous and continuous modes. In the batch mode of operation, the substrate is fed once and let to degrade over time. Achieving steady-state conditions is quite difficult in this

mode since biodegradable substrate concentration decreases with time and results in a growth limiting environment for the anaerobes. In contrast, semi-continuous and continuous operations allow steady-state conditions to be achieved under constant substrate concentrations (achieved by constant feeding or feeding at predetermined intervals), thus facilitating maximum microbial growth. Semi-continuous operation is reported to have higher substrate degradation efficiency than continuous operation due to the longer contact time between the biomass and the substrate (Budiastuti, 2004).

2.4 FACTORS AFFECTING THE ANAEROBIC DEGRADATION OF COMPLEX ORGANIC INDUSTRIAL WASTEWATERS

There are a variety of factors which can influence the end products of fermentation and ultimately methane generation from anaerobic degradation of complex organic industrial wastewaters. Wastewater characteristics; operational parameters such as hydraulic retention time (HRT) and/or solids retention time (SRT), temperature and pH are just but a few of the parameters which can influence the outcome of the process along with a few other physico-chemical parameters (Banerjee *et al.*, 1998). To engineer the anaerobic reactors to produce proper metabolic intermediate products that are easily degraded by the methanogens, it pays to have first hand knowledge of factors which may inhibit/enhance process performance of the anaerobic system. Figure 2.3 elucidates the carbon substrate flow in an anaerobic degradation system and what is the individual percentage contribution of each product.

Figure 2.3 - Schematic representation of generalised carbon flow in an anaerobic degradation system and individual percentage composition of intermediates

2.4.1 Seed organisms in reactor system

The seed organisms present in a reactor during the startup of an anaerobic degradation system is very important as it directly influences the degradation rates of organic compounds. In practice, acclimatization of seed sludge with the intended substrate is considered important for enriching certain appropriate microbial communities and thereby ensuring maximum substrate utilization. Past research studies have demonstrated this aspect using lactose- and gelatin-acclimatised cultures on various organic compounds. Cultures when fed with glucose and/or lactate exhibited faster substrate utilisations as a result of acclimatisation to lactose hydrolysis products during the acclimatisation period (Gavala and Lyberatos, 2001). In contrast, longer adaptation period was required for gelatin-acclimatised cultures due to the time needed to produce necessary enzymes since glucose and lactate are not intermediate products of gelatin (protein) biodegradation. A similar research study was performed by Perle *et al.*, (1995) which exhibited that solubilisation of protein was faster when casein-acclimatised sludge was used in place of unacclimatised sludge due to the existing presence of necessary proteolytic enzymes. However in the face of complex wastewaters which are not composed of a single product, it is

difficult to acclimatise a seed to all the components. Instead it would be prudent to choose a seed sludge which can tolerate maximum possible components i.e. a sludge degrading wastewater closest to chosen feedstock, and hence not compromising on the overall process performance.

2.4.2 Type of wastewater feedstock and different high strength industrial wastewaters

Wastewater composition forms the second most important component which may affect the process performance. Wastewaters are markedly different from one another in their COD-contributing organic components viz. carbohydrates, proteins and lipids in the particulate (insoluble) and soluble forms. Bioconversion of the COD components to intermediate products and their subsequent distribution is influenced by the operational conditions and physico-chemical parameters which are conferred upon the anaerobic reactors such as pH, HRT, temperature and OLR. Each condition elicits a different response in the degradation process and it is important to have an optimal range. Synthetic and real organic wastewaters require optimal conditions for degradation depending on the type of organic compounds (substrates) present in them. Glucose and other relatively simple carbohydrates like lactose are degraded to VFAs and alcohols at ≤ 1 -day HRT under optimum pH of 5 to 5.5 (Fang and Yu, 2001). The product compositions were not influenced by HRT but even the slightest changes in pH caused a marked decrease in the product formation as well as methane generation. In the case of protein substrates like gelatine or beef extract, generation of metabolic intermediates were not significantly influenced by HRT but were sensitive to pH; maximum degradation and generation occurring at pH 6.8 to 7 (Yu and Fang, 2003). In a study conducted for complex organic substrates such as synthetic dairy wastewater prepared from powder milk which contained carbohydrates, proteins and lipids, along with digested primary sludge, the carbohydrate fraction was readily acidified while protein acidified to a lesser degree and lipid the least (Yu and Fang,

2002). It has been reported that at short HRTs of 4 h, 93% of carbohydrate, 57% of protein and 20% lipid were converted to acidified metabolic intermediates (Fang and Yu, 2001). Upon increase of the HRT to 24 h, conversion rate of protein and lipid increased to 86 % and 46% respectively.

High strength industrial wastewater is typically difficult to define. It can originate from different food industries, agricultural industries as well as a variety of chemical industries. Basically the term originated from the fact that these wastewaters possess very high COD levels (Robinson, 2001). Some high strength wastewaters may also contain elevated levels of ammonia and metal ions. Industries generally do not use BOD₅ test for determining the strength of their wastewaters due to inherent deficiencies of the BOD test. COD is then most common parameter used for determining the strength of wastewater. A wastewater is deemed low strength by the industries if the COD levels are <1000mg/L (Ganesh *et al.*, 2007). Certain chemical industries may also produce effluents containing 'hard COD' wastewaters (wastewaters where COD contribution is more from inorganic substances, phosphate and ammonia). The most common examples of high strength industrial wastewaters include Tannery effluent wastewater (Robinson, 2001), Textile industry wastewater (Brik *et al.*, 2006), Dairy industry wastewaters including whey permeate (Robinson, 2001), Palm oil industry effluent, petrochemical industry effluents (Ganesh *et al.*, 2007) and Pharmaceutical industry effluents (Chang *et al.*, 2008).

2.4.3 Temperature of the anaerobic degradation process

As with any biological process, anaerobic degradation is also highly temperature dependent for its microbial community growth rates. Studies have shown that biomass growth increases with increment in temperature up to 60°C (Pohland and Ghosh, 1971). The physical properties of complex wastewaters,

such as viscosity, surface tension and mass transfer, are also influenced by the variation in process temperature. Anaerobic reactors are normally operated at mesophilic (30 to 40°C) or at thermophilic temperature (50 to 60°C). However, higher temperatures may result in the process instability of reactors due to the accumulation of VFAs in the effluent. Under psychrophilic temperatures (below 25°C), the process rates are slow compared to mesophilic and thermophilic temperatures. Recent research studies have cited two optimal temperature ranges with one process vessel at at mesophilic (around 35°C) and the other at thermophilic (55 to 60°C) temperatures with decreasing degradation rates between these two optima. Mackie and Bryant (1981) have however suggested that the low rates between these two reaction vessels might have been due to lack of acclimatization. Anaerobic waste degradation at thermophilic temperature has been reported to be significantly efficient than at mesophilic temperature in the sense that there is a marked increase in the reaction rates of particulate hydrolysis directly leading to higher degradation rate, higher organic loading rates, shorter effective HRT or SRT, smaller reactor volume required to treat the same quantity of waste, greater temperature shock resilience and more importantly, greater reduction of pathogens (Angelidaki *et al.*, 2003). Thermophilic temperatures do possess some disadvantages with the most notable being that thermophilic anaerobic process was less stable to environmental changes and more favourable for the production of propionate than the mesophilic process (Fang and Chung, 1999). Some research studies have also found that no marked improvement in performance occurred in organic substrate degradation within thermophilic reactors in comparison to mesophilic reactors (Yu *et al.*, 2002). The unpopularity of thermophilic anaerobic reactors than their mesophilic counterparts may stem from the contradictory findings of researchers as well as the fact that the whole process needs to be heated up to high temperatures, thereby making the anaerobic degradation net energy consuming if the methane yield is not satisfactory.

2.4.4 Hydraulic retention time (HRT)

Hydraulic retention time (HRT) is another of the process parameters that may affect microbial community performance of the hydrolysis-acidification reactor. HRT determines the extent to which maximum achievable hydrolysis of particulate organic matter and acidification of the soluble organics is probable (Guerrero *et al.*, 1999). The acid-phase reactors two-phase anaerobic degradation systems tend to be operated at short HRTs of 6 to 24 hours to expel the slow-growing methanogens from the reactor vessel (Lettinga and Hulshoff Pol, 1986). However, such short operating HRTs would be ineffective in degrading the particulate organic matter present in complex organic wastewaters such as effluents of livestock and food processing industries. These wastewaters contain undissolved and dissolved organic components consisting of a mixture of carbohydrates, proteins and lipids. Hanaki *et al.*, (1987) reported complete hydrolysis and acidification of carbohydrate at HRT >6 hr while hydrolysis of protein required longer HRT of 30 hr to reach about 60% at pH 6-7 when working with synthetic dairy wastewater.

The study also reported an increase in net acid production with increasing HRT with a concomitant increase in methane production. Studies by Guerrero *et al.*, (1999) exhibited that maximum VSS removal and acidification efficiencies of fish meal processing wastewater occurred when HRT was set to 1day at 55°C (58%) and 37°C (46%). When synthetic and raw dairy wastewater were experimented with, maximum acidification occurred at 12-hr HRT (Demirel and Yenigun, 2004; Fang and Yu, 2001). It was observed that HRT of more than 1 day is widely applicable for the first-phase reactor of a two-phase anaerobic system when animal wastes were used as feedstock. More recently, Ahn *et al.*, (2004) adopted a long HRT of 5-day and alkaline pH of 9 for the treatment of a slurry-type swine waste with ~2.0% total solids content. Certain studies have also adopted a 4-day HRT for the first-phase thermophilic (55°C) treatment of raw cattle manure to maximise the hydrolysis of recalcitrant

organics and destroy pathogenic organisms (Harikishan and Sung, 2003). However despite variations in applied HRT, methane production was reported in acidogenic anaerobic reactor all cases irrespective of the loading rates, suggesting that complete phase separation of acidogenesis and methanogenesis was not probable to achieve.

2.4.5 Toxicants and inhibitors

Toxicants and inhibitors in the wastewaters may irreversibly damage the efficacy of an anaerobic process. Therefore it is imperative to identify the potential inhibitors present in the substrate before feeding into the anaerobic system. The substrate concentration by itself can act as a potential inhibitor by building up hydrogen partial pressure, thereby inhibiting hydrogen utilizing bacteria. This in turn would cause buildup of propionic and butyric acid which would stall the anaerobic process via the product inhibition mechanism (a form of feedback inhibition) (Fox and Pohland., 1994). A review study by Chen *et al.*, (2008) shows that various other factors may also play a role in inhibiting the anaerobic process namely; ammonia content in substrate, pH, light and heavy metal ions, halogenated products, phenols, long chain fatty acids, ketones, alkylated compounds as well as certain types of complex branched alcohols. Other potential inhibitors may include dyes and high molecular weight organics not readily amenable for biological transformation.

2.4.6 pH and buffering capacity of the anaerobic reactor system

In an anaerobic system, methanogens are generally active within a pH range of 6.8 to 7.2 (Novaes, 1986). However, with decreasing pH, methanogens are severely inhibited whilst the fermentative bacteria become more active, producing more fatty acids. The fatty acids, though essentially weak acids in nature, can eventually overcome wastewater alkalinity thereby decreasing pH of

the medium. This can lead to inhibition of methanogens. When change in wastewater pH occurs in the anaerobic system, the resulting condition alters the chemical structure of waste compounds to ionised/nonionised form. Thus a compound originally non-toxic to the microbial communities could become toxic on the account of alteration in its chemical structure arising from the change in pH of the medium (Gerardi, 2006). For example, ammonia (NH_3) and hydrogen sulphide (H_2S) in their non-ionised/undissociated forms are more potent than their ionised forms NH_4^+ and HS^- . This is because the non-ionised forms diffuse through the cell membrane of the microorganisms more rapidly. The buffering capacity or alkalinity of a biological system is shown by its degree of resistance to changes in pH. In an anaerobic system, alkalinity is derived from the degradation of organic-nitrogen compounds, such as amino acids and proteins, as well as the generation of carbon dioxide from organic compounds' degradation. Amino acid and protein degradation releases amino groups ($-\text{NH}_2$) which in turn form ammonia (McInerney, 1988). The ammonia later dissolves in water along with CO_2 generated, to form ammonium bicarbonate ($\text{NH}_4\text{HCO}_3^-$) which accounts for the alkalinity. This is very important in lieu of process stability since it neutralizes the organic acids produced during degradation of organics.

Enzyme-catalysed biochemical reactions form an important part in the degradation step. They are prone to disruption caused by drastic changes in pH (McGilvery, 1983). There exists differing optimum pH for ensuring near complete hydrolysis and acidification of the complex wastewater, since each feed contains differing amounts of carbohydrate, protein and lipid components. Slightly acidic pH is favoured for hydrolysis of carbohydrate while optimum hydrolysis of protein occurs at a neutral or weakly alkaline pH (McInerney, 1988). Complex substrates with a mixture of organic compounds (carbohydrates, proteins, lipid) present a trickier scenario with maximum acidification of the synthetic and real complex wastewaters occurring at an optimum pH range of 5.5 and 6.3 at mesophilic temperature of 35-37°C (Yu and

Fang, 2002). Carbohydrate conversion was reduced by five-fold at pH below 5, while hydrolysis of lipid to LCFA was reduced 2-fold (Hanaki *et al.*, 1987). Increase in the conversion of carbohydrate and protein components is only slight when pH is increased from 5.5 to 6.5 but a two-fold increase in lipids hydrolysis can be achieved. Above pH 5.5, more methane was produced with reduction in VFA concentration due to the onset of methanogenesis. This elucidates that complete phasing of an anaerobic reactor to a standalone acidogenic phase is near improbable.

2.4.7 Hydrogen sulphide

Reduction of sulphate (SO_4^{2-}) and fermentation of sulphur-containing proteins lead to the formation of hydrogen sulphide within the anaerobic degradation system. Undissociated H_2S is a toxic compound to not only methanogens but towards sulphate-reducing bacteria as well. It combines with iron of cytochromes (electrons transfer proteins) and other essential iron-containing compounds in the microbial cells leading to a disruption of the electron transport system, which in turn causes loss of function and ultimately cell death (Brock *et al.*, 1994). It has been reported that total hydrogen sulphide concentrations of 100 to 300 mg/L or free H_2S of 50 to 150 mg/L causes severe inhibition and in some cases even complete cessation of the biogas production. Gallert and Winter (2005) exhibited that at total sulphide concentrations of 270 mg/L, methanogenesis was reduced by 50% while a concentration of 85 mg/L inhibited sulphate-reducing bacteria. In another study, an anaerobic system treating waste with high ammonia concentration such as animal manure was found to record a 40% reduction in methane production at a sulfide concentration of 23 mg/L (Hansen *et al.*, 1999). Large amounts of H_2S can lead to precipitation of essential heavy metal ions, such as iron, nickel, molybdenum, cobalt etc as metal sulphides, thus causing deficiencies in heavy metal bioavailability for the microbial communities. Figure 2.4 illustrates the

influence of pH and temperature on the dissociation of hydrogen sulphide and ammonia. At low pH values and low temperature, formation of undissociated hydrogen sulfide is favoured over the ionised sulphide form (Stams *et al.*, 2005, 2003).

Figure 2.4 - Effects of pH and temperature on the dissociation of hydrogen sulphide and ammonia, (.... 25°C, ---- 37°C and ____ 60°C) Source: Stams *et al.*, 2003

2.4.8 Organic overload

Organic overloading, simply put, is too much feed at any given point of time. It can occur either by increasing feed concentration at a given HRT or decreasing HRT at a given feed concentration. This causes organic influent to the reactor to exceed the bioconversion capability of the seed microbial communities. Anaerobic reactors treating intermediate to high concentrations of soluble biodegradable organic substrates are most prone to organic overload. Substrates that have low buffering capacity such as carbohydrates-rich wastes from sugar, starch, potato, distilleries and fruit processing factories also cause organic overload in the system. During overloading, accumulation of hydrogen released by fermentative bacteria from glucose degradation occurs and therefore results in an elevated hydrogen partial pressure ($>10^{-4}$ atm). This causes a shift in the fermentative pathway from acetate, hydrogen and carbon dioxide production,

and the system produces ethanol and longer-chain VFAs such as propionic, butyric acid and valeric acid (Gallert and Winter, 2005; Harper and Pohland, 1987). High concentration of these compounds causes inhibition to the methanogens. When organic overloading is caused by HRT reduction, accumulation of VFAs occurs due to the HRT being shorter than the generation time of slow growing VFA-degrading acetogens and acetoclastic methanogens. This is commonly observed in the acid-phase anaerobic reactors with very short HRT of less than 24 hrs (Yu and Fang, 2001).

2.4.9 Hydrogen

Thermodynamics inside of an anaerobic degradation vessel plays an important role in the bioconversion of various organic compounds (Harper and Pohland, 1986; Thauer *et al.*, 1977). In thermodynamic terms, oxidation-reduction reactions are described by the biochemical standard Gibbs free energy change values (ΔG) of the substrate catabolism at standard conditions of pH 7, 1 atm. and 1 kg/mol activity. Energetically favourable redox reactions occur only if the net free energy change ΔG has a negative value. Anaerobic oxidation reactions of the VFAs and alcohol are thermodynamically possible only when the reduced product (hydrogen) is maintained at low concentrations (done by the hydrogen-utilising microorganisms). Increasing the biological hydrogen removal capacity of the reactor via hydrogen-utilising methanogens, sulphate reducing and nitrate-reducing bacteria, and/or the physical hydrogen removal capacity through gas release can maintain hydrogen levels below 10^{-4} atm (10^{-8} M). This keeping of hydrogen at bay allows the continuous oxidation of propionic and higher organic acids (Fox and Pohland, 1994).

2.4.10 Effects of macronutrients, trace and heavy metals on anaerobic degradation

Methanogenesis is generally considered the rate-limiting step in the anaerobic degradation of soluble wastes. It is only prudent that research has focused on the nutritional requirements for the growth of methanogens, aiming at optimizing the overall anaerobic digestion process. Macro-nutrients viz. carbon, nitrogen, hydrogen, sulfur, potassium, phosphorus, calcium and magnesium are required at minimal concentrations ranging from 1 to 10mM (Archer and Kirsop, 1991). Trace metals like cobalt, iron, nickel, selenium and molybdenum are required for co-enzymes and enzymes involved in catalysing reactions in methanogenesis (Mulrooney and Hausinger, 2003). Nickel is a key component of important methanogenic coenzymes such as F420 and F430 which are key enzymes in all methanogens. It is also present in the enzymes like dehydrogenases and acetyl-coenzymeA decarboxylase/synthase. Iron, cobalt, selenium and molybdenum form the cofactors in many of the anaerobic microbial enzymes. Regarding the toxicity of heavy metals, nickel, copper, lead and zinc are toxic to the anaerobes at differing concentrations. Demirel and Yenigun (2002) reviewed the inhibitory/toxic effect of heavy metals on acid-phase anaerobic reactor. Copper was reported to be most toxic in the range of 1-10 mg/L to VFA-producing microorganisms. Zinc was found to be toxic at 5-40 mg/L. In comparison between cadmium and chromium, cadmium over 20 mg/L was more toxic while chromium at dosages over 5 mg/L was more toxic to acidogenic microbial communities. In the case of organic substances, pentachlorophenol concentrations up to 35 mg/L were found to partially inhibit the acidification of glucose in the acidogenic reactor.

2.5 MICROBIAL VARIATION IN AN ANAEROBIC DEGRADATION SYSTEM

As discussed previously, the anaerobic degradation process is divided into four distinct phases. In reality however, each phase is performed concurrently by a diverse community of Bacteria and Archaea that are interdependent. For example, the conversion of propionate to acetate is thermodynamically unfavorable unless the hydrogen partial pressure in the reactor system is kept to a minimum. To ensure equilibrium, there exists cooperation between syntrophic propionate oxidizing genus *Smithella* with hydrogenotrophic methanogens; *Methanospirillum* (de Bok *et al.*, 2001). Various studies have been conducted over time investigating effects of operational parameters on the microbial diversity in anaerobic systems. The organic loading rate, indicating the amount of organic carbon fed to the system is co-joined with the hydraulic retention time, and subsequently impacts the microbial diversity. If the organic loading rate is too high, the growth and metabolism of fermentative bacteria exceed the growth and metabolism of syntrophic bacteria and methanogens, leading to accumulation of VFAs and subsequent acidification of the system. Other studies examining effects of sulfur and nitrogen compounds on the microbial diversity of anaerobic degradation system have turned up with differing conclusions. Anaerobic degradation systems which receive waste feedstocks with high sulfate loads have been found to have either a reduction or in other cases no significant change in methane production (Pender *et al.*, 2004). Transition metals (e.g. Ni, Co, Cu) on the other hand have been found to positively impact the system with increased methane production. This is most probably due to availability of necessary metal cofactors for the microbial communities (Speece *et al.*, 2006).

A special case of how a system operation affects the microbial diversity is the temperature at which the process is carried out. Majority of the anaerobic degradation systems globally are operated at mesophilic temperatures (~27-40°C). The operation of psychrophilic (<15°C) and thermophilic (>50°C) degradation systems has however been on the rise. The main reason for

operation at psychrophilic or thermophilic temperatures varies depending on parameters involved. Psychrophilic systems negate the need to heat the feedstock to higher temperature than at which it was discharged from its source (also at the same temperature range as that of the anaerobic system) (O'Reilly *et al.*, 2010). The microbial communities in psychrophilic systems tend to possess a similar composition to mesophilic systems; fermentative *Bacteroidetes* and syntrophic *Proteobacteria* being dominant (O'Reilly *et al.*, 2010). Thermophilic systems on the flip side require an energy input to heat the influent to thermophilic temperatures. However, thermophilic anaerobic degradation has been in consideration for some time due to its superior degree of pathogen reduction, significant reduction in retention time, and faster and higher rate of biogas production compared to systems operating at mesophilic temperatures (Ahring, 2003). The bacterial communities in thermophilic systems are dominated by thermophilic genera such as *Thermoanaerobacter*, *Anaerolinea*, and some members of the phylum *Thermotogae* (Yamada *et al.*, 2006). However, thermophilic systems have their own inherent disadvantages. One such negative of thermophilic anaerobic degradation is the higher probability of system failure due to an increase in VFA accumulation, especially propionate, which in turn results in marked reduction in biogas production (Speece *et al.*, 2006). Recent research efforts have been undertaken to investigate “phasing” of the anaerobic systems with the acidogenesis phase operating at thermophilic temperatures and the methanogenic phase operating at mesophilic temperatures (Lv *et al.*, 2010). The use however, is still in its nascent stages and has yet to be proven as being commercially viable for large scale operation.

2.5.1 Uncertainty of microbial community dynamics

Microbial consortia play the most important part in any anaerobic degradation process. Hence a conscious effort has been made over the years to study the diversity and distribution of microbial communities in an anaerobic system. However due to the unpredictable nature of microbial flora, the dynamics of microbes within the anaerobic systems have been termed as a “black box” by researchers (Rivière *et al.*, 2009). Numerous studies have examined the microbial diversity, their responses to differing process parameters as well as differing feed inputs in anaerobic degradation systems. However, there is still more unknown about the performance and responses of microbial communities within these systems than what is currently known at this point of time in anaerobic degradation research. Variety of methods has been developed and is available for the investigation of the microbial diversity in anaerobic degradation systems. Each method has its own inherent advantages coupled with disadvantages. Thereby selection of a technique to analyse the microbial communities play an important role too. The following sections discuss the various methods used for microbial analysis within an anaerobic degradation system along with their use and application as well as the relevance to this thesis.

2.5.2 Molecular analysis methods for analysis of microbial communities in anaerobic degradation systems

Traditionally, cultivation-based methods such as dilution plating and most probable number (MPN) estimates of anaerobic bacteria on selective enrichment culture media were common techniques used to isolate, identify and enumerate the different types of viable microbial populations present in wastewater and environmental samples. The major limitations of these traditional methods are obvious in the sense that they were slow, time consuming, laborious and substantially under-estimate the complex microbial populations due to their bias of selecting a small fraction of cultivable

organisms alone on the chosen media whilst majority of the uncultivable yet viable communities were excluded. The uncultured microbial diversity ranges from 70-99% of actual microbial species, a substantial number, highlighting a large number of uncharacterized species, some of which may play an important role in anaerobic degradation (Hugenholtz *et al.*, 1998). Over the years however, the molecular based analysis methods took prevalence over the culture based methods, which were slowly phased out for analysis of environmental samples. Also the cost of operations for use of molecular methods came down due to rising popularity and newer easier inventions. Analysis of microbial communities in anaerobic degradation systems were also slowly starting to be analyzed with the help of culture independent molecular based analysis.

The ribosomal RNA genes (rRNA genes) are a well established cluster of genes found in every genome of *Prokaryotes* including *Bacteria* and *Archaea*. These rRNA genes are the target site for molecular based analysis of microbial communities in anaerobic degradation systems. The ribosome in a cell is comprised of two subunits; the large and small subunits. The prokaryotic ribosome has the 50S large sub-unit and 30S small subunit. The 50S sub-unit consists of two RNA components, the 5S and 23S rRNAs, while the 30S sub-unit has only one RNA component, the 16S rRNA. Studies investigating microbial diversity have made use of all three rRNAs. However the 16S rRNA gene has become the standard target for molecular analysis. It is preferred because its length (~1600 nucleotides) provides a large degree of phylogenetic information while being short enough to be adequately sequenced using standard sequencing technologies. A traditional reason for the use of 16S rRNA gene dates all the way to Carl Woese, who used 16S rRNA gene sequences and determined that *Archaea* were a separate branch of life from the *Eubacteria* (Woese and Fox, 1977). This combination of positive factors led to the later usage of DNA homology as a basis for bacterial taxonomy. This was and is still considered superior in comparison to traditional measures such as metabolic

activity profiling or analysis of cell envelope structure. Deoxy-ribonucleic acid (DNA) is the prime requirement for molecular analysis. Early methods of extraction did not yield good quality as well as quantity of DNA. Over the period of time, methods for the extraction of DNA from mixed microbial communities have improved. Concurrently, a variety of DNA analysis methods have also been developed and applied in research. This has gained fast ground in anaerobic degradation as well. The most common and powerful molecular analytical methods include single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), fluorescent in situ hybridization (FISH), quantitative real-time PCR (qPCR), and DNA sequencing methods. Each of these analysis methods has a set of known advantages and disadvantages for community analysis which are explained in the latter part of this section.

2.5.3 Community profiling methods

Two of the most commonly used methods for investigating microbial diversity in anaerobic systems are SSCP and DGGE. Both methods rely on separation of the 16S rRNA gene fragments in an acrylamide gel during which fragments with different nucleotide sequence composition form distinct bands. In the process of SSCP single stranded fragments of the 16S gene are utilized which assume a unique structural conformation depending on the nucleotide sequence of the template DNA strand. The sequences form unique bands in an acrylamide gel due to differences in their migratory ability in an electric field which is caused by differences in secondary structure conformation. SSCP was first used in the field of anaerobic degradation by Leclerc et al. (2004) to examine diversity of *Archaeal* microbial communities in 44 different anaerobic degradation systems. The study reported 23 unique species bands out of which two of the most prominent bands were identified as *Methanosaeta concilii* and a *Methanobacterium* belonging to the vadin cluster first described by Godon et al.

(1997). SSCP has been recently used by other groups to examine the microbial diversity in AD systems (Zhao *et al.*, 2008). However its use has so far been limited as more advanced methods of analysis have been developed. This limitation arises from the complications in the ability to accurately determine the taxonomic classification of a specific band after separation. Band classification using SSCP is based on comparison to profiles of known microbial communities (Leclerc *et al.*, 2004). Due to this reason, classification in SSCP is highly dependent on the accuracy and reliability of this comparison, which unfortunately not always turns out to be accurate.

In comparison to SSCP, DGGE uses double stranded 16S rRNA gene fragments amplified from one of the hyper-variable regions of the template. When amplified hyper variable fragments are run through an acrylamide gel containing a denaturing gradient achieved by urea and formamide, the DNA fragments partially denature as the hydrogen bonds between nucleotides of the two strands are disrupted. This disruption transforms the DNA fragment thereby exceeding the pore size of the gel. This prevents further migration along the gel and forms a band which is later visualized in a manner similar to the SSCP. However, DNA bands from the DGGE gel can be excised and sequenced to know the genus and species of the organism from which the template DNA was extracted. This allows a direct identification of the DNA band without relying on a proxy community as in the case of SSCP, thereby conferring a distinct advantage on DGGE over SSCP. Also, DGGE is relatively cheaper to run in comparison to SSCP or RFLP. Hence, numerous studies have been conducted to examine the microbial diversity in anaerobic degradation systems using DGGE. These studies were primarily conducted to examine the effects of various operating conditions, feedstocks, and system designs on microbial community development. For example, Akarsubasi *et al.* (2005) reported differing abundance of *Archaea* in response to changes in feedstock composition.

The third profiling method, RFLP, differentiates between 16S rRNA sequences by digesting PCR amplified 16S fragments with a set of restriction enzymes. This is done in order to generate a set of small and varied DNA fragments which are later visualized on an agarose gel. Different band patterns arise for different sequences which stem from changes in the number and location of restriction sites across the 16S gene which are cut by the restriction enzymes. These are then used to determine if an obtained sequence is unique in comparison to another. A variant of RFLP known as terminal RFLP (T-RFLP) that analyzes only one or two fragments of the 16S gene, termed the terminal fragment, has been developed for large scale analysis of complex communities. In this particular method, near full length 16S rRNA gene sequences are amplified using PCR. The templates are from a mixed community DNA sample. The amplification is performed using either one, or two, primers that are labelled with a fluorescent marker. This mixed PCR product is then digested using one or more restriction enzymes. During analytical phase, the length and quantity of the fluorogenic labelled fragments are determined by capillary gel electrophoresis. The resultant profiles of different fragment lengths are then compared between samples to determine individual relative abundance and diversity within and between samples. It can also be compared to a database of fragments generated from previously known species to tentatively identify a fragment in an unknown sample (Abdo *et al.*, 2006).

2.5.4 Species and family specific profiling and quantitative methods

Community profiling methods are extremely useful in determination of the occurrence of broad changes in microbial diversity. However they are limited in their ability to specifically identify and quantify a desired species or family target. Two novel techniques of FISH probing and qPCR analysis allow for the identification and quantification of only certain desired targets. The basic

principles they are based on and different methods used define them to be suitable for verily different tasks. FISH technique is based on utilizing a fluorogenic nucleotide probe that hybridizes to pre-defined genomic sequence targets within the microbial cells obtained from an environment sample. The probe is then visualized using fluorescent microscopy, which allows for location of cells which contain the hybridized probe. The cells possessing the hybridized probe can then be enumerated either visually or with specific software provided with the visualization device. FISH probes are designed to specifically target the 16S rRNA gene cluster for a number of different taxa. By using probes labelled with different fluorescent molecules it is possible to semi-quantitatively compare the abundance of targets to each other or to the total number of cells in the sample. One particular useful application of FISH to anaerobic degradation samples is the determination of the physical structure of microbial biomass that is generated within anaerobic systems. Past studies by Angenent *et al.*, (2004) made use of FISH probes which were directed towards certain members of methanogens to determine that the genus *Methanosaeta* was involved in anaerobic granule formation. Yamada *et al.*, (2005) also used the FISH technique for the examination of the physical distribution of *Chloroflexi* species in anaerobic granules. Microautoradiography FISH (MAR-FISH), combines the principle of FISH probing along with microautoradiography allowing for the codetermination cell identity and metabolic function (Lee *et al.*, 1999). FISH has been successfully applied by Ariesyady and colleagues (Ariesyady *et al.*, 2007a; 2007b) in studying the syntrophic bacteria which participate in the conversion of non-acetic acid VFAs to acetate, to provide further insight into the metabolism and physical distribution of syntrophic bacteria.

Another technique which helps is species and family quantification is the quantitative real time PCR (qPCR). This technique takes advantage of recent advancements in PCR technology to monitor the production of DNA during the PCR cycle in real time conditions. There are two primary methods for qPCR with one method using the SYBR green, a non-specific fluorogenic

molecule that binds to double stranded DNA. The other method uses a dual-labeled TaqMan[®] probe which binds to a specific DNA target and has a fluorescent marker at the 3' end and a quencher molecule that absorbs the fluorescence at the 5' end. In the SYBR green method, quantification is done by the relative fluorescent intensity of each sample releases after each amplification cycle. In the case of the TaqMan method, the dual labeled probe binds to a target DNA along with the standard PCR amplification primers. During the extension phase, the probe degrades into individual nucleotides on the account of the 5'-3' exonuclease activity of the Taq polymerase enzyme. Since the fluorescent marker is no longer quenched, the fluorescence is detected by a detector instrument. For either assays, a critical or cycle threshold (Ct) value is determined as the relative cycle at which the fluorescence of a sample increases above background value. By comparing the Ct values of samples to those of standards with known starting quantities of template DNA, quantification of the abundance of a particular gene sequence in a mixed community DNA sample is accurately determined.

Both assays have been in extensive use offlate in the analysis of methanogens in particular inside the anaerobic systems. Yu et al. (2005) have designed and tested a set of TaqMan qPCR primers and probes specific for the 4 primary orders of methanogens witnessed in major anaerobic degradation systems. These were also used in monitoring the dynamics of acetoclastic methanogens when faced with varying acetate concentrations (Yu *et al.*, 2006). In another research study, Franke-Whittle et al. (2009) designed a newer and refined set of primers for quantifying *Methanoculleus*, *Methanosarcina*, and *Methanothermobacter* based on the SYBR green based assays. Apart from focusing on the 16S rRNA gene, qPCR has also been adapted to target functional genes as an alternative method for enumeration of methanogens. Based on this modification, Steinberg et al. (2009) developed a set of alternate TaqMan probes to target the *mcrA* gene, which codes for the methyl-CoM

reductase alpha subunit, and succeeded in applying them to the examination of methanogen diversity in several anaerobic degradation samples as well as methanogenic sediments.

2.5.5 Drawbacks of molecular analytical techniques

Each microbial community profiling method has its own specific set of drawbacks that must be considered before selection for analytical purposes. In SSCP and TRFLP, the primary limitation of the method is its inherent inability to accurately identify a band or peak of interest to a known species. This stems from the inability to sequence the band or fragment itself. Hence instead must be compared to known bands for SSCP or fragments for T-RFLP. Another disadvantage with T-RFLP is that often multiple species can mistakenly be assigned to a single fragment. This is because the comparison is solely based on the presence and location of a restriction enzyme site in a given sequence of template. False or “pseudo” peaks may also inhibit the proper interpretation of a T-RFLP profile (Egert and Friedrich, 2003). In the case of DGGE, the limitation encountered is with the reproducibility of band patterns arising from the complexities of casting a denaturing gradient gel. In some cases, the formation of heteroduplex sequences lead to formation of a single band instead of two rightful bands (Gafan and Spratt, 2005). Another criticism of DGGE by Muyzer *et al.*, (1993) is that the technique is not sensitive enough in detection or representation of changes in rare species which may significantly influence the microbial community function and dynamics. A common issue which affects all the profiling methods is the PCR induced bias. Previous studies have elucidated that PCR amplification sometimes results in biased amplicon pools which may reflect the true microbial community composition (Suzuki and Giovannoni, 1996). FISH and qPCR possess the ability to identify, locate, and enumerate specific microbial taxa or functional genes. However both require foreknowledge of potential diversity present in the sample in order to design the

specific primers and probes. Also the development of primers and probes to specifically target only a particular taxon of interest is difficult due to sequence similarity between related taxa.

2.5.6 Relevance of molecular analytical techniques to this study

As described in Section 2.4.1., the uncertainty of microbial community dynamics in an anaerobic degradation systems stem from a variety of factors. As evinced from the literatures cited in sections 2.5.1 and 2.5.2, there exists considerable conflict in terms of concluding the microbial community structure in general. One particular reason which can be attributed to this variance in terms of community structure can stem from the design of the anaerobic system itself. Another cause could arise from the type of feed used for the degradation process. In some cases, there exists considerable washout of the important niche members due to inhibitors present in the feed. Therefore, it is of prime importance to conduct a profound study of the microbial community members to obtain a reasonable picture of the niche members which may in turn give us the pathway of degradation involved. The knowledge of microbial community structure would also prove beneficial in terms of deciding the operational parameters of the anaerobic degradation system. In spite of the limitations listed, community profiling methods nevertheless prove a useful tool in the screening of a large number of samples. Out of the techniques listed, DGGE is currently the preferred methodology for most researchers since the technique allows upto strain level sequence comparison of the DNA band generated with very minimal errors involved. DGGE in combination with the quantitative family specific profiling would form a potent analytical tool in understanding the microbial community profile of anaerobic systems treating high strength industrial wastewater.

2.6 CONCLUSIONS

Though anaerobic processes are frequently controlled with the help of modifying physico-chemical parameters, it is noted that a fairly extensive if not a thoroughly comprehensive microbial analysis would be imperative from a successful operations point of view. This arises from the anaerobic process being heavily dependent on the nature of microbial communities and their responses to the waste substrate being fed into the process system. A review of the molecular microbial techniques used to study and observe the microbial communities revealed that real-time PCR combined with PCR-DGGE technique would be the most suitable, especially in a time bound study. This would help in better understanding the community dynamics and would also be less time consuming. Moreover, these two techniques are the least affected by PCR bias unlike other techniques like SSCP, TRFLP or even clone libraries. Hence for this project study, real-time PCR and PCR-DGGE techniques would be used in conjunction with physico-chemical parameters and biochemical analyses, to provide an in-depth understanding of the microbial communities and their effect on the outcome of an anaerobic degradation process.

CHAPTER 3 MATERIALS AND METHODS

3.1 SEED COLLECTION AND INDUSTRIAL WASTEWATER ANALYSIS

3.1.1 Seed Collection

For the anaerobic degradation experiments, anaerobic sludge was collected from a mesophilic anaerobic digester at a local municipal sewage treatment plant treating primary and secondary sludges. This seed sludge was identified as AnSL. The long term parameters of the seed biomass obtained from the digester are illustrated in Table 3.1.

Table 3.1 - Properties of the anaerobic seed sludge AnSL

Parameters	Value
TCOD	23.58 ± 0.11 g/L
SCOD	4.32 ± 0.17 g/L
Total solids (TS)	22.40 ± 0.97 g/L
Volatile solids (VS)	18.53 ± 1.02 g/L
Total suspended solids (TSS)	19.49 ± 0.73 g/L
Volatile suspended solids (VSS)	16.64 ± 1.15 g/L
Total VFA [*]	0.18 g/L [*]

* Only acetic acid detected

3.1.2 Industrial wastewater selection

Three different industrial wastewaters, designated INDWW, WW1 and WW2 were profiled according to various parameters stated in Table 3.2.

Table 3.2 - Physicochemical parameters of three different industrial wastewaters

Parameters	INDWW	WW1	WW2
TCOD	343.12 ± 3.56 g/L	90.27 ± 2.52 g/L	40.40 ± 1.49 g/L
SCOD	294.35 ± 2.78 g/L	85.72 ± 1.35 g/L	37.87 ± 0.21 g/L
VFA	32.17 ± 0.93 mM	1.02 ± 0.03 g/L	8.73 ± 0.68 g/L
Major organics	Acetate, Valerate, Glutarate, adipate, and succinate	Acetate	Acetate, Valerate, Butyrate
pH	9.23	9.27	9.01
Colour	Deep red	Buff colour	Amber

The wastewaters were selected on the basis of their COD content (roughly translatable into the amount of potential methane) and VFA content (amount of easily degradable organics). Out of the three however, INDWW was the primary feed of choice due to its highest COD content as well as the high VFA content.

3.2. ANALYTICAL METHODS

3.2.1. pH measurement

Sample pH was measured using a portable Thermo Scientific pH meter.

3.2.2. Total solids, volatile solids, total suspended solids and volatile suspended solids (APHA Standard Methods 2540 B)

For the determination of TS, VS, TSS and VSS, mixed liquor with a sample size of between 3 to 5 mL was used for analysis. The analysis was performed according to the standard methods 2540 B.

3.2.3. Chemical oxygen demand (APHA Standard Methods 5220 C closed method)

For the analysis of total COD (TCOD), whole mixed liquor sample was used. For soluble COD (SCOD) determination, mixed liquor sample was centrifuged at 13,000g for 5 min and the clear supernatant was decanted and filtered through 0.2µm pore size nylon filter. The filtrate was then used for SCOD testing. The samples were diluted suitably and were treated with sulphuric acid reagent by gentle mixing. The well-mixed sample was digested in a block digester preheated to 150°C and refluxed for 2 hr inside the fume cupboard.

3.2.4. Volatile fatty acids (VFAs by Gas Chromatography method)

A 0.9 mL of centrifuged (13,000g for 5 min) and pre-diluted sample was acidified with 0.1 mL 10% formic acid prior to VFA analysis. For the analysis, a 1 µL of the acidified sample was used for detection of acetate, propionate, i- and n-butyrate, i- and n-valerate, i- and n-caproate and heptanoic acid by Gas Chromatograph Agilent Systems, model 7890A, Palo Alto, CA equipped with a flame-ionisation detector (FID). Temperatures of the column, injector and detector were 250°C, 60°C and 250°C respectively. The initial oven temperature was 60°C with a first ramp of 120°C and a final ramp of 240°C. The VFA concentrations are then determined from a pre-prepared four-point standard mixed VFA calibration graphs.

3.2.5. Biogas volume and composition (CH₄, CO₂, H₂ by Gas Chromatography method)

Biogas production was measured by a digital hand held Mark III Manometer to calculate the pressure of gas in headspace of vials/serum bottles. In the case of reactor experiments, the biogas produced was collected in 5L or 10L Tedlar gas bags. The biogas produced was sampled and analysed for methane, carbon dioxide and hydrogen using Gas Chromatograph Agilent Systems, model

7890A, Palo Alto, CA equipped with thermal conductivity detector (TCD). High purity Argon was used as the column carrier gas. The front inlet temperature was set at 40°C while the columns PCM A1 at 250°C, PCM C1 at 400°C and PCM C2 at 275°C. The front and back detectors were set at 150°C. Concentrations of methane, carbon dioxide and hydrogen were determined from their respective 4-point standard calibration graphs.

3.2.6 DNA Extraction and qPCR analysis

DNA was extracted from diluted sludge samples using Roche MagNA Pure DNA extraction kits. The extraction procedure was performed in a Roche MagNA Pure DNA extractor (Roche Diagnostics, Mannheim, Germany). Sample volume for the extraction was 100 µL with the same eluted volume of DNA. The probes and primers (Yu *et al.*, 2005) used for q-PCR were obtained from TIB MolBiol (Berlin, Germany) and are listed in Table 3.3. q-PCR reaction was performed in a Roche Lightcycler 480-II (Roche Diagnostics, Mannheim, Germany). The reaction volume for q-PCR was set at 20 µL with each reaction mixture containing 2 µL of template DNA (concentrations not shown), 1 µL (final concentration, 5×10^{-7} mol/L) of the forward and reverse primers along with 2 µL (final concentration 2×10^{-7} mol/L) of the TaqMan probe corresponding to each primer and probe set, 10 µL of Roche Lightcycler Mastermix and PCR-grade sterile water, to a final volume of 20 µL.

Two-step amplification of the target DNA, combining the annealing and the extension steps, was performed as described in a previous study (Yu *et al.*, 2005) with modification and applying the following conditions: an initial 10 min incubation at 95°C for Taq DNA polymerase activation; 55 cycles of denaturation at 95°C for 10 sec; and simultaneous annealing and extension at 60°C for 30 sec. The fluorescence response data obtained during annealing and extension period was in the “single” mode with the channel setting at F1. The

fluorescent signal data was then processed using Roche Lightcycler software (version 4.0).

Table 3.3 - List of primers and probes used for the 16S rRNA gene copy number quantification

Target group	Primer name	Primer Sequence (5'→3')
<i>Eubacteria</i>	BAC338F ⁺	ACTCC TACGG GAGGC AG
	BAC516F [*]	TGCCA GCAGC CGCGG TAATA
	BAC805R [#]	GACTA CCAGG GTATC TAATC
<i>Methanobacteriales</i>	MBT857F ⁺	CGWAG GGAAG CTGTT AAGT
	MBT929F [*]	AGCAC CACAA CGCGT GGA
	MBT1196R [#]	TACCG TCGTC CACTC CTT
<i>Methanomicrobiales</i>	MMB282F ⁺	ATCGR TACGG GTTGT GGG
	MMB749F [*]	TYCGA CAGTG AGGRA CGAAA
	MMB832R [#]	CACCT AACGC RCATH GTTTA C
<i>Methanosaetaceae</i>	MST702F ⁺	TAATC CTYGA RGGAC CACCA
	MST753F [*]	ACGGC AAGGG ACGAA AGCTA
	MST862R [#]	CCTAC GGCAC CRACM AC
<i>Methanosarcinaceae</i>	MSC380F ⁺	GAAAC CGYGA TAAGG GGA
	MSC492F [*]	TTAGC AAGGG CCGGG CAA
	MSC828R [#]	TAGCG ARCAT CGTTT ACG

⁺ Forward Primer, ^{*} TaqMan probe, [#] Reverse Primer

3.2.7 Denaturing gradient gel electrophoresis (DGGE) profiling and phylogenetic analysis

The DGGE test was conducted to investigate Eubacterial and Archaeal community structures targeting the 16S rRNA gene, in a Biorad D-Code Universal Mutation Detection System (Biorad, Hercules, California, United States of America). A conventional PCR was performed with domain-level universal primers: BAC338F (5'-ACTCCTACGGGAGGCAG-3') and BAC805R (5'-GACTACCAGGGTATCTAATCC-3') (Lee *et al.*, 2008) for Eubacteria; ARC787F (5'-ATTAGATACCCSBGTAGTCC-3') and ARC1059R (5'-GCCATGCACWCCTCT-3') for Archaea (Shin *et al.*, 2010).

The 5' ends of forward primers were capped with 40-bp GC-clamps, 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG -3' for Eubacteria and 5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3' for Archaea, to stabilize the melting behaviour of PCR products (Muyzer *et al.*, 1993). The PCR mixture of 20 µL was prepared by GoTaq Mastermix (Promega, Madison, Wisconsin, United States of America) 10 µL, forward primer 1 µL, reverse primer 1 µL, template DNA 2 µL and PCR grade water 6 µL. The mastermix contained dNTP's, MgCl₂, Taq polymerase enzyme and buffer solution. The working concentrations of the forward and reverse primers were 500 nmol/L. A touch-down PCR was conducted according to the protocol described by Shin *et al.* (2010).

The touchdown PCR products were used as template which was loaded onto an 8% (W/V) acrylamide gel with a denaturing gradient of 30%-70%. Here the gradient of 100% was defined as 7 M urea with 40% formamide. The gel was subjected to electrophoresis at 85 V for 13hr in 1× TAE buffer. After staining with ethidium bromide, the bands visible to naked eye were excised and eluted in distilled water. The eluted DNA samples were further amplified using primers without the corresponding GC clamps. The PCR product was then purified from 1% agarose gel and cloned onto p-GEMT Easy vector (Promega, Madison, Wisconsin, United States of America). The cloned 16S rRNA gene fragments were then sequenced by capillary sequencing (AIT biotech, Singapore) and the sequenced results were compared with reference sequences generated in the GenBank database using the BLAST program (BLAST: Basic Local Alignment Search Tool -- <http://blast.ncbi.nlm.nih.gov>). Sequences were deposited in the GenBank database.

CHAPTER 4 EFFECT OF A HIGH STRENGTH INDUSTRIAL WASTEWATER ON THE MICROBIAL COMMUNITY DYNAMICS DURING A MESOPHILIC BIOCHEMICAL METHANE POTENTIAL TEST

4.1 ABSTRACT

A high strength chemical industry wastewater was assessed for its impact on anaerobic microbial community dynamics and consequently mesophilic methane generation. Cumulative methane production was 251 mL/g total chemical oxygen demand removed at standard temperature and pressure at the end of 30 days experimental period with a highest recorded methane percentage of 80.6% of total biogas volume. Volatile fatty acids (VFAs) analysis revealed that acetic acid was the major intermediate VFAs produced with propionic acid accumulating over the experimental period. Quantitative analysis of microbial communities in the test and control groups with quantitative real time polymerase chain reaction highlighted that in the test group, Eubacteria (96.3%) was dominant in comparison with methanogens (3.7%). The latter were dominated by Methanomicrobiales and Methanobacteriales while Methanosarcinaceae in test groups increased over the experimental period, reaching a maximum on day 30. This increase in Methanosarcinaceae was concomitant with acetic acid being the major VFA intermediate detected in the test groups during the experimental period. Denaturing gradient gel electrophoresis profile was performed, targeting the 16S rRNA gene of Eubacteria and Archaea, with the DNA samples extracted at 3 different time points from the test groups. A phylogenetic tree was constructed for the sequences using the neighborhood joining method. The analysis revealed that the presence of organisms resembling Syntrophomonadaceae could have contributed to increased production of acetic and propionic acid intermediates while decrease of organisms resembling Pelotomaculum sp. could have most likely contributed to accumulation of propionic acid. This study suggested that the degradation of organic components within the high strength industrial

wastewater is closely linked with the activity of certain niche microbial communities within eubacteria and methanogens. Also the decrease in certain specific members of the microbial communities (Eg: *Pelotomaculum* sp.) and increase of certain communities (Eg: *Methanosarcinaceae*) could have a direct bearing on the efficiency of the anaerobic degradation process.

4.2. INTRODUCTION

Anaerobic degradation has been used as a high strength organic waste and wastewater treatment process for several decades (Ahring, 2003). The degradation process is complex and includes sub-processes like hydrolysis, acidogenesis and methanogenesis, each of which is delicately balanced with each other for optimum substrate degradation. A consortium of Eubacteria converts organic carbon into acetic acid and/or carbon dioxide, which is then further converted to methane by a group of specialized microbes, the methanogens (Ueno *et al.*, 2001). The conversion of organics to methane under anaerobic conditions is reliant on the actions of microbial communities on the substrate. Therefore, it is useful to study the microbial flora and its members which are involved not only in order to improve the overall anaerobic process but also to track process changes which may lead to failure of system (Fernandez *et al.*, 1999).

Anaerobic degradation has widespread applications in today's pursuit of renewable energy sources. But before determining its effective potential, it would be better served if a key element of the process i.e. the ultimate biogas generation potential of a given substrate can be better understood. In the past and up until a few years ago, research was focused more on solving the issue of determining the biogas generation potential. The biochemical methane potential (BMP) test does indeed provide a sufficient base for determining the potential of a particular substrate to generate methane. Past research on the BMP test had often focused on either optimizing the substrate to inoculum ratio (Fernandez *et al.*, 2001; Neves *et al.*, 2004; Raposo *et al.*, 2006) or had used specific

substrates (Raposo *et al.*, 2006). Some research has also been dedicated to understanding the various factors which may affect the degradation process like temperature, pH and particle size of substrate (Pabon-Pereira *et al.*, 2012). However, substantial research has not been reported on understanding the microbial community dynamics during the process of a BMP test.

Microbial community shifts occur over a period of time during the anaerobic degradation process with niche members possibly growing to dominate over the other members. However, tracking these changes may be difficult with the standard practices of culturing organisms. This may arise from the niche organism's inability to propagate ex-situ and hence leading to false results. Recent developments in molecular techniques targeting the 16S rRNA gene can possibly provide greater insight into such anaerobic community shifts in response to different process settings (Lee *et al.*, 2009). Monitoring microbial communities in an anaerobic degradation process can possibly provide information for process optimization and system configuration. The quantitative real time polymerase chain reaction or q-PCR test allows for targeting microorganisms in an anaerobic degradation process and so can facilitate tracking of community dynamics and shifts as the process undergoes change (Yu *et al.*, 2005). The changes in microbial community structure can be monitored using denaturing gradient gel electrophoresis (DGGE) in combination with investigation of formation and degradation of certain reaction products. The DGGE technique has proven effective in detecting microbial community shifts and also identifying the phylogenetic affiliates of microbial populations in mixed culture systems (Ueno *et al.*, 2001, Calli *et al.*, 2005). Lee *et al.*, (2008) also elucidated that DGGE and qPCR are ideal techniques to study microbial transitions in batch systems operating with mixed microbial cultures. The phylogenetic analysis would help in the in-depth better understanding of how the communities at the species or strain level react to the presence of a particular substrate. This would prove especially helpful in the study of acidogens, whose community dynamics are relatively unknown.

This study uses the BMP test coupled with microbial community analysis, to investigate anaerobic degradation process challenged with a high strength chemical industry wastewater at mesophilic (35°C) temperature. Process performance was investigated in terms of chemical oxygen demand (COD) reduction, biogas production, and microbial community responses. Duration of the BMP test in this study was 30 days. Community shifts of individual methanogen families were monitored with the q-PCR technique and DGGE combined with phylogenetic analysis provided the community structure within the systems. The research also provided an insight on impact of wastewater on methane yields with changes in community structure.

4.3. MATERIALS AND METHODS

4.3.1. Wastewater characteristics

The chemical industry wastewater was initially profiled in terms of parameters shown in **Table 4.1**. The wastewater was defined as high strength in terms of its high total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) value.

Table 4.1 - Chemical industry wastewater (INDWW) properties

Parameter	Value
Total chemical oxygen demand	(343.12 ± 3.56) g/L
Soluble chemical oxygen	(294.35 ± 2.78) g/L
Volatile fatty acids (VFA's)	(3.6 ± 0.02) g/L
Acetic acid	0.83 g/L
Valeric acid	1.23 g/L
Other organic components	Glutarate, adipate, and
pH	9.23
Total dissolved solids (TDS)	(9914 ± 46) mg/L
Sodium	44.1 g/L
Colour (visual)	Deep red

4.3.2. Seed biomass

Anaerobic sludge was collected from a mesophilic anaerobic digester at a local municipal sewage treatment plant treating primary and secondary sludges. This seed sludge, identified as AnSL, was profiled as shown in **Table 4.2**.

Table 4.2 - Properties of the anaerobic seed sludge AnSL

Parameters	Value
TCOD	23.58 ± 0.11 g/L
SCOD	4.32 ± 0.17 g/L
Total solids (TS)	22.40 ± 0.97 g/L
Volatile solids (VS)	18.53 ± 1.02 g/L
Total suspended solids (TSS)	19.49 ± 0.73 g/L
Volatile suspended solids (VSS)	16.64 ± 1.15 g/L
Total VFA*	0.18 g/L*

*Only acetic acid detected

4.3.3. Pre-experimental phase

AnSL was “degassed” at 35°C in a manner as described in a previous study (Angelidaki *et al.*, 2009). This preparatory stage was performed for 10 days for depletion of residual substrate originally present in the sludge.

4.3.4. EXPERIMENTAL PHASE

4.3.4.1. BMP tests

The test was performed in a manner similar to Angelidaki *et al.* (2009) but with modifications. Volume of the serum bottle was 150 mL -- with 90 mL working volume and 60 mL headspace. A sterile mineral salts medium (MSM) was prepared with the following components (g/L): NH_4Cl 1.5, $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.8, NaHCO_3 1.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3, NaCl 3.0 and 5mL trace elements solution. The trace elements solution contains the following components (g/L): $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.6, H_3BO_3 0.38, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.20, $(\text{NH}_4)_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.10, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$

0.10, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.10, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.23, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.3, and $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.05.

The 750 mL of seed biomass was mixed well with 75 mL wastewater and 175 mL MSM to maintain a substrate to inoculum ratio of 2 g SCOD:1 g volatile suspended solids (VSS). For the blank controls, sludge was mixed with MSM to maintain the final VSS equal to the test group. Then 90 mL of homogenous test mixture was added to serum bottles, sealed with rubber septa, crimped and the headspace flushed with N_2 gas for 2 minutes. After flushing with N_2 gas, the serum bottles were incubated in shaker incubators at 150 r/min, and 35°C . Serum bottles containing the wastewater were identified as AD/IND-WW and blank control bottles named AD/Control. All the analyses were performed in triplicate. The experimental period was considered as 30 days due to no further methane production and VFA degradation between day 30 and day 35 (data for day 35 not illustrated).

4.3.4.2. Physicochemical characterization

For initial testing of total solids (TS), volatile solids (VS), total suspended solids (TSS), VSS, TCOD, SCOD and VFAs, samples were drawn from the homogenous bulk mixture. Later during the experimental stages, for TCOD, SCOD and VFA analyses, 1mL samples were withdrawn from the serum bottles and diluted suitably for analysis. The total volume changes during the sampling period were taken into consideration for calculation purposes. VSS (taken to determine microbial growth) and pH measurement were performed only on day 0 and day 30 due to limited sample volume. Tests for TCOD, SCOD, TS, VS, TSS, and VSS were performed in accordance with standard methods (APHA, 1998). For VFA analysis, the liquid part of diluted samples was filtered through a $0.2\ \mu\text{m}$ syringe filter and 900 μL of the filtered sample was mixed with 100 μL of 10% formic acid for testing the VFA's. Samples were analyzed using a

gas chromatograph equipped with flame ionization detector (GC-FID) (Agilent Systems, model 7890A, Palo Alto, California, United States of America).

4.3.4.3. Gas measurement

Gas measurements were performed as mentioned in Section 3.2.5.

4.3.4.4. DNA extraction and q-PCR analysis

DNA extraction and qPCR analysis were performed as mentioned in Section 3.2.6

4.3.4.5. Denaturing gradient gel electrophoresis (DGGE) profiling and phylogenetic analysis

The DGGE test was conducted to investigate Eubacterial and Archaeal community structures targeting the 16S rRNA gene, in a Biorad D-Code Universal Mutation Detection System (Biorad, Hercules, California, United States of America). Time points chosen for the DGGE profile were at day 0, 15 and 30. The analysis was performed for the test groups alone to investigate the effect of the high strength industrial wastewater on the microbial communities present in anaerobic biomass. DGGE was performed as mentioned in Section 3.2.7. Sequences analyzed were deposited in the GenBank database with the accession numbers from **KF511593** to **KF511611**. Neighbour joining trees were constructed for phylogenetic analysis using the MEGA-5.1 software (Tamura *et al*, 2011).

4.4. RESULTS

4.4.1. Physicochemical characterization

The changes in VSS, pH, TCOD and SCOD are listed in **Table 4.4**. The relatively unchanged pH levels in the test group on day 30 indicated a likely self buffering system, with possibly no need for external pH control. The VSS profiles of the test group on day 30 (11.56 ± 0.45 g/L) indicated a slight increase in the biomass content (day 0: 10.29 ± 0.84 g/L). This was a likely indicator that the seed biomass was able to propagate in the presence of the wastewater. However, other parameters such as VFA production and degradation profile, COD reduction and methane generation need to be considered for studying biomass activity and efficacy. The results of VFA analysis for the test groups are illustrated in **Figure 4.1**. VFA concentration for the test groups was 3.6 g/L on day 0 and reached a maximum of 7.46 g/L on day 20 but subsequently decreased till day 30 (3.93 g/L). Acetic acid was the major intermediate VFA with a highest concentration of 3.34 g/L on day 20. Propionic acid was also found to increase throughout the experimental period with a maximum of 2.51 g/L on day 30. VFA values in control groups were below detectable limits and hence not illustrated.

Table 4.3 - COD profiles during mesophilic (35°C) BMP tests

Group	Day 0	Day 15	Day 30
TCOD (AD/Control)	(15.68 $\pm$ 0.12)	(13.70 $\pm$ 0.22)	(9.05 $\pm$ 0.07)
TCOD (AD/IND-)	(39.22 $\pm$ 0.07) g/L	(38.96 $\pm$ 0.08)	(34.94 $\pm$ 0.10)
SCOD (AD/Control)	ND	0.23g/L	N.D
SCOD (AD/IND-)	(21.26 $\pm$ 0.15) g/L	(23.43 $\pm$ 0.32)	(17.09 $\pm$ 0.11)
VSS (AD/Control)	(10.32 $\pm$ 0.17) g/L	NA	(9.18 $\pm$ 0.24)
VSS (AD/IND-WW)	(10.29 $\pm$ 0.84) g/L	NA	(11.56 $\pm$ 0.45)
pH (AD/Control)	7.45	NA	7.11
pH (AD/IND-WW)	7.34	NA	7.41

*N.D – Not Detected, NA - Not Analyzed

Figure 4.1 - VFA profile of AD/IND-WW at 35°C

4.4.2. Biogas measurement and profile

Biogas measurement was done every 5 days except between day 20 and 30 where measurements were made on day 27 and day 30. Gas production was calculated as volume produced over every 5 days after pressure in headspace was equalized to 1 atm at room temperature following each measurement. The cumulative methane production in test group was 233 mL at standard temperature and pressure (STP) while the control group produced a cumulative methane volume of 98 mL STP (highlighted in **Figure 4.2**). Maximum methane production in the mesophilic test group occurred between day 27 and day 30 where it had produced 60.5 mL within 3 days. The highest recorded methane percentage of total biogas in the test group was 80.6% while those in control recorded 66.8%.

Figure 4.2 - Volume of methane produced over 30 days period for AD/IND-WW at 35°C. Error bars indicate standard deviation of triplicate measurements

4.4.3. q-PCR analysis

The shifts in the numbers of 16S rRNA gene copies of Eubacteria and different Methanogenic communities are illustrated in **Figure 4.3**. It was observed in the test groups that, Eubacterial populations gradually decreased till day 20 after which their numbers became more stable. However, the Eubacteria still accounted for 96.3% of the total microbial population with the Archaea contributing only 3.7%. Eubacteria in the control groups decreased continuously during the test period. Within the Methanogenic populations of both test and control groups, Methanomicrobiales and Methanobacteriales were most dominant. While Methanosarcinaceae increased over the 30-day period in the test groups, it decreased in copy numbers over the 30 days period in the control groups.

Figure 4.3 - Differences in the shifts in microbial populations during the 30 days period in a) AD/IND-WW at 35°C and b) Control. Error bars indicate standard deviation of triplicate measurements

4.4.4. DGGE profile and phylogenetic analysis

DGGE profile and phylogenetic analysis was conducted to characterize the microbial community structure in response to the presence of wastewater. Both Archaeal and Eubacterial PCR products were subjected to DGGE profiling and phylogenetic analysis. This was performed with DNA samples collected during day 0, day 15 and day 30 from the test groups. The DGGE profile is illustrated in **Figure 4.4**. The profile indicates certain DNA bands of the Eubacteria faded in intensity over the 30 days period and so suggesting the wastewater may have stressed the microbes, resulting in a decrease of copy numbers of DNA. However, the Archaeal profile had remained relatively stable through the 30 days period, suggesting Archaea were more resistant to the stress. The phylogenetic trees constructed are illustrated in **Figure 4.5** for Eubacteria and **Figure 4.6** for Archaea.

Figure 4.4 - DGGE profile of microbial DNA isolated from AD/IND-WW at 35°C. L1, L2 and L3 represent the Eubacterial DNA isolated from Day 0, Day 15 and Day 30 samples while L4, L5 and L6 represent Archaeal DNA isolated from Day 0, Day 15 and Day 30 respectively

Figure 4.5 - Neighbor-joining method used for constructing phylogenetic tree, highlighting the identities of 16S rRNA genes of Eubacteria at 35°C. Taxon marked with • indicates the organisms analyzed. Numbers at nodes are bootstrap values derived from 100 analyses

Figure 4.6 - Neighbour-joining method used for constructing phylogenetic tree, highlighting the identities of 16S rRNA genes of Archaea at 35°C. Taxon marked with ▲ indicates the organisms analyzed. Numbers at nodes are bootstrap values derived from 100 analyses

In the DGGE profile, band 1 of Eubacteria was the most prominent band which did not visibly decrease in intensity (refer to band 2). When the sequences were analyzed, it resembled closely (98-99%) to the genera *Pelomonas*, belonging to the phylum Proteobacteria. Another interesting band development was band 4 in the Eubacteria which closely resembled (95%-97%) the genera *Pelotomaculum* belonging to the phylum Firmicutes. This band had decreased in its visual intensity as evidenced in lanes 2 and 3. Another band belonging to the phylum Firmicutes was band 3 which was present only on day 30 which closely resembled (99%-100%) the genus *Sedimentibacter* as well as the thiosulfate reducer, *Dethiosulfatibacter*. Bands 5, 7 and 6 closely resembled (96%-99%) the genera *Syntrophomonas* and *Pelospira* respectively which belong to the phylum Firmicutes as well.

The Archaeal bands fell within three main orders: Methanomicrobiales, Methanosarcinales and Methanobacteriales. The Methanomicrobiales had 4 band profiles with bands 8 and 9 similar (98%-100%) to *Methanospirillum hungatei* JF-1 strain while bands 10 and 11 closely resembled (99%-100%) *Methanolinea tarda* NOBI-1. The Methanosarcinales had 6 bands out of which band 12 and band 13 were similar to the genus *Methanosaeta*. Bands 14 and 15 resembled closely (97%-99%) to *Methanosarcina barkeri* strain DSM-800 while bands 16 and 17 closely resembled (96%-99%) *Methanosarcina mazei* strain DSM-2053 and OCM-26. The Methanobacteriales had just two bands, band 18 and band 19 which closely resembled (97%) *Methanobacterium formicicum* strain DSMZ-1535.

4.5. DISCUSSION

There have been many studies reporting use of BMP test for various organic compounds. Studies by Owens and Chynoweth (1993) and Hansen et al. (2004) proposed protocols for determination of BMP while recent studies by Angelidaki et al. (2009) have refined the methodology involved. However, past

studies have not given due importance to understanding the microbiology behind the degradation process involved during the BMP test. As mentioned earlier, the degradation process would be underpinned by microbial communities involved and it would only be prudent to study the microbial community dynamics in tandem with the process parameters to get a better picture of the degradation process. Therefore, this study focused on the relation between the microbial communities, COD reduction, VFAs generation and the CH_4 produced during the anaerobic degradation process of a high strength chemical industry wastewater at mesophilic (35°C) temperature.

VFA intermediates are generated in majority of anaerobic degradation processes. They serve as precursors for the production of methane, especially acetic acid. The wastewater investigated in this study was inherently rich in VFA's. Valeric acid along with glutaric and acetic acid formed its major components. Hence it was initially assumed that anaerobic process (especially methanogenesis) would perform better due to readily available VFA for degradation. According to Speece (1996), acetate is the most important VFA intermediate, contributing to more than 70% of the methane produced during an anaerobic degradation process. However, acetate can be used by only a small group of organisms, the "acetoclastic methanogens", belonging to the order Methanosarcinales which comprises the families Methanosaetaceae and Methanosarcinaceae. These use acetate as a substrate to generate methane as the end-product. Considering the VFA data from the test group, the acetate levels had increased till day 20 after which it rapidly decreased till day 27. This was concomitant with the q-PCR results which highlight that Methanosarcinaceae had increased till day 30 although Methanosaetaceae remained relatively constant. Previous studies have shown Methanosaetaceae have a relatively low acetate $K_s = \sim 5\text{--}70\text{ }\mu\text{M}$ for conversion to methane while Methanosarcinaceae have a higher acetate $K_s = \sim 1\text{ mM}$ (Hori *et al.*, 2006; Sekiguchi *et al.*, 1999; Jetten *et al.*, 1992). Thus Methanosaetaceae would likely predominate under low acetate concentration while Methanosarcinaceae would prevail under higher

acetate concentration conditions, with the latter also having a higher growth rate compared to Methanosaetaceae. The results of this study are in correlation with previous findings wherein organisms resembling Methanosarcina had been found more prominent from day 15; the point where acetate was on the verge of reaching its highest concentration. However, from day 20 there was rapid drop in acetate concentration which was likely caused by the effective aceticlastic action of Methanosarcinaceae. The most logical hypothesis would be that Methanosarcinaceae need a desired acetate concentration combined with a particular population level for effective aceticlastic methanogenesis. Methanospirillum, Methanolinea and Methanobacterium-like organisms were also detected in the test group. These methanogens are obligately linked with the Syntrophomonadaceae (also present in the system) to produce methane through the hydrogenotrophic pathway. However, the hydrolytic and acidogenic action of Syntrophomonadaceae could have occurred at a faster rate than the consumption rate of acetate or hydrogen to produce methane by the Methanosarcina or the hydrogenotrophic methanogens. This could have led to the initial accumulation of acetate and then followed by the decrease and could also explain the slow initial rate of methane generation.

Aside from the above discussed phenomena, there was also accumulation of propionic acid in the test group. Propionate is an important part of the anaerobic metabolism and contributes to 6%--35% of the carbon balance in the anaerobic degradation system (Scholten and Conrad, 2000). In the test group, propionic acid accumulation was continuous and reached a maximum on day 30. Past research has shown that when n-valerate was present in the substrate, the acidogenic degradation produced propionate as the major intermediate, rather than acetate, thereby leading to propionate accumulation (Gallert and Winter, 2008). It had also been reported that if odd number of carbon atoms were to be present in VFAs or organics in the substrate, propionate would be the final end product of the acidogenic degradation pathways instead of butyrate or acetate (Weng and Jeris, 1976). The wastewater

used in this study has n-valerate as the major VFA and sodium salts of glutaric acid which contains odd number of carbon atoms and hence these may have caused the propionic acid accumulation. However, band 4 in the DGGE profile has corresponding similarity to the genus *Pelotomaculum* which as reported in earlier studies is an important genus in propionate oxidation (Imachi *et al.*, 2007) along with species of the genera *Desulfotomaculum*. However, propionate oxidizers (including *Pelotomaculum* and *Desulfotomaculum*) are slow growers and highly sensitive organisms and therefore are easily susceptible to even low levels of high strength organics (Gavala *et al.*, 2003). The DGGE profile shows that band 4, showing similarity to propionate oxidizers, had decreased in intensity on day 15 and day 30, indicating a probable reduction in their copy numbers. This reduction of population numbers could have been one of the major factors leading to the accumulation of propionic acid.

From the various parameters analysed, it is clear that the degradation process was not comprehensive and a significant amount of residual COD was observed at the end of day 30. This may have been due to a partial inhibition of the anaerobic degradation process (E.g.: accumulation of propionic acid possibly due to reduction in copy numbers of *Pelotomaculum* sp.). This inhibition could have been caused by the presence of certain organic compounds present in the wastewater not amenable to biological transformation (recalcitrants) and which could have been toxic for certain microbial genera (Duran and Speece, 1999). They also showed that mass transfer limitations influence the degradation of a particular biodegradable compound. This could have been an underlying factor for incomplete propionic acid degradation. The propionic acid may have been generated at a faster rate by organisms closely resembling *Syntrophomonas* and *Pelospora* which might have led to propionate itself inhibiting the propionate degraders, possibly through a feedback mechanism. This build up of propionate could have also adversely affected other microbial communities as well. In support of this argument, it has been

reported earlier that imbalance of microbial populations causes build-up of intermediates unfavourable to the methanogenic populations (McMahon *et al.*, 2001), which could very well be the case in this study. Leclerc *et al.* (2001) also state that the imbalance between population members could very well be the underlying reason behind the inhibition of anaerobic processes.

This study demonstrated the changes in microbial community dynamics as well as diversity during over a period of time during the batch operation of BMP test. The study was conducted with no changes in operating conditions over the experimental time period. However, it is impossible to determine which specific component of the wastewater would have actually affected the microbial community dynamics and in turn, the bioprocess, due to the complex nature of the wastewater involved. It is also highly unlikely that the microbial community dynamics could be completely explained as a function of one or a few factors since the seed used in this study consisted of a complex set of micro-organisms. Hence a much more profound study is needed in order to completely understand the role that the microbial communities play in the anaerobic degradation process.

4.6. CONCLUSION

The anaerobic degradation process at mesophilic temperatures is closely linked to its microbial community dynamics. In the test group, increased VFAs production was marked by the presence of *Syntrophomonas*, *Pelospora* and *Sedimentibacter* like Eubacterial species. Propionic acid accumulation was observed throughout the experimental period which could have stemmed from reduction in the population of *Pelotomaculum* sp. in combination with a possible feedback inhibition mechanism. Methanogens remained relatively unaffected by the presence of wastewater. *Methanosarcinaceae* population increase could have contributed to effective acetoclastic methanogenesis. Complex chemical nature of wastewater in conjunction with complex microbial community structure in seed biomass makes it difficult to explain process as function of one or more physical parameters involved.

CHAPTER 5 INFLUENCE OF ONE STEP TEMPERATURE INCREASE ON MICROBIAL COMMUNITY DYNAMICS DURING THERMOPHILIC BIOCHEMICAL METHANE POTENTIAL TEST OF AN INDUSTRIAL WASTEWATER

5.1. ABSTRACT

In the anaerobic degradation industry, thermophilic anaerobic reactors are considerably less common than mesophilic anaerobic reactors. Consequently biomasses used to seed thermophilic anaerobic reactors are often derived from mesophilic reactors. This mesophilic seed biomass may then be subjected to gradual temperature change or the more extreme one step change. In the present study, a mesophilic seed biomass was subjected to a direct one step temperature rise acclimation (35°C to 55°C) for 10 days. This seed was further used in a thermophilic (55°C) biochemical methane potential test of a high strength industrial wastewater to observe the microbial community dynamics and thermophilic process performance following the temperature change. Cumulative methane production was 138mL/g total chemical oxygen demand (TCOD) removed adjusted for standard temperature and pressure (STP) with the highest recorded methane content of 32.0% of total biogas volume. Volatile fatty acids (VFAs) analysis indicated an initial production of VFA's intermediates, especially acetic acid, but no subsequent degradation. Methanomicrobiales were the dominant methanogenic flora with variable numbers of Methanosarcinaceae. Phylogenetic analysis highlighted the presence of true thermophilic and certain eurythermal organisms in Eubacterial and Methanogenic populations. It can be stated that the one step temperature increase had subjected the mesophilic seed biomass to selection pressures by narrowing of microbial diversity and consequently caused a community imbalance. This community imbalance then led to excessive buildup of intermediates (VFAs) which further curtailed methanogenesis via loss of function of biosynthetic pathways, especially in aceticlastic methanogens (Methanosarcinaceae). Hence the one step temperature increase acclimation

method could adversely affect startup of a thermophilic anaerobic process and may require more careful monitoring and control of feeding pattern (organic loading rate), pH and intermediates buildup.

5.2. INTRODUCTION

Thermophilic anaerobic degradation (50°C-60°C) has gained interest over mesophilic process (35°C-40°C) due to higher potential for increased destruction rate of organic solids and other substrates, better solids liquid separation, and increased removal of pathogenic microbes (Krugel *et al.*, 1998; Peddie *et al.*, 1996; Ziemba and Peccia, 2011). Previous studies indicated that thermophilic anaerobic degradation has a higher substrate utilization rate, higher microbial decay rate, capability for treatment of higher organic loadings and higher specific growth rate (Zabranska *et al.*, 2000; De la Rubia *et al.*, 2002; Gavala *et al.*, 2003b). Notwithstanding these positive reports, some contradictory reports cited thermophilic systems producing poor supernatant quality, lower process stability, and high levels of VFA accumulation depending on the manner in which the thermophilic seed biomass was obtained (De la Rubia *et al.*, 2006).

For the initiation of thermophilic anaerobic degradation process, a thermophilic seed biomass is usually suggested. However operational constraints and past studies have shown that mesophilic seed may serve as a good source of thermophilic homologues provided the biomass is acclimated to the higher temperature in a suitable manner. De la Rubia *et al.*, (2005) reported that selective growth of populations at higher temperature is imperative to obtain good process performance under thermophilic conditions. Aitken and Mullenix (1992) also reported that some mesophilic organisms may remain dominant at thermophilic temperatures due to their wide thermal range, causing an imbalance thereby leading to process failure. From the past studies, it can be deduced that mesophilic seed contains thermophilic homologues and could be

used for initiating a thermophilic process. However, temperature, along with organic substrates, played a role in determining the outcome of desired anaerobic process. Hence this study was focused on determining the impact of a one step thermal shock on a mesophilic seed and its subsequent process performance under thermophilic conditions.

Microbial community changes are intertwined in the anaerobic degradation process and occur over a period of time with some members dominating in numbers or degrading efficacy of substrates. Recent developments in molecular techniques targeting the 16S rRNA gene could possibly provide greater perspective into the anaerobic community shifts in response to different process settings (Lee *et al.*, 2009). The quantitative real time polymerase chain reaction or q-PCR test allows rapid tracking of community dynamics and shifts as and when process change occurs (Yu *et al.* 2005). Denaturing gradient gel electrophoresis (DGGE) can be used in combination with investigating the formation and degradation of certain reaction products to provide a comprehensive outline of the functionality of the microbial communities involved. The DGGE technique has been used to detect microbial community shifts and identify certain phylogenetic affiliates of mixed microbial populations' complex culture systems (Ueno *et al.* 2001).

This study coupled the biochemical methane potential (BMP) test of a high strength industrial chemical wastewater with microbial community analysis. A mesophilic seed biomass was subjected to a one step temperature increase procedure (Bouskova *et al.* 2005) which was further used in the BMP test under thermophilic conditions (55°C). Process performance was investigated in terms of chemical oxygen demand (COD) reduction, volatile fatty acids (VFAs) production, biogas production which was further co-related with the microbial community responses. Changes within individual methanogen families were monitored using q-PCR technique. DGGE combined with phylogenetic analysis provided the community structure. The study also

provided an insight on whether one step temperature increase was conducive for obtaining a thermophilic seed biomass for anaerobic degradation of high strength chemical industry wastewater.

5.3. MATERIALS AND METHODS

5.3.1. Wastewater characteristics

The chemical industry wastewater was initially profiled in terms of parameters shown in **Table 4.1**.

5.3.2. Seed biomass

Anaerobic sludge was collected from a mesophilic anaerobic digester at a local municipal sewage treatment plant treating primary and secondary sludges.

5.3.3. Pre-experimental phase

The seed sludge underwent one step temperature increase from 35°C to 55°C in a manner as described in a previous study (Angelidaki *et al.* 2009, Bouskova *et al.*, 2005). This process would acclimate, select and scale up the thermophilic homologues while degrading the inherent organics present in the seed sludge.

5.3.4. EXPERIMENTAL PHASE

5.3.4.1 BMP tests

BMP tests were performed according to the protocol described in Section 4.3.4.1. All sample analyses were performed in triplicate.

5.3.4.2. Physicochemical characterization

Physicochemical characterization was performed as described in Section 4.3.4.2.

5.3.4.3. Gas measurement

Gas measurement was performed as described in Section 3.2.5

5.3.4.4. DNA extraction and q-PCR analysis

DNA extraction and q-PCR analysis was performed as described in Section 3.2.6

5.3.4.5. Denaturing gradient gel electrophoresis (DGGE) profiling and phylogenetic analysis

DGGE profiling and phylogenetic analysis was performed as described in Section 3.2.7. All nucleotide sequences analyzed were submitted to GenBank under the accession numbers **KF511612-KF511631**. Phylogenetic trees were constructed using the neighbourhood joining method (Tamura *et al.*, 2011).

5.4. RESULTS

5.4.1. Physicochemical characterization

VSS, pH values on day 0 and day 30 along with residual COD profiles on day 0, day 15 and day 30 are listed **Table 5.1**. The results of VFA analysis for the test groups are illustrated in **Figure 5.1**. The VFA levels reached a maximum of 5.95 g L⁻¹ on day 10 and thereafter did not show significant change (day 30, 6.21 g L⁻¹). VFA values in blank controls were below detectable limits and hence not illustrated.

Table 5.1 - COD profiles during thermophilic temperature (55°C) BMP tests

Group	Day 0	Day 15	Day 30
TCOD (AD/Control)	14.71 ± 0.03 g/L	12.62 ± 0.32 g/L	8.80 ± 0.01 g/L
TCOD (AD/IND-WW)	39.14 ± 0.10 g/L	38.37 ± 0.32 g/L	38.90 ± 0.29 g/L
SCOD (AD/Control)	N.D	1.22 ± 0.01 g/L	N.D
SCOD (AD/IND-WW)	21.16 ± 0.08 g/L	26.11 ± 0.17 g/L	26.42 ± 0.12 g/L
VSS (AD/IND-WW)	10.13 ± 0.88 g/L	N.A	9.01 ± 0.52 g/L
VSS (AD/Control)	10.27 ± 0.64 g/L	N.A	8.26 ± 0.44 g/L
pH (AD/IND-WW)	7.38	N.A	7.02
pH (AD/Control)	7.45	N.A	7.13

*N.D-Not Detected, NA - Not Analyzed

Figure 5.1 - VFA profiles of AD/IND-WW at 55°C

5.4.2. Biogas measurements

Biogas measurement was done after every 5 days except for the reading between day 20 and 30 where readings were taken on day 27 and day 30. Gas production was calculated as volume produced over every 5 days after pressure

in headspace was equalized to 1atm at room temperature following each measurement. The cumulative methane production in test group at thermophilic temperatures was 36mL at STP in comparison to the blank control which produced 75.4mL at STP (highlighted in **Figure 5.2**). Maximum methane production in the test group occurred between day 15 and day 20 (10.1mL) after which the rate of methane production remained relatively unchanged but with a cumulative increase in volume. Highest recorded methane quality in the test group was 32.0% while those in the blank control were 61.5%.

Figure 5.2 - Volume of methane produced over 30 days period for AD/IND-WW and AD/Blank Control at 55°C. Error bars indicate standard deviation of triplicate measurements

5.4.3. q-PCR analysis

Results of 16S rRNA gene copies of Eubacteria and different Methanogenic communities in test and blank control are illustrated in **Figure 5.3**. It was observed that in test groups that even though organisms had remained viable at 55°C, the anaerobic degradation process proceeded at a slow rate. Eubacteria were the dominant microflora in both groups albeit decreasing copy numbers over the test period. *Methanomicrobiales* dominated the methanogenic communities. The copy numbers of *Methanosaetaceae* in the test group gradually decreased over the 30 days period while *Methanobacteriales* and *Methanomicrobiales* remained relatively constant after day 10. The levels of *Methanosarcinaceae* in the test group dropped on day 20 but later stabilized towards the end of the study period. In the control groups, *Methanosarcinaceae* copy numbers increased sharply from day 10 to day 15 after which there was a gradual decrease till day 30.

Figure 5.3 - Differences in the shifts in microbial populations of a) AD/IND-WW at 55°C and b) Control during the 30 days period. Error bars indicate standard deviation of triplicate measurements

5.4.4. DGGE profile and phylogenetic analysis

DGGE profile and phylogenetic analysis were conducted to characterize the microbial community structure in response to a thermophilic anaerobic degradation of a high strength chemical industry wastewater. Both Archaeal and Eubacterial PCR products were subjected to DGGE profiling and phylogenetic analysis were performed with DNA samples collected during day 0, day 15 and day 30 from the test group. The DGGE profile is illustrated in **Figure 5.4** which indicated that a majority of the bands in the Eubacteria fade in intensity over the

30-day test period. This correlates with the real time PCR results which highlighted a gradual decrease in copy numbers of the Eubacterial populations in the test samples. However, the Archaeal profile had remained relatively stable through the 30-day period, although there was impaired methanogenesis. This could likely indicate a loss of function in certain methanogenic groups. The phylogenetic trees constructed are illustrated in **Figure 5.5** for Eubacteria and **Figure 5.6** for Archaea.

Figure 5.4 - DGGE profile of microbial DNA isolated from AD/IND-WW at 55°C. L1, L2 and L3 represent the Eubacterial DNA isolated from Day 0, Day 15 and Day 30 samples while L4, L5 and L6 represent Archaeal DNA isolated from Day 0, Day 15 and Day 30 respectively

For the DGGE profile at 55°C, band 1 was prominent in its intensity though visibly decreasing in its intensity over the experimental period as evidenced by band 2. When the sequences were analyzed, it was found to closely resemble (97-99%) to the genera *Schlegelella* and *Tepidimonas* - both belonging to *Proteobacteria*. Band 3 was found to resemble (98%) the genera *Gracilibacter* and *Thermotalea*, while bands 4 and 5 closely resembled the genus *Pelotomaculum*. All of them belong to the order *Clostridiales*. Bands 6 and 7 closely resembled (99%) the genus *Thermosyntropha* while band 8 resembled (96%) the genus *Syntrophothermus*, all belonging to *Firmicutes*. The Archaeal bands fell within 3 main orders, *Methanomicrobiales*, *Methanosarcinales* and *Methanobacteriales*. Bands 9 and 10 closely resembled (98%) the genus *Methanoculleus* while bands 11 and 12 resembled (99%) the genus *Methanolinea*. All of them belong to *Methanomicrobiales*. Bands 13 and 14 resembled (97-99%) the genera *Methanosaeta* while bands 15 and 16 resemble (97%) the genus *Methanosarcina*. Bands 17 and 18 resemble (96-98%) the genera *Methanomethylovorans* and *Methermicoccus*. All of them belong to *Methanosarcinales* while bands 19 and 20 closely resemble (99%) the genus *Methanothermobacter* which belong to *Methanobacteriales*.

Figure 5.5 - Neighbour-joining method used for constructing phylogenetic tree, highlighting the identities of 16S rRNA genes of Eubacteria at 55°C. Taxon marked with • indicates the organisms analyzed. Numbers at nodes are bootstrap values derived from 100 analyses

Figure 5.6 - Neighbour-joining method used for constructing phylogenetic tree, highlighting the identities of 16S rRNA genes of Archaea at 55°C. Taxon marked with ▲ indicates the organisms analyzed. Numbers at nodes are bootstrap values derived from 100 analyses

5.5. DISCUSSION

Past studies concerned with obtaining thermophilic seed biomass from mesophilic seed have had two vastly different proponents regarding the acclimation method. One was a stepwise increase in temperature over a period of time (De la Rubia *et al.*, 2005; De la Rubia *et al.*, 2006). However the multistep process was time consuming. The other suggested a one step direct increase of mesophilic to the thermophilic condition (Griffith and Alvarez, 2002; Bouskova *et al.*, 2005; Palatsi *et al.*, 2009). According to Bouskova *et al.*, (2005), the time required for adaptation of mesophilic seed in the one step process was less than half the time required in the multistep process. However several process imbalances were caused due to accumulation of certain VFA intermediates, especially an increase in the propionate to acetate ratio. Therefore, it would be beneficial from a process control point of view to elucidate the consequences of one step temperature increase, which may influence microbial community shifts as well as process imbalances.

This study coupled the degradation of a high strength (high TCOD, SCOD, VFA, and sodium levels) chemical industry wastewater at thermophilic (55°C) temperature. The seed for thermophilic process was obtained by one step temperature increase method of a mesophilic anaerobic biomass. The BMP test was used for evaluating process performance based on TCOD reduction as well as cumulative methane production. This study also sought to evaluate the response of microbial communities towards the presence of industrial wastewater after being subjected to a one step temperature increase. The results seem to indicate an impaired process performance. Methane levels in the test group were less than those of the blank controls in conjunction with negligible TCOD reduction as well as VFA degradation over the 30-day test period. One possible cause is the wastewater had stressed the microbes under thermophilic condition. This could have led to failure of multiple biosynthetic pathways, and thereby compromising the overall anaerobic process. Previous studies have

shown that anaerobic degradation process had failed under thermophilic conditions when challenged with certain organic compounds like vegetable tanning agents, polyhydroxy phenolic compounds and slimicides (Disley *et al.*, 1992). Results of this study suggest that a similar situation may have occurred.

In this study, the acetate levels in the test group did not reduce after day 10, in concurrence with very low methane production, indicating that acetate was not degraded by the methanogens. Speece (1996) reported that acetate is the most important intermediate of the anaerobic degradation process and contributes to more than 70% of methane produced. In this study, acetate was found to be the major VFA intermediate generated. However, acetate can be used only by the “aceticlastic methanogens”, which belong to the order *Methanosarcinales* and comprise of *Methanosaetaceae* and *Methanosarcinaceae*. The non degradation of acetate in this study indicates that there is likely impairment of aceticlastic methanogens, especially *Methanosarcinaceae*. It may also be said that certain methanogenic communities in the seed may not have survived the one step temperature increase or may have experienced a loss of function when confronted with certain compounds present in the high strength industrial wastewater. This could have contributed to improper methanogenesis, leading to impaired process performance. The presence of the high strength industrial wastewater might have also affected the function of certain eubacterial communities which in turn caused improper degradation of organics along with VFA accumulation in the system. A similar finding was reported during the degradation of municipal sludge in a thermophilic system (De la Rubia *et al.*, 2006) wherein the accumulation of VFA was attributed to the manner in which the thermophilic seed was obtained. Chen, (1983) reported that only 25% of the acetate utilizing methanogens which grow at 35°C can actually utilize acetate at 50°C and above. The aceticlastic methanogens (especially *Methanosarcinaceae*) in the test samples of the present study could have lost

the capability to use acetate under thermophilic conditions due to various factor(s).

The phylogenetic analysis of DGGE bands from the test system revealed that certain organisms found in both Eubacteria (*Schlegelella*, *Gracilibacter* and *Tepidimonas*) as well as Archaeal communities (*Methanolinea tarda* and *Methanosaeta harudinaceae*) were “eurythermal” which can exist within a temperature range (25°C-60°C) with optimum growth rates in the mesophilic temperature region (25°C-40°C). Organisms resembling *Schlegelella thermodepolymerans* being facultative anaerobes, performed depolymerising reactions on complex polymers (Elbanna *et al.*, 2003) while *Tepidimonas thermarum* assimilated organic acids (Albuquerque *et al.*, 2006). The organisms closely resembling *Thermotalea metallivorans* and *Gracilibacter thermotolerans* converted organics into acetate as the end-product whilst remaining tolerant of alkaline conditions (Ogg and Patel, 2009). The organism which closely resembled *Pelotomaculum thermopropionicum* degraded propionic acid while in co-culture with *Methanothermobacter thermoautotrophicus* which was also found at 55°C (Imachi *et al.*, 2002). Other organisms in the Eubacterial ecosystem include *Thermosyntropha lipolytica* which is able to degrade fatty acids and higher lipids (Svetlitsnyi *et al.*, 1996) while there is also *Syntrophothermus lipocalidus* which is able to oxidize fatty acids which have 4-10 carbon atoms (Sekiguchi *et al.*, 2000). However, the bands resembling *Syntrophothermus*, *Schlegelella*, *Thermotalea* and *Gracilibacter* faded away towards the end of the experimental period (Figure 4), indicating that it had encountered some form of inhibition, thereby causing a reduction in its copy numbers. This inhibition most likely caused an organic overloading on the other organisms which was most likely why the VFAs production/degradation at 55°C could have stopped after day 10. An interesting observation is the presence of two methanogens resembling *Methanolinea tarda* and *Methanosaeta harudinaceae*, both of which are very commonly observed in the anaerobic degradation systems operating at 35°C. These could have caused

some community imbalances within the archaeal community structure via the syntrophic nature of many Eubacterial communities with its corresponding Methanogenic homologues. Due to certain Methanogenic homologues being absent, it would hinder the efficient carbon transfer from one community to the other, thereby leading to impaired process performance. This would also explain the decrease of Eubacterial communities (as seen in real time PCR results, **Figure 5.3**). Another interesting observation is the decrease in band intensities of organisms resembling *Methanosarcina thermophila* and *Methanomethylovorans thermophila* at day 15 through till day 30, which could explain the decrease of copy numbers of *Methanosarcinaceae* on day 20. Hori *et al.*, (2006) reported that a well developed Archaeal taxonomy structure was vital to achieve maximum VFA degradation rates under thermophilic conditions and any imbalance in the community structure would lead to impaired process performance. Iranpour *et al.*, (2005) stated that to obtain a robust thermophilic culture, it would be better served to increase the temperature sharply to promote the development of true thermophiles rather than developing the thermotolerant mesophiles when a multistep temperature increase is employed (Aitken and Mullenix, 1992). The one step temperature increase method used in this study however, caused the scale up of certain thermotolerant mesophiles rather than true thermophilic communities (as evidenced by the taxa in the phylogenetic analysis). Aitken and Mullenix (1992) also stated that thermotolerant mesophiles cause community imbalances when subjected to a one step temperature increase due to their optimum growth rates being closer to mesophilic temperatures. In this study, it is observed that there is no degradation of VFA after day 10 as well as negligible methane production. This could be attributed to an improper Eubacterial and Archaeal taxonomic structure within the systems coupled with a loss of function of certain aceticlastic methanogenic groups (likely within *Methanosarcinaceae*). These eurythermal communities also may not be resilient to the stress imparted by the high strength wastewater coupled with high temperature, thereby leading to a

collapse in the process due to community imbalance and inhibition. This is further supported by very little COD reduction over the test period. It could be said that one step temperature increase might have reduced niche community members (tolerant to high strength organics of the industrial wastewater) by imparting a thermal shock which led to poor process performance under thermophilic conditions.

The real time PCR results also reveal that though methanogenic communities were relatively unaffected by the one step temperature increase, there might have been some form of inhibition to certain biosynthetic pathways from the wastewater. This could have caused loss of function of some methanogenic members. The phylogenetic analyses also suggested that the seed biomass used in this study was comprised of a complex community of microbes which adapted accordingly to the temperature during the one step temperature increase process. It can be said that the initial one step increase from 35°C - 55°C could have removed niche microbial communities tolerant to the organics of industrial wastewater in the form of a thermal shock. In order to obtain a much better performing thermophilic seed biomass for high strength wastewater from this mesophilic seed, it would be prudent to subject it to a multistep temperature increase (with shorter time intervals or broader temperature gradients) rather than a one step rapid temperature increase. Finally, care must be taken during process control so as not to allow too much VFA accumulation since it may affect the methanogenic capability of the acclimated methanogens.

5.6. CONCLUSION

One step temperature increase of mesophilic seed biomass might have removed some communities governing effective anaerobic degradation in the form of a rapid thermal shock coupled with presence of high strength industrial wastewater had a direct bearing on anaerobic degradation process. Phylogenetic analysis reveals presence of eurythermal organisms which may have caused

community imbalances, in turn leading to poor process performance. Certain acetoclastic methanogenic communities especially *Methanosarcinaceae* could have experienced a loss of function, probably caused by initial thermal shock. Care must be taken during process control so as not to allow excess VFA accumulation, especially acetic acid. Feeding pattern (OLR, HRT) must be monitored when using a thermally acclimated seed to avoid build up of VFA's intermediates during the process since it may affect the microbial communities especially methanogens.

CHAPTER 6 TEMPORAL MICROBIAL COMMUNITY DYNAMICS AND BIOCHEMICAL RESPONSES IN A MESOPHILIC BATCH ANAEROBIC REACTOR TREATING A HIGH STRENGTH CHEMICAL INDUSTRY WASTEWATER

6.1 ABSTRACT

Microbial community shifts and biochemical responses were monitored in a mesophilic anaerobic single stage batch reactor treating a chemical industry wastewater. The dynamics of microbial populations were monitored using quantitative real-time PCR and phylogenetic analysis, both of which exhibited good concurrence with physical process data. Bi-nodal production of methane, associated with initial hydrogenotrophic methanogenesis followed by acetoclastic methanogenesis, was observed during first two batches of wastewater feeding. Bacterial community structure analysis by targeting the 16S rRNA genes in a denaturing gradient gel electrophoresis (DGGE) revealed several members of *Clostridiales* were responsible for acidogenic reactions in the system. A rapid increase in *Methanosarcinaceae* populations concomitant with rise in acetic acid levels was detected during first batch of wastewater feeding. Further analysis indicated hydrogenotrophic methanogenesis as the likely pathway for initial methane production followed by late onset of acetoclastic methanogenesis, thereby indicating reason for bi-nodal methane production. Statistical analyses of DGGE profiles using the non metric multidimensional scaling (NMS) analysis highlighted spatial changes in bacterial community structure during three batches of wastewater feeding. Preliminary analysis of microbial biochemical responses showed significant extracellular protein production with concomitant increase in total lipid (intracellular + extracellular) levels over the three batches of wastewater feeding. Extracellular glycolipid bio-surfactant production was not observed, indicating different mechanism of substrate utilization and stress mitigation. These results suggest that analysis of microbial community responses with respect to spatial migration and biochemical responses towards operational

conditions would prove helpful in diagnosis of process performance of an anaerobic system especially during dynamic states such as process start-up or recovery.

6.2 INTRODUCTION

Anaerobic degradation in a batch process is considered useful due to simple process requirements like inexpensive equipment and ability to operate with wastes containing high total solids concentration. It has proven useful in past laboratory-scale studies to assess the degradability rate of a substrate and to determine the obtainable biogas yields (Lastella *et al.*, 2002). Other attractive features include simple design and process control, low energy consumption and generally good process robustness (Brummeler, 2000). As in other anaerobic systems, a batch system is governed by microbial processes as well. A variety of microbes co-exist in batch systems even though a single substrate is utilized (Fernandez *et al.*, 2000). Hence, an understanding of microbial community dynamics is necessary to improve and for better control of the anaerobic degradation process.

Recent microbial techniques targeting 16S rRNA gene has allowed for culture independent analysis of microbial communities (Calli *et al.*, 2005). Studies by Akarsubasi *et al.*, (2005) have indicated understanding of microbial communities through quantitative and qualitative methods could help in understanding of the functional attributes of microbial communities within the anaerobic system. Studies have been undertaken in the past which either focused on the role of methanogens alone (Yu *et al.*, 2006), methanogenic community response to different substrates (Lee *et al.*, 2009) or the role of acidogenic populations (Lee *et al.*, 2008). A combined study of acidogenic and methanogenic community responses over successive feeding cycles has not been attempted in batch processes mainly due to the complex nature of the microbial seed used. This study aimed at understanding the acidogenic

community dynamics together with methanogenic community dynamics occurring in a single stage batch anaerobic reactor fed with a high strength chemical industry wastewater. Qualitative and quantitative methods were used and correlated with physical process parameters to better understand the functional attributes of acidogenic and methanogenic communities.

Industrial wastewaters can be high in organics but low in essential nutrients. Some of the organics may also be inhibitory to certain microbial communities, especially to those which have niche functions. These extraneous factors are usually countered by the communities producing certain extracellular proteins as osmotic solutes (Vyrides and Stuckey, 2009) as well as production of lipids to strengthen the cellular membrane structure (Sikkema *et al.*, 1994). A batch process is highly likely to produce certain metabolites during the stationary phases as its microbial communities attempt to adapt better to environmental conditions including increased substrate accumulation. Therefore it could be prudent to observe biochemical responses of microbial communities during the anaerobic process since this may provide a better understanding of how microbial communities cope with stress.

For experimental purposes, an anaerobic reactor was operated in batch mode over three successive wastewater feeding cycles to treat a high strength chemical industry wastewater. The temporal dynamics of bacterial and archaeal communities were ascertained using quantitative real time PCR targeting the 16S rRNA gene. Furthermore, phylogenetic changes in the bacterial and archaeal community structures were envisaged using the denaturing gradient gel electrophoresis (DGGE) technique. Statistical analysis of DGGE profiles were performed using non metric multidimensional scaling analysis (NMS) which is a multivariate ordination technique and provides insight into the behavior of microbial communities within an anaerobic environment. A preliminary analysis was also performed to understand the microbial biochemical responses to reactionary changes occurring in the system over time.

6.3 MATERIALS AND METHODS

6.3.1 Wastewater characteristics

The chemical industry wastewater, designated INDWW was initially profiled in terms of parameters shown in **Table 6.1**.

Table 6.1 - Chemical industry wastewater (INDWW) properties

Parameters	Value
Total chemical oxygen demand	(343.12 $\pm$ 3.56) g/L
Soluble chemical oxygen demand	(294.35 $\pm$ 2.78) g/L
Volatile fatty acids (VFA's)	(3.6 $\pm$ 0.02) g/L
Acetic acid	0.83 g/L
Valeric acid	1.23 g/L
Other organic components	Glutarate, adipate, and succinate
pH	9.23
Sodium	44.1 g/L
Colour (visual)	Deep red

Anaerobic sludge was collected from a mesophilic anaerobic digester at a local municipal sewage treatment plant treating primary and secondary sludges and designated as AnSL.

6.3.2 Reactor Operation

An anaerobic completely stirred tank reactor (Winpact fermenter system, Major Sciences, California, USA) of 12.5L total volume and 10L working volume was operated in batch mode. The reactor was seeded with the anaerobic seed biomass and INDWW in the ratio of 4gm substrate total chemical oxygen demand (TCOD): 1gm seed volatile suspended solids (VSS). A mineral salts

supplement medium (MSM) was prepared according to Section 4.3.4.1 and added to the wastewater to provide essential trace metal ions. Operating temperature was set at 35°C with continuous stirring of 150rpm. The pH of the system was set to a band of 5.5 to 7.5, with 2N NaOH solution and 1M HCl solutions used for maintaining pH. Following feeding, the reaction phase was continued till maximum TCOD degradation was achieved and no further methane production was observed over two consecutive samplings. At the end of each batch, the reactor was decanted and then topped up to the 10L working volume with a new batch of feed+MSM mixture. Batch 1 lasted for 80 days, Batch 2 for 50 days and Batch 3 for 15 days. Possible inhibition was encountered in Batch 3 as evidenced by no reduction of TCOD, methane production or VFA accumulation over 3 consecutive samplings after which reactor operation was stopped (Day 145). For physicochemical analysis, 50mL of well mixed samples were collected every 5 days. For VFAs analysis and DNA extraction, the samples were diluted suitably before analysis. Physicochemical analysis was performed in accordance with Standard Methods (APHA, 1998). Biogas and VFA analysis was performed as described in Section 4.3.4.2.

6.3.3 DNA extraction and quantitative realtime PCR analysis

DNA was extracted from mixed liquor samples and quantitative realtime PCR analysis was performed as described in Section 3.2.6.

6.3.4 Denaturing gradient gel electrophoresis (DGGE) profiling and phylogenetic analysis

DGGE technique was used for the phylogenetic analysis of samples withdrawn every 10 days, and was performed as described in Section 3.2.7. The sequences

were deposited in GenBank under the accession numbers of **KT070952-KT070994**.

6.3.5 Statistical analysis of DGGE profiles

The DGGE band patterns were transformed into binary matrices using the Quantity One software of Bio-Rad GelDoc system (Bio-Rad, Hercules, California, United States of America). The presence or absence of bands was scored as either 1 or 0, thus avoiding bias of any PCR discrepancy. The matrices representing bacterial and archaeal community structure profiles were analyzed by non-metric multidimensional scaling (NMDS). According to McCune and Grace (2002), NMDS provides an effective ordination method for ecological community data. It condenses complex data sets into single points set in a low dimensional space. Furthermore, data points of highest similarity are clustered together on an axis. The plot of the ordination visualizes relationships between analyzed communities and their spatial migration. NMDS ordination was performed based on Sorenson (Bray-Curtis) distance measure using PAST Software version 2.17 (University of Oslo, Oslo, Norway).

6.3.6 Biochemical analysis

Biochemical analyses were performed on the mixed liquor samples withdrawn from the test groups. Protein extraction from supernatant of mixed liquor samples was done using sodium hydroxide and formaldehyde method (Liu and Fang, 2002) followed by purification using dialysis membrane (Sigma Aldrich 12 KDa cutoff cellulose tubing membranes) against 0.1M phosphate buffer, pH 7.0. The purified protein samples were reconstituted in 0.1M phosphate buffer, pH 7.0 and used for further analysis. A modified Lowry's Assay (Lowry *et al.*, 1951) was then performed with the purified extra cellular protein (ECP) using bovine serum albumin as standard. Lipid extraction and quantification was performed by gravimetry using solvent extractions (Chloroform:Methanol

mixture 33:66 v/v) (Bligh and Dyer, 1959). The mixed liquor was analyzed for extracellular glycolipid biosurfactant production by incubating in wells of a blue agar plate containing hexacetyltrimethyl ammonium bromide (0.2mg/mL), methylene blue (0.005mg/mL) and agar (1.5%) (Seigmund and Wagner, 1991).

6.4 RESULTS

6.4.1 Reactor operation

The reactor was operated for a total of 145 days over three batches of wastewater feeding. **Figure 6.1** illustrates the reactor process performance in terms of cumulative methane production versus COD reduction. The spikes in COD concentration indicate new batch of wastewater substrate feeding. A distinct lag phase in methane production was observed initially in Batch 1 (**Figure 6.1**) followed by appreciable methane production from Day 20 onwards in a bi-nodal manner (first spike from day 25 to day 50 and second spike from day 50 to day 75) and reaching a maximum of 91L on Day 75 in Batch 1. In Batch 2, there was methane production right from Day 80 (again in a bi-nodal manner) and maximum volume of methane (20L) was produced in the time period between Day 115 and Day 120. However gas production tapered off towards the end of the second batch (Day 130) and there was no gas production in the third batch of feeding till the end of Day 145. This could be attributed to inhibition of microbial communities. The methane yield coefficient was calculated to be 0.33L CH₄ produced/gm COD reduced in Batch 1 and 0.31L CH₄ produced/gm COD reduced in Batch 2. The COD removal percentages were 40.91% in Batch 1 and 26.80% in Batch 2 of the initial amounts. There was no COD removal observed in Batch 3 over three successive samplings. The maximum methane percentage measured was 79.93% of total biogas in Batch 1 and 70.34% of total biogas in Batch 2. There was also a reduction in VSS content at the end point in comparison to Day 0 (Day 0: 10.04±0.73g/L, Day 80: 11.81±0.16g/L, Day 130: 10.17±0.59 g/L and Day 145: 8.89±0.62 g/L) with

final VSS content measured being just over 11% lesser than initial VSS amount. However, this reduction in VSS was unlikely to significantly impact process performance, thereby indicating that process failure in Batch 3 may be due to inhibition of certain biosynthesis pathways within them.

Figure 6.1 - Changes in total COD profile along with cumulative methane production

VFA's analysis over the experimental period revealed that acetate and propionate were the major acidogenic products (**Figure 6.2**). These two VFAs made up more than 66% of the total VFAs concentration. The other major VFA, valerate, was present in the INDWW substrate and was degraded during the process. A maximum acetate concentration of 3.73 g/L was observed on Day 40 in Batch 1 and 2.52 g/L on Day 115 in Batch 2. Propionate was retained within the system at high concentrations due to it being thermodynamically unfavorable for anaerobic degradation (Lee *et al.*, 2008). The VFAs profile suggested a diauxic utilization of acetate i.e. initial production and

accumulation of acetate followed by subsequent degradation and methane production. This would also better explain the bi-nodal manner of methane production in Batches 1 and 2 of wastewater feeding.

Figure 6.2 - Major VFA intermediates produced over three batches of reactor operation

6.4.2 Quantitative microbial community analysis

Quantitative changes in microbial populations were determined by analysis of the 16S rRNA gene concentrations via the real time PCR technique (**Figure 6.3**). The profiles highlighted significant temporal variations in bacterial and methanogenic populations with respect to changes in process performance. The bacterial 16S rRNA concentration initially decreased from 5.99×10^{10} copies/mL on Day 0 to 3.30×10^{10} copies/mL on Day 55 (~45% decrease), but later increasing to 9.27×10^{10} copies/mL on day 95 (64% increase). This decrease-increase in relative abundance of bacterial community members could be

attributed to changes in the degradation pattern of substrates - i.e. decrease stemming from loss of function of community members or loss of niche community organisms and hence loss of ability to degrade the wastewater components effectively; and thereafter increase in population of microbes which had adapted to the substrate components.

Figure 6.3 - Quantitative changes in bacterial and methanogenic 16S rRNA gene concentrations over three batches of reactor operation. I, II and III indicate the batch of operation.

The total methanogenic population decreased in the initial phase of Batch 1 from Day 0 to Day 35 after which it was relatively stable. Considerable variation at the inter species level, was observed with an increase in the population of *Methanosarcinaceae* from Day 0 to Day 40. The decrease in other methanogens such as *Methanomicrobiales*, *Methanobacteriales* and

Methanosaetaceae coincided with the rapid population increase of *Methanosarcinaceae* in Batch 1. This trend was concomitant with the VFA levels wherein initial accumulation of acetic acid and propionic acid was observed. The K_s value for acetate of *Methanosarcinaceae* being higher i.e. utilizes acetate only at concentrations of $\geq 1\text{mM}$ (Yu *et al.*, 2006) explains why there was initial accumulation of acetic acid followed by subsequent degradation. The relationship between acetic acid accumulation and *Methanosarcinaceae* increase is illustrated in **Figure 6.4**. After Day 35, no significant population growth of other methanogenic species was observed.

Figure 6.4 - Relationship between Methanosarcinaceae 16S rRNA gene concentration and acetic acid concentration over the period of reactor operation. I, II and III indicate the batch of operation

6.4.3 Bacterial and archaeal community shifts

PCR-DGGE targeting was performed on bacterial and archaeal 16S rRNA genes to analyze the microbial community shifts which occurred during reactor operation. The DGGE profile showed a continuous shift in the bacterial community structure during the three batches of reactor operation (**Figure 6.5a**). The archaeal community shift occurred only during the early period of reactor operation in Batch 1 after which the patterns remained relatively stable (**Figure 6.5b**). Due to the number of sampling points, DGGE was performed on different batches of gels. After band excision and comparison with the GenBank database, the gels were aligned suitably for representation. The 26 bacterial and 17 archaeal 16S rRNA gene fragments were excised from the gels and compared with the GenBank database. The nearest similar members are highlighted in **Table 6.2** (for bacteria) and **Table 6.3** (for archaea) along with their accession numbers and similarity percentages.

Figure 6.5 - DGGE profiles of A) Bacterial and B) Archaeal 16S rRNA gene. Seed represents the original biomass and the numbers at the top of the lanes indicate sampling time

Table 6.2 - Phylogenetic affiliation of bacterial 16S rRNA gene sequences from DGGE bands

DGGE number sequence similarity)	band (%)	Closest species (accession number)	Phylum/class
B1, B17 (93%)		<i>Paludibacter propionici</i> genes strain WB4 (AB078842)	<i>Bacterioides/Bacteroidia</i>
B2, B3, B18 (95%)		<i>Clostridium ultunense</i> strain DSM 10521 (AB524740)	<i>Firmicutes/Clostridia</i>
B4, B5, B19 (88%)		<i>Alkaliflexus imshenetskii</i> strain Z-7010 (AJ784993)	<i>Bacterioides/Bacteroidia</i>
B6, B7, B20 (96%)		<i>Sedimentibacter hydroxybenzoicus</i> strain JW/Z-1 (L11305)	<i>Firmicutes/Clostridia</i>
B8, B9, B10, B21(87%)		<i>Treponema zuelzeri</i> strain DSM 1903 (FR749928)	<i>Spirochaetes/Spirochaetales</i>
B11, B12, B22 (96%)		<i>Syntrophomonas sapovorans</i> strain OM (AF022249)	<i>Firmicutes/Clostridia</i>
B13, B23 (89%)		<i>Desulfobulbus elongatus</i> strain FP (X95180)	<i>Proteobacteria/δ-Proteobacteria</i>
B14, B24 (96%)		<i>Ottowia shaoguanensis</i> strain J5-66 (KC222642)	<i>Proteobacteria/β-Proteobacteria</i>
B15,B16, B25, B26 (99%)		<i>Azospira restricta</i> strain SUA2 (DQ974114)	<i>Proteobacteria/β-Proteobacteria</i>

Table 6.3 - Phylogenetic affiliation of archaeal 16S rRNA gene sequences from DGGE bands

DGGE number sequence similarity)	band (%)	Closest species (accession number)	Order/family
A1, A9 (99%)		<i>Methanospirillum hungatei</i> JF-1 strain (CP000254)	<i>Methanomicrobiales/Methanospirillaceae</i>
A2, A10 (99%)		<i>Methanoregula boonei</i> strain 6A8 (DQ282124)	<i>Methanomicrobiales/Methanoregulaceae</i>
A3, A11 (97%)		<i>Methanolinea mesophila</i> strain TNR (AB447467)	<i>Methanomicrobiales/Methanoregulaceae</i>
A4, A5, A12 (100%)		<i>Methanosaeta concilii</i> strain GP6 (CP002565)	<i>Methanosarcinales/Methanosaetales</i>

A6, A15 (99%)	<i>Uncultured Methanosarcina sp.</i>	<i>Methanosarcinales/Methanosarcinaceae</i>
A7, A16, A17 (99%)	<i>Uncultured Methanosarcina sp.</i>	<i>Methanosarcinales/Methanosarcinaceae</i>
A8, A13, A14 (99%)	<i>Uncultured Methanosarcina sp.</i>	<i>Methanosarcinales/Methanosarcinaceae</i>

In the bacterial DGGE profile (**Figure 6.5a**), it was observed that majority of the bands had close affiliations with *Clostridiales*. Some members resembling *Sedimentibacter hydroxybenzoicus* (B6, B7, B20) were not present in the seed biomass as well as on Day 0 but were present later on till Day 70, absent on Day 80 and again appearing from Day 100. This organism may have been responsible for the majority of the acidogenic reactions (Breitenstein *et al.*, 2002) along with *Syntrophomonas sapovorans* (B11, B12, B22) which hydrolyzed long chain fatty acids (Roy *et al.*, 1986). These members may have adapted suitably and so efficiently degraded the substrate components in INDWW, thereby becoming the major acidogenic species. A syntrophic acetate oxidizer, *Clostridium utunense* (B2, B3, B18) was observed which could convert acetate to methane syntrophically in conjunction with hydrogenotrophic methanogens (Schnurer *et al.*, 1996). Interestingly, bands B13 and B23 resembled the propionate oxidizing bacteria *Desulfobulbus elongatus* which had decreasing band intensities throughout the initial phase of the experimental period (Day 0 to Day 30) and stabilizing from Day 70. This organism belongs to the family of propionate oxidizing bacteria (POB) which syntrophically oxidizes propionic acid to acetate and hydrogen in conjunction with hydrogenotrophic methanogens (Samain *et al.*, 1984). The organism was present throughout the experimental period, and this suggested possibility of inhibition to biosynthetic pathways, consequently leading to accumulation of propionic acid within the system. Other organisms observed included bands B1 and B17 resembling *Paludibacter propionigenes*, a carbohydrate consuming

bacterial species producing acetate and propionate as major products (Ueki *et al.*, 2006) and bands B8, B9, B10, B21 resembling *Treponema zuelzeriae*, a major acetogenic bacterial species (Veldkamp., 1960). These organisms had low band intensities throughout the experimental period, suggesting they might not have adapted efficiently to presence of the wastewater organics, and so subsequently not becoming the major acidogenic species. A similar trend was observed with bands B4, B5 and B19 resembling *Alkaliflexus imshenetskii*, a propionate producing species of the *Bacteriodes* phylum (Zhilina *et al.*, 2004) which disappeared on Day 30 and reappeared on Day 50. Two other bacterial species found were *Ottawia shaoguanensis* (B14, B24), responsible for metabolizing various amino acids via a chemolithotrophic pathway (Geng *et al.*, 2014) and *Azospira restricta* (B15, B16, B25, B26), which could metabolize ethanol (Bae *et al.*, 2007). Both these organisms were present throughout the experimental period. Based on substrate composition, it was highly unlikely that these organisms could have played a major role in the acidogenesis reactions, and so would have been a niche community.

In the archaeal DGGE profile (**Figure 6.5b**), it may be observed that certain shifts/variations in band patterns occurred in the early stages of the reactor operation in Batch 1 (day 10 to day 70). The overall profile, however, remained stable throughout the reactor operation except for a few bands which appeared after day 30. From **Table 6.3**, it may be observed *Methanomicrobiales* formed the majority of methanogenic species. Bands A1, A9 resembled closely the hydrogenotrophic methane producing archaeon *Methanospirillum hungatei* (Patel *et al.*, 1976) while A2 and A10 resembled *Methanoregula boonei*, another hydrogenotrophic methanogen (Brauer *et al.*, 2011). Bands A3, A11 closely resembled *Methanolinea mesophila*, a mesophilic hydrogenotrophic methanogen also able to metabolize formate to generate methane (Sakai *et al.*, 2012). In the *Methanosarcinales* family, there were three species of *Methanosarcinaceae* (A6, A7, A8, A13, A14, A15, A16, A17) which utilizes H₂, CO₂, acetate, formate and methanol to produce methane. These organisms,

however, possess highest affinity towards utilizing acetate as substrate for methane production, especially at concentrations $\geq 1\text{mM}$ (Yu *et al.*, 2006). Also present was one species of *Methanosaetaceae* with bands A4, A5 and A12 closely resembling *Methanosaeta concilii*, an acetotrophic methanogen which would consume acetate when present at low concentrations (Patel *et al.*, 1990). In spite of *Methanobacteriales* being successfully quantified in the quantitative real-time PCR, there were no bands of *Methanobacterium* detected in the DGGE profile. Studies by Shin *et al.*, (2008) postulated that *Methanobacteriales* have a relatively low abundance within the domain archaea ($< 13\%$ of the total sum of methanogenic populations), which might lead to the organism going undetected in the DGGE profile. In this experimental study, the maximum copy numbers of *Methanobacteriales* was observed on Day 85 (0.95×10^8 copies/mL), which was 9.39% of the total methanogenic population. This could have been the reason for its non-detection in the DGGE profile.

6.4.4 Ordination

NMS ordination was employed to visualize the changes in bacterial and archaeal community structures. **Figure 6.6a** and **Figure 6.6b** illustrates the changes in bacterial profile over Batches 1, 2 and 3 while **Figure 6.6c** illustrates the changes in archaeal profile in Batch 1. The changes in archaeal profile were not evident in Batches 2 and 3 and hence not illustrated. The results met the general criteria of good NMS ordination with stress levels below 10 (McCune and Grace, 2002). Both bacterial and archaeal NMS profiles were assigned on a 2 dimensional axis with 95% of the variability being explained by original band patterns of the DGGE profile. The initial shift in bacterial and archaeal profiles during batch 1 of reactor operation, tended away from the seed profile. However during the later stages of Batch 1, there was a gradual shift back towards the seed profile with slight variations, albeit in the same quadrant (quadrant 4). The bacterial NMS profile for Batches 2 and 3 were distinctly

different from Batch 1 and the end point was significantly away from the starting point of the reactor operation (quadrant 3 to quadrant 1). However the pattern of migration was similar to Batch 1 (quadrant 3 to quadrant 2) indicating that subtle changes in community shifts may have impacted the reaction pattern of organisms towards the INDWW wastewater.

6.4.5 Biochemical analyses

ECP and total lipid quantification profiles are illustrated in **Figure 6.7**. The ECP profile indicated a gradual increase throughout the time period of reactor operation in Batches 1 and 2. In Batch 3, there was a sharp spike of protein concentration in the first 5 days of operation after which it remained constant till the end of the batch and subsequently end of reactor operation. ECP concentration reached a maximum on Day 80 in Batch 1 (1172.58 mg/L) and Day 130 in Batch 2 (1091.37 mg/L). The total lipid quantification (both intracellular and extracellular) showed an increase in total lipid levels throughout the period of reactor operation. However, there was a sharp spike in lipid levels from Day 20 till Day 55 (5.26 g/L) in batch 1 of the reactor operation. A maximum of 6.88 g/L of total lipid was observed on Day 125.

Figure 6.8 highlights the Blue Agar plate for detection of glycolipid biosurfactant production. From the figure, it may be observed that there were no dark blue haloes around the wells containing the mixed liquor. Hence it could be concluded there was no production of extracellular glycolipid biosurfactants. It should be noted a reactor operated in batch mode would have had stationary phases in the microbial growth cycle (which favours production of secondary metabolites like biosurfactants) while treating complex substrates. However, the absence of such substances would indicate a different mechanism used to cope up with stress imparted by substrate components and different methods of substrate accumulation.

Figure 6.6 - Nonmetric multi dimensional scaling ordination plot of A) Bacterial DGGE band patterns (Batch 1), B) Bacterial DGGE band patterns (Batch 2 and 3), and C) Archaeal DGGE band patterns (Batch 1). Labels indicate time point and seed biomass used. Proportion of variance for each axis is indicated in parenthesis. Dotted lines indicate transition pattern of communities via different time points of reactor operation

Figure 6.7 - Extracellular protein (ECP) concentration and Total Lipid concentrations at different time points over the time period of reactor operation.

Figure 6.8 - Blue agar confirmatory test for glycolipid biosurfactant production. Numbers indicate the time point of samples tested during period of reactor operation. NOTE: Absence of dark blue haloes indicate negative result for glycolipid production

6.5 DISCUSSION

In this study, a continuous stirred tank reactor was operated in batch mode for degrading a complex high strength chemical industry wastewater. The substrate degradation rates were monitored by measuring COD degradation over the time period of the reactor's batch operation. The batch degradation process in this study could be roughly divided into two major phases. In the early acidogenic phase, the complex bio-macromolecules of the wastewater were acidified into volatile fatty acids (VFAs). In the methanogenic phase, by-products of acidogenic reactions such as acetate, CO₂ and H₂ were converted to methane.

Clostridiales-like organisms appeared to have been involved in the acidogenic reactions to produce VFAs. The VFAs profile clearly indicated acetate was the major intermediate VFA produced and this was concurrent with earlier reported findings (Speece, 1996). Valeric acid was a major component in INDWW wastewater and this was converted to acetate and propionate during the acidogenic process. Previous reports have shown that when n-valerate is present in the substrate, the acidogenic degradation produced propionate as a major intermediate and subsequently leading to propionate accumulation (Gallert and Winter, 2008). Unlike acetate, propionate oxidation is energetically unfavorable. It has to be converted to acetate and hydrogen before being converted to methane. Under anaerobic conditions, this would be achieved with the help of the syntrophic association of acetogenic bacteria and hydrogenotrophic methanogens (Stams *et al.*, 1998). During the reactor run, propionate was never degraded completely even in the presence of the propionic acid oxidizing bacteria (POB) within the system (B13, B23). It is likely that excess propionate production, due to high rate of acidogenesis by organisms like *Syntrophomonas* within the system, coupled with inherently slow substrate utilization rate of POB contributed to propionate accumulation. The POB have slow growth and substrate utilization rates coupled with a high sensitivity to high strength organics (Gavala *et al.*, 2003). The organics present in the INDWW wastewater could have contributed to inhibition of certain biosynthetic pathways within the POB, thereby further curtailing the utilization rate of propionate within the system thereby contributing to high residual COD levels. Propionate was virtually the only VFA remaining after 80 days of reaction in Batch 1. Another likely hypothesis is that the high acetate levels could have inhibited the rate of propionate degradation. Fukuzaki *et al.*, (1990) demonstrated that in a batch system at pH 6.5, when the total acetic acid levels were increased from 0.6 g/L to 3 g/L, the q_s of propionic acid decreased from 0.5 to almost 0. The pH level in this reactor system was maintained at 6.8 and the acetate level reached a maximum of 3.73 g/L on Day 40. This might have

triggered a feedback inhibition wherein excess of end-product hampered the degradation rates of propionic acid.

For the conversion of acetate to methane, a specialized group of methanogens known as the 'aceticlastic methanogens' are involved. Accordingly, the members of *Methanosarcinales* are expected to be the most dominant species (Kobayashi *et al.*, 2008) but contrary to previous studies, *Methanosarcinales* were not the dominant group during reactor operation. The DGGE profile as well as the real time PCR profile showed *Methanomicrobiales* followed by *Methanobacteriales* to be the major methanogenic species. However, there was a sharp increase in *Methanosarcinaceae* members from Day 20 till Day 40. *Methanosarcinaceae* possesses maximum affinity towards acetate when performing methanogenesis, though they can utilize other substrates to generate methane. It is highly likely that a particular acetate concentration combined with a certain population level of *Methanosarcina sp.* could have caused the phenomenon of concurrent increase in acetate concentration along with *Methanosarcinaceae* levels. This is better explained in the second batch of reactor operation (**Figure 6.4**), where there was no increase in *Methanosarcinaceae* levels but with a rise in acetate concentration, indicating that the inherent population level of *Methanosarcinaceae* was sufficient to degrade the acetate produced. *Methanosaeta concilii*, another aceticlastic methanogen, was also present in the system but was unlikely to be the major aceticlastic methanogen since *Methanosaetaceae* have a higher affinity for acetate only at lower concentrations in comparison to *Methanosarcinaceae* members (Hori *et al.*, 2006). The presence of *Methanosarcinaceae* is necessary for overall reactor efficiency, especially in terms of acetate consumption, since the accumulation of intermediates can often cause system failure (Batstone, 2002). It was noted the seed DGGE profile had very low band intensities of *Methanosarcinaceae* - a commonly observed phenomenon in full scale anaerobic systems in which low acetate concentrations are encountered (Demirel and Scherer, 2008) and the production of acetate as a major VFA

intermediate in this study's system had spurred the growth of *Methanosarcinaceae* members during the reaction period.

Hydrogenotrophic methanogenesis also forms a major methanogenic pathway in anaerobic systems (Ahring, 2003). In this study, hydrogenotrophic *Methanomicrobiales* were the most dominant methanogenic group. The presence of *Methanospirillum hungatei*, *Methanoregula boonei* and *Methanolinea mesophila* throughout the experimental period coupled with the presence of a high acetate concentration environment suggested a syntrophic removal of hydrogen in conjunction with *Syntrophomonas*-like organisms (B11, B12, B22). Past studies have given due importance to the aceticlastic methanogenesis pathway (Kobayashi *et al.*, 2008; Karakashev *et al.*, 2005). However, recent reports have demonstrated that hydrogenotrophic methanogens are equally prevalent in anaerobic systems (Song *et al.*, 2010). By analyzing the methane production profiles in Batches 1 and 2, hydrogenotrophic methanogenesis was likely the initial contributor to methane production in this study. An increased *Methanosarcinaceae* population, combined with a spike in methane production from day 50 till day 70 would suggest aceticlastic methanogenesis also contributed to methane production at a later stage once acetate degradation began. It can therefore be postulated that in a single stage batch system treating high strength industrial wastewaters of similar substrate composition, it is highly likely that bi-nodal methane production with initial hydrogenotrophic methanogenesis followed by aceticlastic methanogenesis would be the mode of methane generation.

In this study, NMS was applied to better visualize the pattern of microbial community shifts during batch anaerobic degradation of INDWW wastewater. The bacterial community shifts in Batch 1 suggested migration occurred distinctly away from the seed profile during the initial phases of reactor operation. The DGGE profile showed certain bacterial species decayed during the process (reducing in band intensities or disappearing completely) and

might have acted as "noise bands", causing this particular pattern of migration. Towards the end of Batch 1, the migration profile slowly shifted back towards the seed profile, suggesting a shift to the condition of low residual acetate concentration and high methanogenic activity similar to that occurring in the large scale digester which provided the seed biomass. This indicated a batch process could enrich acidogens and methanogens for a successful startup of a large scale process. Similar findings have been reported by Lee *et al.*, (2009) when treating three different sources of wastewater with a batch process. In this study, the NMS profiles for bacteria also highlights that as the numbers of feeding cycles increased, the bacterial community migration pattern veered away from the seed to evolve distinctly. From a qualitative aspect, these results imply that methanogenic community members evolved in a pattern similar to the seed inoculum along with the production of methane while bacterial acidogenic community members were affected by the presence of weak and/or redundant bands. The noising of these weak bands was likely caused by some form of inhibition to the biosynthetic pathways of the acidogenic communities in the first two batches of substrate feeding, subsequently leading to process failure in the third batch. Therefore qualitative analysis of microbial communities could prove useful in interpreting microbial community shifts with respect to significant operational challenges posed to a large scale reactor, treating high strength industrial wastewaters. Similar studies on acidogenic (Falk *et al.*, 2009) and methanogenic process (Lee *et al.*, 2009) have explained the relationship between qualitative analysis of microbial communities with respect to operational parameters but in a single batch of substrate loading. This study not only validated the findings of previous studies but also showed that subsequent batches of substrate loading could impact degradation patterns of acidogenic bacteria towards same substrate. This study also showed that in a single stage process, acidogenesis and not methanogenesis could be the rate limiting step in the anaerobic degradation process. This was clearly observed in

the third batch of substrate loading wherein lack of acidogenesis over three consecutive time points led to no methane production.

Protein forms a major component in the prokaryotic cell (nearly half of the dry weight) while lipids form an integral component of the cellular membrane structure (Madigan and Martinko, 2006). In this study, a constant increase in protein levels was observed (**Figure 6.8**) in the absence of specialized proteolytic *Clostridia*, which would indicate that microbial communities experienced stress from the substrate components, leading to excess production of proteins. Osmolytic shock is a major form of stress encountered by microbial communities. Microbial cells adapt to this osmolytic shock by maintaining a concentration gradient across membranes by secreting proteins in the form of osmotic compatible solutes into extra cellular space (Vyrives and Stuckey, 2009). In this study, the microflora within the system likely experienced osmolytic stress from the substrate components which had led to increased production of extracellular proteins as osmotic compatible solutes to maintain cellular integrity. In this study, it was also observed that there was an increase in total lipid concentrations during the experimental period. This could have resulted from presence of lipophilic compounds in the substrate which could have altered the membrane structure of cells and thereby triggering increased production of membrane lipids to maintain rigidity and fluidity of cell membranes (Sikkema *et al.*, 1994). Another cause could have been the production of extracellular glycolipid biosurfactants to better capture substrate components, as well as cellular community encapsulation for protection from toxic compounds. However, the blue agar confirmatory test for glycolipid surfactant production resulted in a negative reaction. The blue agar plate consisted of cationic hexa-cetyltrimethylammoniumbromide (CTAB) and methylene blue complex in an agar-agar solid medium. A liquid sample containing anionic glycolipid biosurfactants, when incubated in the blue agar medium would react with CTAB to release methylene blue from the complex, thereby creating a dark blue halo (Siegmund and Wagner, 1991). The absence

of dark blue haloes after incubation of samples drawn following different time periods, indicated an absence of glycolipid production within the system. It was more likely that an increase in membrane lipid content had occurred to provide increased cellular stability in the presence of certain substrate components. Further studies are needed to ascertain the type of proteins and lipids produced during the process. However, to provide a complete picture of the type of proteins and lipids along with the organism which produces the lipid would be difficult due to the complex nature of the seed source involved.

6.6 CONCLUSIONS

Microbial community shifts and responses were linked to reactor performance during a batch anaerobic degradation of a high strength chemical industry wastewater. The batch process was operated over three batches of wastewater substrate loading with an organic removal efficiency of 40.56% in Batch 1 and 25.55% in Batch 2. Microbial community analysis revealed a shift in acidogenic community profile throughout the three batches of feeding while methanogenic community shifts occurred only during the early period of Batch 1. Propionate accumulation was observed in all three batches of feeding due to excess rate of acidogenesis and increased acetate levels combined with slow substrate utilization rate of propionate oxidizing bacteria and some form of inhibition to biosynthetic pathways. Bi-nodal methane production was observed in the first two batches of substrate feeding with hydrogenotrophic methanogenesis as likely contributor towards initial methane production followed by late onset of acetoclastic methanogenesis. NMS ordination studies reveal migration of archaeal communities similar to that of seed profile but bacterial communities diverged possibly due to decay of certain species originally present in seed biomass and likely inhibition from substrate components. Elevated ECP and total lipid levels were observed, indicating microbial response to overcome

certain level of stress imparted from substrate components. Glycolipid production was ruled out due to a negative reaction in the blue agar plate test. Complex nature of seed makes it difficult to determine the organisms responsible for increased protein/lipid production.

CHAPTER 7 OPTIMISING FEED MIXING RATIO FOR HIGH STRENGTH INDUSTRIAL WASTEWATERS WW1 AND WW2 AND EVALUATING THE BIOCHEMICAL METHANE POTENTIAL OF INDIVIDUAL BLENDS

7.1. BACKGROUND

The basis of this study stemmed from two main reasons: 1) The shutting down of operations of the source industry of INDWW and 2) The excessive propionic acid accumulation in the system during the anaerobic degradation of INDWW. Propionic acid is extremely detrimental to the anaerobic degradation process. The VFA profile of the single stage batch process highlights that even if staging/ phasing of reactor vessels are to be done, there is every chance that high levels of propionic acid accumulation may still occur in either reaction vessel. Nevertheless with the source industry also shutting down their operations, utilizing the other two remaining industrial wastewater feedstock was considered the best option. However since the other two wastewaters did not have a COD as high as INDWW nor high concentrations of inherent usable VFA, it was necessary to consider mixing WW1 and WW2 to achieve high TCOD levels and subsequently used in anaerobic process to maximise the methane output and COD degradation. This chapter deals with the process of optimizing the feedstock mixing ratio of WW1 and WW2 and also evaluating the biochemical methane potential of the individual wastewater blend ratio.

7.2. OBJECTIVE

The objective of this study was to evaluate the optimal mixing ratio for mixing the feedstock WW1 and WW2. The study also incorporated the evaluation of BMP of the mixed ratios using the Automated Methane Potential Testing System (AMPTS) (Bioprocess Control, Sweden).

7.3. ANALYTICAL PHASE

7.3.1. Wastewater feedstock

The wastewater feedstock used in this study was WW1 and WW2, the properties of which are highlighted in **Table 7.1**.

Table 7.1 - Properties of industrial wastewater feedstock WW1 and WW2

Parameters	WW1	WW2
TCOD	90.27 $\pm$ 2.52 g/L	40.40 $\pm$ 1.49 g/L
SCOD	85.72 $\pm$ 1.35 g/L	37.87 $\pm$ 0.21 g/L
VFA	1.02 $\pm$ 0.03 g/L	8.73 $\pm$ 0.68 g/L
Major organics	Acetate	Acetate, Valerate
pH	9.27	9.01
Colour (visual)	Buff colour	Amber

7.3.2. Orthogonal experimental design for mixing wastewater

The orthogonal experimental design was used to devise different mixing ratios of COWW and CSWW. The main assumption made in this case was that the individual wastewaters do not react with each other. For mixing, 1 unit of COD loading was set to 0.5gCOD/gVSS of seed biomass. The design is elucidated in **Table 7.2**.

Table 7.2 – Orthogonal experimental design for the mixing WW1 and WW2

Test group	Units of WW1	Units of WW2	Total loading COD/g VSS
Blank	0	0	0
WW1 WW2	1	1	1
WW1 2(WW2)	1	2	1.5
2(WW1) WW2	2	1	1.5
2(WW1) 2(WW2)	2	2	3

7.4. EXPERIMENTAL PHASE

7.4.1. Evaluation of BMP of determined mix ratio

The exact volumes of seed biomass, wastewater and MSM used to keep all the VSS to near accuracy is highlighted in **Table 7.3**.

Table 7.3 - Exact volumes of wastewaters, seed sludge and MSM used for BMP experiment

Test group	Volume of WW1 (mL)	Volume of WW2 (mL)	Volume of MSM (mL)	Volume of seed sludge (mL)	Total wastewater volume (mL)
Blank	0	0	150	250	0
WW1 WW2	22.15	49.5	78.35	250	71.3
WW1 2(WW2)	22.15	99	28.85	250	121.15

2(WW1) WW2	44.3	49.5	56.19	250	93.8
2(WW1) 2(WW2)	44.3	99	6.7	250	143.3

7.4.2. Automated Methane Potential Testing System (AMPTS)

The AMPTS differs from conventional BMP testing systems. The biggest advantage is that it is fully automated, provides stirring quite similar to that of a reactor impeller, has a buffer to absorb carbon dioxide, and has a wet flow meter to measure only methane coming out of the buffer tank. Since the whole process is automated, minimal effort is needed to measure the total volume of methane produced during the test period. In this experiment, the system volume was set to 400mL, temperature of process 35°C, and a starting pH of 7.1. The reaction vessels were immersed in a water bath to maintain the temperature. Sampling for analysis was done every three days with 5mL of MLVSS being withdrawn through the sampling port. Volume changes during sampling were taken into account for calculation purposes.

7.4.3. Chemical oxygen demand (APHA Standard methods 5220C closed reflux method)

Analysis of total COD and soluble COD of the mixed liquor sample of all test groups was done as described in Section 3.2.3.

7.4.4. VFA analysis

Analysis of VFA's was done as described in Section 3.2.4

7.4.5. Biogas composition

Analysis of Biogas composition was done as described in Section 3.2.5

7.4.6. Microbial Community analysis

Microbial community analysis was performed only for those test groups which produced significantly more methane volume in comparison to the blank control. The analysis was performed as described in Section 3.2.6.

7.5. RESULTS

7.5.1. Methane Volume and quality

Maximum methane volumes over a 30day test period was observed in blend ratios 2(WW1) WW2 (887.98mL) and WW1 WW2 (663.64mL) respectively. In comparison, the Blank produced only 279.83mL of methane over 30days while WW1 2(WW2) and 2(WW1) 2(WW2) produced 208.06mL and 272.25mL respectively. The profile is elucidated in **Figure 7.1**. Based on the methane production profile, it was safe to assume that 2(WW1) WW2 and WW1 WW2 were the best blend ratios to produce maximum methane. The maximum recorded methane content for different ratios were WW1 WW2 – 80.75%; WW1 2(WW2) – 41.5%; 2(WW1) WW2 – 80.20% and 2(WW1) 2(WW2) – 41.64%. A very important observation was that in blend ratios containing 2 units of WW2, there was excess production of carbon dioxide, leading to a hypothesis that there might be some very easily degradable organics in WW2 which subsequently led to excess production of carbon dioxide.

Figure 7.1 - Total methane volumes generated over the 30day test period by different wastewater ratios. The volumes indicated are cumulative volumes over the test period

7.5.2. Chemical oxygen demand

The TCOD and SCOD profiles of different blend ratios are illustrated in **Figure 7.2** and **Figure 7.3**. Maximum TCOD reductions were observed in 2(WW1) WW2 and WW1 WW2 in comparison to the Blank . The methane produced to TCOD reduced is elucidated in **Table 7.4**.

Figure 7.2 - TCOD profile of different wastewater blending ratios during the BMP test period

Figure 7.3 - SCOD profile of different wastewater ratios during the BMP test period

Table 7.4 - Comparison of methane produced to TCOD reduced over the experimental period

Test group	Mean CH ₄ (mL)	Mean TCOD reduction (gm)
Blank	279.83 ± 3.58	1.48 ± 0.13
WW1 WW2	663.64 ± 4.96	2.88 ± 0.29
WW1 2(WW2)	208.06 ± 0.62	2.28 ± 0.69
2(WW1) WW2	887.98 ± 1.59	3.69 ± 0.04
2(WW1) 2(WW2)	272.25 ± 2.02	2.74 ± 0.10

Error represents standard deviation of triplicate analyses

7.5.3. VFA profile

The VFA profiles indicated that all blend ratios produced very high amounts of VFA especially acetic acid. However, only WW1 WW2 and 2(WW1) WW2 were able to degrade the acetic acid to methane. It was interesting to note that

blend ratios containing 2 units of WW2 produced high amounts of VFA but led to no subsequent degradation. The profile is further illustrated in **Figure 7.4**.

Figure 7.4 - VFA profiles of the different test groups a) WW1 WW2, b) 2(WW1) WW2, c) WW1 2(WW2) and d) 2(WW1) 2(WW2) over the 30day experimental period

7.5.4. Microbial community analysis

Previous results indicated that WW1 WW2 and 2(WW1) WW2 were the best blending ratios in terms of methane generation and TCOD reduction. Hence the community responses analysis was performed for these two test groups. The

profiles of the blend ratios were remarkably similar except for a minor change in the time period of response. Also *Methanosarcinaceae* tended towards becoming the dominant methanogenic flora during the test period. This was concomitant with the rise in levels of acetic acid. Since acetic acid was the major VFA produced, it was only logical that *Methanosarcinaceae* be the dominant methanogenic microbial flora. The profiles are illustrated in **Figure 7.5**.

Figure 7.5 - Microbial community responses towards a) WW1 WW2 ratio and b) 2(WW1) WW2 ratio over the 30day experimental period

7.6. CONCLUSIONS AND RECOMMENDATIONS

- The methane levels and COD reduction rates of different blend ratios indicated that WW1 WW2 and 2(WW1) WW2 were the best possible mixing ratios for optimal process conditions of maximum methane production and maximum COD degradation.
- Microbial community analysis indicated only a partially slower response of the communities in case of 2(WW1) 1WW2. Otherwise the community response profile was similar to the other test group WW1 WW2. *Methanosarcinaceae* profile was co-relatable with the profile of acetic acid being generated in blend ratios of WW1 WW2 and 2(WW1) WW2 during the BMP test.
- It was interesting to note that blending ratios containing 2units of WW2 produced high levels of total VFA but did not undergo subsequent degradation into methane.
- WW1 WW2 was considered best blending ratio due to faster rate of methane generation combined with very high methane quality, lesser propionic acid accumulation and faster total VFA degradation
- Staging/phasing of the anaerobic degradation process of selected wastewater ratio could help in maximising COD reduction, methane production, reducing of propionic acid accumulation and vastly improving effluent quality.

CHAPTER 8 COMPARATIVE STUDY OF MICROBIAL COMMUNITY DYNAMICS AND BIOCHEMICAL RESPONSES DURING BATCH ANAEROBIC DEGRADATION OF HIGH STRENGTH CHEMICAL INDUSTRY WASTEWATERS SEEDED WITH DIFFERENT SEED BIOMASS

8.1 ABSTRACT

Community dynamics and biochemical responses of three different microbial seed biomass obtained from three different sources (designated R1SL, FSL and AnSL respectively) were examined during anaerobic degradation of an optimized mixture of high strength industrial wastewaters. A Biochemical methane potential (BMP) test was performed in single batch mode till maximum possible methane production was achieved. AnSL and R1SL exhibited similar cumulative methane production and TCOD removal rates while FSL exhibited negligible methane production. Biochemical response tests showed the production of extracellular proteins and biological metal chelators (siderophores) by all test groups. Biosurfactant production was also observed in R1SL, FSL and AnSL test groups. During batch operation, rate of methanogenesis in AnSL and R1SL increased with elevated levels of *Methanosarcinaceae*. *Clostridiales* formed majority of Eubacteria in all test groups and *Methanomicrobiales* formed the majority of methanogenic species. Eubacterial community dynamics in R1SL and AnSL were varied over the experimental period while Methanogenic community structure exhibited very little or no change at all. Phylogenetic analysis revealed very little Methanogenic diversity in FSL; a likely cause for impaired methanogenesis. Overall, this could imply that changes in Eubacterial community structure of R1SL and AnSL had less impact on development and progressive activity of Methanogenic community structure. Increased methane production in R1SL and AnSL test groups were likely caused by higher numbers of aceticlastic methanogens (*Methanosarcinaceae*). Differences in methane production between AnSL and R1SL test groups could be attributed to difference in

diversity of *Methanosarcinaceae* in the seed biomass rather than change in community structure.

8.2 Introduction

Anaerobic degradation has gained attention for treating organic pollutants like industrial wastewaters due to its ability to produce methane whilst reducing pollution load (Appels *et al.*, 2011). Anaerobic degradation is a multi-stage reaction governed by different groups of microbial populations where acidogens hydrolyze and ferment complex organic molecules to carbon dioxide, hydrogen and acetate which are utilized by methanogens for their growth and methanogenesis. Microorganisms of varied physiological and biochemical activities coexist in anaerobic degradation and their synchronized activity is necessary for maximum degradation of organic pollutants. Overall, the performance of anaerobic degradation is heavily reliant on proper functioning and interaction of acidogens and methanogens involved.

During startup of a batch mode of anaerobic process (acclimation period), the reaction rates increase till the system reaches a semi steady state (Wilderer *et al.*, 2001). Such changes in reaction rates could arise from changes in community structure or population size. However previous attempts to examine these changes in detail have been hampered by the complexity of mixed culture systems in an anaerobic process. Therefore limited information is available on the microbial community dynamics, its biochemical responses as well as its overall impact on process performance. This particular knowledge gap, especially the biochemical responses of microbial communities, deserves more attention since anaerobic processes have slow reaction rates and prolonged startup periods as its major drawbacks.

The aim of this study was to assess the response of different seed biomass in terms of microbial community structure and biochemical responses, along with the changes in physicochemical reaction profiles in a biochemical methane

potential test. For this purpose, test groups were seeded with three different biomasses obtained from three different sources. The experiment was operated over a total period of 46 days, using an optimized mixture of two high strength industrial wastewaters as feed. Akarsubasi *et al.*, (2005) state that it is desirable to have quantitative and qualitative information of microbial responses towards a substrate for reliable linkage with process data. In this study, denaturing gel gradient electrophoresis (DGGE), a qualitative technique, and real-time polymerase chain reaction (PCR), a quantitative technique, were used. Non-metric multidimensional scaling (NMS) was also used to envisage the microbial community structure shifts during the experimental period. The present study reports both qualitative and quantitative changes in microbial community structure during a biochemical methane potential test co-degrading two different wastewaters. Biochemical tests were performed to detect presence of "siderophores"; biological metal chelators and increase/decrease of extracellular protein (ECP) to better understand the microbial community mechanisms in degrading high strength industrial wastewaters.

8.3 Materials and Methods

8.3.1 Wastewater characteristics

Two chemical industry wastewaters, designated as WW1 and WW2 were initially profiled in terms of parameters shown in **Table 8.1**. The wastewaters were defined as high strength in terms of its high total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) value.

Table 8.1 - Chemical industry wastewater properties

Parameter	WW1	WW2
Total chemical oxygen demand	(122.72 ± 2.16) g/L	(154.26 ± 1.71) g/L
Soluble chemical oxygen	(117.27 ± 1.83) g/L	(151.53 ± 2.78) g/L
Volatile fatty acids (VFA's)	--	--
Major organic components present	Monoethylene glycol and Diethylene glycol	Diethylene glycol and Triethylene glycol
pH	7.19	6.98
Colour (visual)	Colourless	Amber

8.3.2 Seed biomass

R1SL was collected from a lab scale continuous stirred tank reactor treating the aforementioned wastewaters in conjunction with two industrial secondary biosludges. FSL was a mixture of primary and secondary sludge while AnSL was collected from a local anaerobic digester treating primary and secondary sludges.

8.4 Experimental Phase

8.4.1 BMP Tests

The wastewaters and different seed biomass were mixed as shown in **Table 8.2**. The wastewaters and biomass were mixed with an initial F/M ratio of 1.5g TCOD:1g volatile suspended solids (VSS). For the blank controls, de-ionized (DI) water was added to maintain the same VSS concentration to that of test group. The wastewaters were mixed in the ratio of 1g TCOD WW1 : 1g TCOD WW2. The experiment was performed in triplicate rubber stopped serum bottles (0.3L total volume, 0.25L working volume and 0.05L headspace volume) under

mesophilic conditions (35°C) in a shaker incubator maintained at 150 rpm till maximum possible methane was produced. Anaerobic conditions were maintained by flushing the headspace of serum bottles with N₂ gas. Blank controls were maintained for each test group to analyze the residual methane produced by seed biomass without addition of wastewater substrate.

Table 8.2 - Experimental setup

Sample group	Biomass volume (mL)	Initial approximate VSS (g/L)	Volume of WW1	Volume of WW2	Volume of DI water	Total working volume
R1SL	161	8	12	10	67	250
FSL	105	8	12	10	123	250
AnSL	131	8	12	10	97	250
R1SL	161	8	-	-	89	250
FSL	105	8	-	-	145	250
AnSL	131	8	-	-	119	250

8.4.2 Analytical methods

Testing of total solids (TS), volatile solids (VS), total suspended solids (TSS) and pH were performed on Day 0 and Day 46 in accordance with standard methods (APHA, 1998). During the experimental stages, for VFA's and other analyses, 10mL of mixed liquor sample was withdrawn periodically through fine needle aspiration. One milliliter of sample was diluted 10 times for VFA analysis and 1mL was diluted 5 times for microbial, ECP, and siderophore analyses. The total volume changes during the sampling period were taken into consideration for calculating methane generation. VFA analysis was performed as described in Section 3.2.4. pH measurement was performed only on day 0 and day 46 due to limited sample volume. Individual biogas components were

analyzed as described in Section 3.2.5 with care taken not to allow the headspace pressure to fall below zero.

8.4.3 DNA Extraction and q-PCR analysis

DNA extraction and q-PCR analysis was performed as described in Section 3.2.6.

8.4.4 DGGE analysis

DGGE analysis was performed on the DNA samples extracted from test groups as described in Section 3.2.7. All nucleotide sequences were deposited in GenBank with the following accession numbers **KJ590965 - KJ591006**

8.4.5 Statistical analysis of DGGE profiles

Statistical analysis of DGGE profiles were performed by the NMDS method as described in Section 6.3.6.

8.4.6 Biochemical analysis

A modified Lowry's Assay (Lowry *et al.*, 1951) was performed on the supernatant of the mixed liquor to test the extra cellular protein (ECP) content. A test for production of "siderophores"; biological metal chelators, was performed on all the test groups. A confirmatory Chrome-azurol sulphonate assay or CAS Assay (Shwynn and Neilands, 1987) followed by a qualitative analysis to confirm the type of siderophore present [Catecholate/phenolate assay (Arnou, 1937); Hydroxamate assay (Neilands, 1981)] was conducted using the supernatant of mixed liquor samples withdrawn from the test groups. Glycolipid biosurfactant production was analyzed using a modified version of the

Seigmund and Wagner test (1991) by incubating MLVSS samples in the wells of a blue agar plate containing hexacetyltrimethyl ammonium bromide (0.2mg/mL), methylene blue (0.005mg/mL) and agar (1.5%).

8.5 RESULTS AND DISCUSSIONS

8.5.1 Process performance and evaluations

The performance profiles of the three different seed biomass with respect to VFA profile and methane generation is illustrated in **Figure 8.1** and **Figure 8.2**, respectively. All test groups produced acetate, propionate and butyrate (in order of amount accumulated). A similar observation was made by Kim *et al.*, 2013 when treating whey permeate in repeated feeding batch systems. In all the test groups, acetate was the major intermediate and increased production was observed from Day 5 (R1SL - 5.00 g/L; FSL - 0.73 g/L; AnSL - 2.70 g/L). The use of different seed sludges had a marked effect on the process performance which was reflected primarily in the VFA production and degradation rates. AnSL test group had the maximum amount of VFA produced (7.48 g/L on Day16) while R1SL test group and FSL test group produced 6.62 g/L on Day 16 and 6.94 g/L on Day 34 respectively. The increased VFA production during the earlier periods may be attributed to enhanced acidogenesis with favourable community structure. However, the degradation rate of the VFA's entirely depends on the presence of a robust methanogenic community structure as well. This could explain the reason why there was only VFA accumulation but no subsequent degradation in FSL test group. Additionally, AnSL test group and FSL test group produced butyrate as one of its major VFA intermediate, indicating that the difference in acidogenic community structure was responsible for the differing intermediates produced.

Figure 8.1 - VFAs profile of A) R1SL; B) FSL and C) AnSL test groups over the experimental period

There was considerable TCOD reduction in the R1SL (1.15gm) and AnSL (1.59gm) test groups but negligible TCOD reduction in the FSL test group (0.04gm) over the 46 day experimental period. The comparison of cumulative methane produced to TCOD reduced is illustrated in **Table 8.3**. The cumulative methane production over the test period was dissimilar in the test groups R1SL (266.66 mL at STP) and AnSL (367.46 mL at STP) but not significantly different [$p > 0.05$, t-test]. Markedly different acidogenic reaction profiles as well as methane production profiles (**Figure 8.1** and **Figure 8.2**) were followed in the two test groups. FSL test group had near negligible cumulative methane production (10.19 mL at STP). Maximum production of methane in R1SL and AnSL test groups were observed in between Day 28 and Day 40 (216.81mL and 211.31mL at STP respectively).

Maximum observed methane concentration in AnSL test group was 81.39% of total biogas and in R1SL was 80.61% of total biogas. In spite of the same operating conditions provided, it was observed that there was a difference in the pattern of methane production with no significant difference in the cumulative volume. This difference in the pattern of methane production could be attributed to the differences in the seeding source for the reaction mixtures (Pozdniakova *et al.*, 2012). Another interesting aspect was the absence of hydrogen production in spite of propionic acid accumulation in R1SL and AnSL test groups. It is also observed that the methane production profiles of R1SL and AnSL are in concurrence with VFA degradation rates observed in the respective test groups. This may again be attributed to the difference in the microbial communities arising from the different seeding sources.

Table 8.3 - Comparison of cumulative methane production and TCOD reduced

Test Group	Cumulative methane produced (mL)	TCOD reduction (gm)
R1SL	266.66 $\pm$ 2.48	1.15 $\pm$ 0.26
FSL	10.19	0.04
AnSL	367.46 $\pm$ 3.51	1.59 $\pm$ 0.17

Standard deviation of triplicate analyses

Figure 8.2 - Methane production profiles of A) R1SL; B) FSL and C) AnSL test groups over the experimental period

8.5.2 Quantitative analysis of microbial communities

Eubacterial and methanogenic communities of all the three test groups were quantitatively analyzed using the real-time PCR technique targeting the 16S rRNA gene. The quantification profiles are highlighted in **Figure 8.3**. In the test groups R1SL and AnSL, there was a gradual decrease of bacterial communities till Day 16 after which the copy numbers remained relatively stable till the end of the experimental period. This was concomitant with the reaction rates for VFA production in both the test groups. However in the test group FSL, the Eubacterial community copy numbers decreased from Day 5 till the end of the experimental period. This decrease would explain why there was a lot of other VFA intermediates (valerate and butyrate) in the VFA profile of FSL test as compared to R1SL test and AnSL test wherein acetate and propionate were the major intermediates.

All the target methanogen groups were detected in the three test groups except for the order *Methanococcales*. The growth of this particular methanogenic populations was probably not supported due to their high salinity requirements (0.3 - 9.4% (w/v) NaCl) for propagation (Boone and Castenholz, 2001). It was observed that *Methanomicrobiales* were the most dominant methanogenic flora in R1SL and AnSL test groups at any given time point of the experimental study. It could be said that *Methanomicrobiales* were the major communities responsible for the methanogenic activity in both the test groups. There was also a significant difference in between the *Methanomicrobiales* copy numbers of R1SL (4.97×10^9 copies/mL on Day 46) and AnSL (9.50×10^8 copies/mL on Day 46) ($p < 0.05$, t-test) at the end of the experimental period. However there was also a marked increase in the numbers of the acetoclastic methanogenic family *Methanosarcinaceae* in both the test

groups. This family was the second most dominant methanogen in the R1SL test group while it formed the third most dominant methanogen in the AnSL test group. There was also a significant difference in between the copy numbers of *Methanosarcinaceae* in between R1SL and AnSL test groups ($p < 0.05$, t-test). Therefore, it can be said that the *Methanomicrobiales* along with the *Methanosarcinaceae* were the major methane producing groups via the hydrogen utilizing and acetate utilizing methanogenesis pathways respectively. In spite of significant differences of copy numbers of *Methanomicrobiales* and *Methanosarcinaceae* in between R1SL and AnSL, AnSL produces more methane albeit not significant, than R1SL. This would imply that differences in seeding sources led to difference in diversity of *Methanomicrobiales* and *Methanosarcinaceae* population which in turn led to difference in methane generation profile. These results are also consistent with the VFA profile of R1SL and AnSL groups wherein acetate was the major VFA intermediate (major substrate utilized by the aceticlastic methanogenic group *Methanosarcinaceae*). The FSL test group had decreasing copy numbers of *Methanomicrobiales* and *Methanosarcinaceae* throughout the experimental period which could possibly explain VFA accumulation and negligible methane production.

The dominance of *Methanomicrobiales* within R1SL and AnSL groups during the experimental period was in accordance with the findings of Kim *et al.*, 2013 who postulated that the *Methanomicrobiales* constitute nearly 60 - 91% of the total methanogenic population in anaerobic systems. Here, in the R1SL as well as AnSL test groups, *Methanomicrobiales* composed approximately 75% and 84% of the total methanogenic populations respectively. This dominant nature of *Methanomicrobiales* (hydrogenotrophic methanogens) could indicate that hydrogen utilizing pathway possibly in combination with conversion of acetate into H_2 and CO_2 is likely the major pathway for the production of methane in R1SL and AnSL test groups. This is

in concordance with the results tabulated by Ahring *et al.*, 2001. Also the hydrogenotrophic methanogens possess higher specific growth rate (μ_m) in comparison to the acetoclastic methanogens; 0.3-4.1 d⁻¹ for hydrogenotrophic methanogens in comparison with 0.1-0.7 d⁻¹ for acetoclastic methanogens (Ahring, 2003). Hence a better control of hydrogenotrophic methanogens would help in improving process performance of anaerobic systems treating similar high strength industrial wastewaters.

Figure 8.3 - Real time PCR profile of A) R1SL; B) FSL and C) AnSL test groups over the experimental period

Acetate was the major intermediate VFA produced in by the three test groups. Since the acetate accumulated was quite high in concentration, it is to be expected that *Methanosarcinaceae* would dominate the aceticlastic methanogens due to a higher K_s value for acetate in comparison to *Methanosaetaceae* (Hori *et al.*, 2006). The same trend was followed in the test groups R1SL and AnSL, wherein there was a marked increase in population numbers of *Methanosarcinaceae* and the population number of *Methanosaetaceae* remained relatively unchanged. In spite of a significant difference in the copy numbers of *Methanosarcinaceae* of the two test groups (R1SL>AnSL), there was a marked difference in the methane generation pattern as well as in cumulative methane production. This could be attributed to the difference in the diversity of *Methanosarcinaceae* arising from different seeding sources leading to a difference in the reaction profiles. In the test group FSL, the copy numbers of *Methanosarcinaceae* decreased after Day 5. This could explain the unutilized acetate within the system as well as the negligible methane production. Shin *et al.*, 2011 postulated that aceticlastic methanogens especially *Methanosarcinaceae* are much more sensitive to inhibitors and changes in environmental conditions such as pH, temperature and free ammonia. Here the compounds present in the high strength industrial wastewaters might have inhibited the growth and activity of *Methanosarcinaceae* present inside the FSL test systems, leading to methanogenic process failure via non performance of aceticlastic methanogens.

8.5.3 DGGE and phylogenetic analysis

Bacterial and archaeal community structures in the 3 test groups were investigated by DGGE followed by phylogenetic analysis. Emphasis was placed on the changes in bacterial communities due to its predominant nature inside single stage systems and also acidogenesis being the rate limiting step in a batch system. Five different time points, Seed, Day 0, Day 16, Day 34 and Day 46

Table 8.4 - Phylogenetic affiliation of Bacterial 16S rRNA DGGE bands

DGGE band number (%) sequence similarity	Closest species (accession number)	Phylum/class
R-1 (98%)	<i>Treponema</i> sp. SPIT5 strain (AM182455)	<i>Spirochaetes/Spirochaetales</i>
R-2 (97%)	<i>Sedimentibacter hydroxybenzoicus</i> strain JW/Z-1 (L11305)	<i>Firmicutes/Clostridia</i>
R-3, An-8 (98%)	<i>Desulfobulbus propionicus</i> strain DSM 2032 (CP002364)	<i>Proteobacteria/δ-Proteobacteria</i>
R-4, F-7 (99%)	<i>Syntrophomonas sapovorans</i> strain OM (AF022249)	<i>Firmicutes/Clostridia</i>
R-5 (97%)	<i>Syntrophomonas wolfei</i> subsp. <i>saponavida</i> strain DSM4212 (CP000448)	<i>Firmicutes/Clostridia</i>
R-6, F-5 (99%)	<i>Pelospora glutarica</i> strain WoGl3 (AJ251214)	<i>Firmicutes/Clostridia</i>
R-7, R-8, F-6 (99%)	<i>Syntrophomonas cellicola</i> strain 19J-3 (DQ288691)	<i>Firmicutes/Clostridia</i>
F-1 (99%)	<i>Clostridium acetireducens</i> strain DSM 10703 (X79862)	<i>Firmicutes/Clostridia</i>
F-2 (97%)	<i>Clostridium aciditolerans</i> strain JW/YJL-B3 (DQ114945)	<i>Firmicutes/Clostridia</i>
F-3 (97%)	<i>Gracilibacter thermotolerans</i> strain JW/YJL-S1 (DQ117465)	<i>Firmicutes/Clostridia</i>
F-4 (99%)	<i>Syntrophomonas palmitatica</i> strain MPA (AB274039)	<i>Firmicutes/Clostridia</i>
F-8 (97%)	<i>Propionivibrio limicola</i> strain GolChi1 (AJ307983)	<i>Proteobacteria/β-Proteobacteria</i>
An-1, An-2 (98%)	<i>Clostridium acidisoli</i> strain CK74 (AJ237756)	<i>Firmicutes/Clostridia</i>
An-3, An-4 (99%)	<i>Sulfurospirillum barnesii</i> SES-3 strain (CP00333.1)	<i>Proteobacteria/ε-Proteobacteria</i>
An-5 (97%)	<i>Treponema azotonutricium</i> strain ZAS-9 (AF320287)	<i>Spirochaetes/Spirochaetales</i>
An-6 (99%)	<i>Sedimentibacter saalensis</i> strain ZF2 (AJ404680)	<i>Firmicutes/Clostridia</i>
An-7 (100%)	<i>Acetobacterium wieringae</i> strain DSM 1911 (X96955)	<i>Firmicutes/Clostridia</i>
An-9 (98%)	<i>Syntrophomonas curvata</i> strain GB8-1 (AY290767)	<i>Firmicutes/Clostridia</i>
An-10 (97%)	<i>Clostridium ultunense</i> strain BS (Z69293)	<i>Firmicutes/Clostridia</i>
An-11 (97%)	<i>Acidovorax caenii</i> strain R-24608 (AM084006)	<i>Proteobacteria/β-Proteobacteria</i>

Table 8.5 - Phylogenetic affiliation of Archaeal 16S rRNA DGGE bands

DGGE band number (%)	Closest species (accession number)	Phylum/class
R-9, An-13 (97%)	<i>Methanospirillum hungatei</i> JF-1 strain (CP000254)	<i>Methanomicrobiales/Methanospirillaceae</i>
R-10 (99%)	<i>Methanoculleus bourgensis</i> strain MS2 (AY196674)	<i>Methanomicrobiales/Methanomicrobiaceae</i>
R-11 (98%)	<i>Methanosaeta concilii</i> strain Opfikon (X51423)	<i>Methanosarcinales/Methanosaetaceae</i>
R-12 (99%)	<i>Methanosarcina semesiae</i> strain MD1 (AJ012742)	<i>Methanosarcinales/Methanosarcinaceae</i>
R-13 (97%)	<i>Methanosarcina barkeri</i> strain DSM 800 (AJ012094)	<i>Methanosarcinales/Methanosarcinaceae</i>
F-9 (100%)	<i>Methanolinea tarda</i> NOBI-1 (AB162774)	<i>Methanomicrobiales/Methanoregulaceae</i>
F-10 (99%)	<i>Methanoculleus receptaculi</i> strain ZC-2 (DQ787476)	<i>Methanomicrobiales/Methanomicrobiaceae</i>
F-11 (97%)	<i>Methanosphaerula palustris</i> strain E1-9c (EU156000)	<i>Methanomicrobiales/Methanoregulaceae</i>
F-12 (98%)	<i>Methanosaeta harundinacea</i> strain 8Ac (AY817738)	<i>Methanosarcinales/Methanosaetaceae</i>
An-12 (99%)	<i>Methanocorpusculum labreanum</i> Z strain (CP000559)	<i>Methanomicrobiales/Methanocorpusculaceae</i>
An-14 (99%)	<i>Methanoregula boonei</i> 6A8 strain (CP000780)	<i>Methanomicrobiales/Methanoregulaceae</i>
An-15 (98%)	<i>Methanosaeta concilii</i> GP6 strain (CP002565.1)	<i>Methanosarcinales/Methanosaetaceae</i>
An-16 (100%)	<i>Methanosarcina acetivorans</i> C2A strain (AE010299.1)	<i>Methanosarcinales/Methanosarcinaceae</i>
An-17 (100%)	<i>Methanosarcina mazei</i> strain DSM 2053, OCM 26 (AJ012095)	<i>Methanosarcinales/Methanosaetaceae</i>

In all test groups, organisms resembling *Syntrophomonas cellicola* strain 19J-3 (R-7, R-8, F-6) were responsible for oxidising saturated fatty acids having 4-9 carbon atoms into acetate propionate and butyrate (Wu *et al.*, 2006). However this particular organism might have reduced in copy numbers in FSL test group (as evidenced by lessening intensity of DGGE bands from Day 16). Similarly, bands R-4 and F-7, resembling *Syntrophomonas sapovorans* fade in intensity on Day 16 in R1SL test group and not present on Day 0 in FSL test group. These organisms hydrolyze the long chain saturated fatty acids and also organics containing more than 18 carbon atoms (Roy *et al.*, 1986). Other *Syntrophomonads* present were bands An-9, F-4 and R-5. Band An-9 resembled *Syntrophomonas curvata* which could have converted the synthesized butyrate into acetate (Zhang *et al.*, 2004). F-4, which resembled *Syntrophomonas palmitatica* could have performed the same function (Hatamoto *et al.*, 2007). However in the test group FSL, there was no degradation of butyrate (**Figure 8.1**), which indicated that this organism might have been inhibited by some other organics present in the wastewater mixture. Band R-5 which resembled *Syntrophomonas wolfei* and also performed a similar function to that of *Syntrophomonas palmitatica* and *Syntrophomonas curvata* (McInerny *et al.*, 1981) was found to be persistent throughout the experimental period. This is also reflected in the VFA profile of R1SL test group wherein no butyrate was observed throughout the experimental period. Band An-1 and An-2 were found to resemble closely *Clostridium acidisoli* which most likely performed acidogenic reactions within the test group and forming butyrate, acetate and formate as end products (Kuhner *et al.*, 2000). This would also explain the continued presence of butyrate till the end of the experimental period in the AnSL test group VFA profile. Similar groups of *Clostridia* were found in the FSL test group; F-1 *Clostridium acetireducens* which uses acetate as electron acceptor while degrading pyruvate and succinate (Orlygsson *et al.*, 1996) while F-2 *Clostridium aciditolerans* which performs acidogenic reactions producing butyrate propionate or acetate as byproducts (Lee *et al.*, 2007). There was also a

thermotolerant and eurythermal *Clostridia*, *Gracilibacter thermotolerans* (band F-3) which may have performed acidogenic reactions and producing acetate and butyrate as byproducts (Lee *et al.*, 2006). The presence of so many acidogens in FSL test group could explain the reason for accumulation of VFA intermediates particularly acetate and butyrate. Bands R-6 and F-5 also belonged to a group of *Clostridium* called *Pelospora glutarica* which likely performed the acidogenic reaction of glutarate with butyrate as its main byproduct (Matthies *et al.*, 2000). This would also explain the continued accumulation of butyrate in the FSL test group throughout the experimental period.

Band An-7 and band An-10 were interesting in the aspect that both were *Clostridiales* but follow entirely different reaction patterns. Band An-7 resembling *Acetobacterium wieringae* performed acidogenic reactions either chemoorganotrophically using complex carbohydrates or chemolithotrophically using H₂ and CO₂ as substrates to produce acetate as end product of fermentation reactions (Braun *et al.*, 1981). However band An-10 resembled *Clostridium ultunense*, a syntrophic acetate oxidizing bacterium which produces methane in stoichiometric amounts but also produces acetate as by product when performing acidogenesis (Schnurer *et al.*, 1996). Such differing *Clostridium sp.* in the AnSL test group could actually contribute to the stability of the process as a whole by the way of species diversity in comparison with R1SL test group which had a more specific set of organisms in lieu of already performing the degradation process in a CSTR environment. This would also suggest that the microbial community, especially acidogens, undergo a drastic shift during the process in order to establish a tolerant and robust microflora to better perform under adverse conditions of high strength wastewater treatment.

Of the other bands of interest in the Bacterial DGGE profile, bands An-6 and R-2 belonged to the same genera, *Sedimentibacter*. Though the identified species were different, the general function of the two was similar. Band R-2, resembling *Sedimentibacter hydroxybezoicus* and band An-6 resembling

Sedimentibacter saalensis, perform decarboxylation reactions to produce acetate and butyrate as the end products (Breitenstein *et al.*, 2002). Bands R-3 and An-8 resemble propionate utilizing bacteria *Desulfobulbus propionicus*, which ferments complex carbohydrates into ethanol and uses the same pathway to convert propionate into acetate as well as using sulfate as an electron donor (Stams *et al.*, 1984). However this was interesting in the sense that there was continuous propionate accumulation in both R1SL and AnSL test groups even in the presence of the propionate utilizer. The most likely hypothesis is that the utilization rate of *Desulfobulbus* could have been affected by the presence of certain compounds from the wastewater mixture. Another possibility is that the production rate of propionic acid is faster than the utilization rate which may have led to constant propionic acid accumulation or a combination of the aforementioned factors. Further studies are needed to ascertain the cause of propionic acid accumulation in the two test groups.

Archaeal DGGE profile of R1SL and AnSL showed simple banding patterns with little changes in the profile over the experimental period. However there were marked absences of certain bands in the FSL test group. Majority of the Archaeal members in the three test groups were found to resemble the order *Methanomicrobiales* indicating that these were the dominant methanogenic populations within the total methanogenic population. This was in concordance with the real time PCR results (**Figure 8.3**). The methanogenic species isolated from the three different test groups have been previously reported in various anaerobic test systems (Bialek *et al.*, 2011). A few recent studies have reported the presence of *Methanospirillum*, *Methanocorpusculum* and even *Methanoculleus* as the dominant methanogenic population within the test systems (Cardinali-Rezende *et al.*, 2012). Another interesting aspect was the total absence of detectable *Methanosarcinaceae* in the DGGE profile of FSL test group. This may stem from the fact that *Methanosarcinaceae* are very sensitive to the presence of inhibitors (Shin *et al.*, 2011). This is concurrent with

the real time PCR results (**Figure 3**) in which it is observed that there is a decrease in the total copy numbers of *Methanosarcinaceae* in the FSL test group from Day 5 onwards. The difference in the diversity of *Methanosarcinaceae* in R1SL and AnSL test groups could have contributed to the stability of process as a whole since AnSL test group had greater volume of methane produced and much more stable methane production profile in comparison to R1SL test group. One particular *Methanosarcinaceae* which might have contributed to this difference is *Methanosarcina acetivorans* (An-16). This particular member of the *Methanosarcinaceae* family uses acetate as its sole substrate and consumes it at a better utilization rate compared to other members of the family (Sowers *et al.*, 1984). A better utilization rate in conjunction with a better defined methanogenic community in AnSL might have caused the difference in methanogenesis profile as well as the increased cumulative production. Such differences in the *Methanosarcinaceae* type could directly be attributed to the differences in seeding source. Interestingly, no archaeal band related to the Methanogenic order *Methanobacteriales* was detected during the phylogenetic analysis by means of DGGE, though this order was successfully quantified using the real-time PCR technique. Shin *et al.*, (2008) postulated that the relatively low abundance of this order within anaerobic systems (<13% of total sum of methanogenic species) in combination with a slow and low growth rate of population could be responsible for the lack of visible bands in the domain-level analysis.

8.5.4 Changes in microbial community structure

NMS was used to visualize the changes in microbial community structure of the three test groups. To avoid PCR based errors and multi copy rRNA genes (Fromin *et al.*, 2002), presence or absence of bands was used to measure species abundance rather than DGGE band intensities. The NMS plots shown in **Figure 8.5** explained more than 90% of the variability of Bacterial communities in the

test groups (cumulative $r^2 > 0.9$ for the ordination axes) with a sufficiently low instability, which indicated that the resultant plots provided a meaningful picture of the community variance within the test systems (McCune and Grace, 2002). All test groups demonstrated significant shifting of Bacterial communities during the experimental period. The communities in R1SL test group had a clear shifting of communities from Day 0 till Day 40 from quadrant 3 to quadrant 1 via quadrant 4 which would explain the 73% total variance on axis 1 and 33% on axis 2. The maximum shift is observed between Day 16 and Day 46. In the FSL test group, the shifting was from quadrant 3 to quadrant 1 via quadrant 4, with the maximum shift observed in between Day 0 and Day 34. This would also explain a variance of 27% on axis 1 but such a low variance of 3% on axis 2. This was also in concordance with the VFA profile of FSL test group in which it was observed that there was no production of VFA after Day 14. AnSL test group had a relatively good bacterial community shift through the experimental period starting from quadrant 3 and moving to quadrant 1 via quadrants 2 and 4. This is in line with the total variance of 41% on axis 1 and 18% on axis 2. It is to be noted that while the bacterial communities evolved separately and differently in all the three test groups, there were clustering of points in all three test groups indicating that certain temporal variations persisted either in the beginning of the experimental period (R1SL test) or in the duration of the experiment (FSL and AnSL test groups). Although the interpretation of the results may vary, it can be said that the change in bacterial community structure with the changes in population size may have contributed to increased/decreased acidogenesis rates within the test groups.

Figure 8.5 - Non metric multidimensional plots for Bacterial community dynamics of A) R1SL test, B) FSL test and C) AnSL test. The proportion of variance for each axis is indicated in parenthesis

In contrast, the methanogenic community structure did not exhibit considerable shifting. All the 15 bands of interest exhibited more than 90% similarity (Sorenson's distance measure). This was evident with the DGGE band patterns exhibiting no visible difference in the migration patterns as well as intensities. It can be said that the methanogenic communities were fairly stable throughout the experimental period and the dynamic community shifting of Eubacteria had little to no effect on the formation and development of the methanogenic communities over the experimental period. This is in accordance with Zumstein *et al.*, 2000 who state that methanogenic communities exhibit significantly lesser diversity and dynamic nature in comparison to Eubacterial communities. The dynamic nature may be limited due to the narrow substrate utilization range for the methanogens (Boone and Castenholz, 2001). Therefore it can be concluded that increased methanogenesis in R1SL and AnSL test groups may be attributed to three reasons viz. a) increased methanogenic population

abundance, b) inherent diversity of methanogenic flora and c) increased substrate availability rather than an alteration of methanogenic community structure.

8.5.5 Biochemical analysis

A biochemical response study was performed on all the test groups to test for the presence of extracellular protein in the diluted mixed liquor. FSL test group had the maximum concentration of extracellular protein in the cell separated mixed liquor at the end of the test period (582.18 ± 0.28 mg/L) when compared to AnSL (317.08 ± 0.51 mg/L) and R1SL (236.23 ± 0.65 mg/L) respectively. Interestingly, there was increased protein production during the initial phase of the experimental period (**Figure 8.6**) which suggests that the protein produced may have acted as a line of defense to some stress imparted by the wastewater. Proteins are the first line of defense towards combating stress and generally an increase in the extra cellular content would indicate some form of stress encountered by the organisms (Pace and Righelato, 1980). Studies by Gonzales-Gil *et al.*, (2003) and Barber and Stuckey, (2000) hypothesize that certain soluble microbial products (SMP's) in the form of extracellular polymeric substances (EPS) or ECPs are secreted by microbes for enhancing nutrient uptake for growth and metabolism. They also suggest that SMP's may play an important role in metal uptake and mitigating metal toxicity. Another interesting point was the decrease in protein concentrations in R1SL (after Day 10) and AnSL (after Day 28 till Day 46) which may suggest that once the communities were accustomed to the wastewater compounds, they may have consumed some of the proteins produced in order to further aid methanogenesis.

Figure 8.6 - Extracellular protein concentrations of R1SL, FSL and AnSL test groups over the experimental period

One other form of stress which is encountered by the microbes are from metal deficiency/toxicity. Liu *et al.*, (2001) postulated that certain extra cellular substances play a decisive role in metal uptake by the cell. Hence a test for confirming production of biological metal chelators; "siderophores", was performed on the cell separated mixed liquor samples of all the test groups. The confirmatory CAS assay results elucidate that R1SL test group exhibited the presence of siderophores right from Day 0 unlike FSL (Day 5) or AnSL (Day 10) test groups (**Table 8.6**). R1SL test group was seeded with a biomass already treating the wastewater in conjunction with two other industrial secondary biosludges and hence might have produced siderophores in order to better uptake metal ions. FSL and AnSL test groups also produced siderophores later on during the test period which may indicate a strategy to better capture essential metal ions especially Fe(II) and Fe(III). Siderophores have maximum affinity towards Fe(III) in nature but also have a tendency to bind to other transition elements like Zn, Ni, Co and Sn (Neilands, 1995). Iron is an important cofactor in many biosynthetic pathways as are Zn, Ni and Co. Absence or deficiency of these metal ions may gradually reduce process

performance and in some cases may lead to spontaneous collapse of major pathways. It may be possible that low amounts of these essential metal ions in seed biomass or from wastewater substrate could have contributed to a slow process performance leading to lesser methane production in comparison to the TCOD reduced. To the authors' best knowledge, this is the first time that siderophore production has been reported during anaerobic degradation.

Table 8.6 - Siderophore analysis of test groups and blank controls

Test group	CAS Assay	Catecholate	Phenolate	Hydroxamate
R1SL Test	+ D0	+	-	-
FSL Test	+ D5	+	+	-
AnSL Test	+ D10	+	-	-
R1SL Blank	-	-	-	-
FSL Blank	-	-	-	-
AnSL Blank	-	-	-	-

Figure 8.7 highlights the Blue Agar plate for detection of glycolipid biosurfactant production. From the figure, it is observed that there were dark blue precipitates around the wells containing the mixed liquor samples from the test groups. The blue agar plate consists of cationic hexacetyltrimethylammoniumbromide (CTAB) and methylene blue complex in an agar-agar solid medium. A liquid sample containing anionic glycolipid biosurfactants, when incubated in the blue agar medium would react with CTAB to release methylene blue from the complex, thereby creating a dark blue precipitate (Siegmond and Wagner, 1991). It is interesting to note that R1SL exhibited biosurfactant production right from Day 0, whereas AnSL exhibited from Day 10 and FSL from Day 5. This can be attributed to the difference in seeding sources which led to differing periods of biosurfactant production. It is also worth noting that a reactor being operated in batch mode is bound to have stationary phases in the microbial growth cycle (prone to producing certain secondary metabolites like biosurfactants) while treating complex substrates. In depth studies may help to understand the mechanism of how proteins aid in

defense mechanisms towards stress, how iron and other elements affect and enhance the process performance as well as the role of biosurfactants in anaerobic degradation process.

Figure 8.7 - Blue Agar plate for observing and confirming the production of glycolipid biosurfactants. The blue zone around well of incubation indicates positive for biosurfactant in A) R1SL test, B) FSL test and C) AnSL test

8.6 CONCLUSIONS

Anaerobic degradation process of mixed high strength industrial wastewaters was performed using separate seeding sources. Two test groups (R1SL and AnSL) showed similar methane production but distinctly different reaction patterns. The overall methanogenesis rates increased in R1SL and AnSL test groups with increase in the population sizes of methanogens (aceticlastic methanogens), in spite of *Methanomicrobiales* dominating the total methanogenic population. *Clostridiales* dominated the bacterial species in all test groups. Despite a strong dynamic nature of the bacterial community in all test groups, very little change was observed in the community dynamics of methanogens, as evidenced by the NMDS profile, suggesting that bacterial community dynamics do not significantly affect the community dynamics of methanogens. Siderophore production was observed in all test groups, likely

indicating a deficiency of essential trace metal ions, which might have consequently hampered process performance. Biosurfactant production was also observed in all three test groups, likely produced for better uptake of substrate as well as mitigating stress. Further studies are needed to understand the role of proteins in combating stress, metal ions in enhancing/decelerating anaerobic degradation process as well as role of biosurfactants in anaerobic degradation process.

CHAPTER 9 MONITORING MICROBIAL COMMUNITY DYNAMICS AND BIOCHEMICAL RESPONSES DURING STARTUP OF A TWO-PHASE ANAEROBIC CSTR TREATING AN OPTIMIZED WASTEWATER MIX

9.1 ABSTRACT

Monitoring microbial community dynamics during startup of a reactor is necessary to understand the mechanisms of microbial action to aid in process control and preventing process failure. For this purpose, a two phase continuous feeding stirred tank reactor (CSTR) was operated for a period of 70 days. An acidogenic reactor (AR) of 6L working volume and methanogenic reactor (MR) of 12L working volume were used in this study to investigate and monitor the structure and responses of microbial flora while treating an optimized mixture of high strength industrial wastewaters. Chemical oxygen demand (COD) removal was around 82% towards end of reactor operation. *Clostridiales* formed the majority of the acidogenic community in both acid and methane reactors while *Methanomicrobiales* formed the bulk of methanogenic species. The methanogenic species in AR were most likely functionally impaired due to inhibition from substrate components while the acidogenic species exhibited significant dynamic nature for maximum substrate utilization. Microbial community shift within MR was relatively lesser in comparison to AR. Production of biological metal chelators; "siderophores" were observed in both reactors along with production of glycolipid biosurfactants. Increased extracellular protein concentration and total lipid concentration was observed in the AR in comparison to MR. A Biomass stress index test also revealed that AR experienced more stress in comparison to MR. Overall, it could be said that the performance of the acid reactor would hold key to the stability and proper functioning of the anaerobic process by mitigating stress on methane reactor by way of effective degradation of wastewater components.

9.2 INTRODUCTION

Anaerobic degradation has been a source of clean treatment method for industrial wastewaters due to inherent advantages of minimal sludge production, combustible biogas generation as well as negligent odor production (Speece, 1996). However, the performance of anaerobic reactors is entirely dependent on the composition and relative activity of its microbial flora. The composition and activity of microbial flora within anaerobic systems are directly affected by wastewater characteristics as well as operational conditions maintained. It has been previously reported that any imposed stress may lead to a change in species types and their population levels within anaerobic systems (Harper and Pohland, 1986). It is therefore imperative to identify the dominant acidogenic and methanogenic species within a reactor system, especially those treating high strength industrial wastewaters, which may impart a toxic effect on these species.

Several degradation phases such as hydrolysis, acidogenesis, and then methanogenesis are involved in a completely mixed reactor (Gujer and Zehnder, 1983). In the hydrolytic and acidogenic phases, organic pollutants are hydrolyzed and/or fermented into intermediate short-chain fatty acids (e.g., lactate, butyrate and propionate), which are further degraded to acetate and H_2/CO_2 . In the last phase, acetate and H_2/CO_2 are converted into methane. A single-stage anaerobic system is highly susceptible to reactor perturbations such as rapid increase in volatile fatty acids (VFAs) and subsequent decrease in the pH. These would subsequently inhibit methanogenesis and lead to process failure. To eliminate such an operational setback, two-stage anaerobic processes have been developed as an alternative (Pohland and Ghosh, 1971). In a two stage process, the hydrolysis/acidogenesis and methanogenesis reactions are split into two separate reactor systems, which allows for high rate of acidogenesis as well as reduction in reactor sizing. The phasing of systems also allows for proper

enrichment of microbial communities and these systems have been proven effective for industrial wastewater treatment (Ghosh *et al.*, 2000).

The start-up period in anaerobic systems is an important step in establishing proper community structure in a two-phase anaerobic treatment process. During this period, the reactor has to be continuously monitored for intermediate buildup as well as the reaction profiles of microbial flora. Recent advances in culture independent techniques, targeting the 16s rRNA gene, have paved way for a better understanding of the microbial community within anaerobic systems (Kim *et al.*, 2013). This study aimed to monitor and evaluate the microbial community dynamics within the acidogenic and methanogenic reactors during the startup of an anaerobic CSTR process. Quantitative realtime PCR and denaturing gradient gel electrophoresis (DGGE) techniques were employed to study the population increases as well as phylogenetic changes of the domains *Eubacteria* and *Archaea* within the reactor systems. Nonmetric multidimensional scaling (NMS) profiling was used to visualize the community shifts within the reactors. Biochemical response tests were performed to analyze microbial community responses to presence of wastewater organics.

9.3 MATERIALS AND METHODS

9.3.1 Reactor operation

The two phase reactor set-up had a Plexiglass 6L working volume acidogenic reactor (AR) and 12L working volume methanogenic reactor (MR). The reactor operating conditions were as summarised in **Table 9.1**. Since the reactors were operated in CSTR mode, the HRT=SRT. The working temperature inside the reactors was controlled at 35 °C. The pH of AR was set to a band of 5.2-7.2 while the pH of MR was set to a band of 6.5-7.5. The pH control was done by automated dosing of 1M hydrochloric acid or 1M sodium hydroxide. The

reactors were seeded with biomass collected from an anaerobic digester in a local wastewater treatment plant, treating a mixture of municipal primary and secondary sludges. The wastewaters used in this study were as profiled in **Table 8.1**. The wastewaters were mixed in the ratio of 1gm TCOD WW1 : 1gm TCOD WW2 in order to obtain a high COD loading strength.

9.3.2 Analytical methods

During the first three days of reactor run, samples were collected every day. Subsequently, the samples were withdrawn every 3 days for analysis. Chemical oxygen demand (COD), total solids (TS), total suspended solids (TSS), volatile solids (VS) and volatile suspended solids (VSS) were performed as per standard method protocols (APHA, 1998). Biogas produced was collected in Tedlar gas bags and volumetrically analyzed using a wet flow meter. Individual biogas components were measured as described in Section 3.2.5. Volatile fatty acids (VFAs) were analyzed as described in Section 3.2.4.

9.3.3 DNA extraction and q-PCR analysis

Two millilitres of mixed liquor sample was washed and resuspended in phosphate buffered saline solution to remove presence of inhibitory compounds arising from wastewater components. The duplicate DNA samples were extracted by the phenol:chloroform:iso-amyl alcohol method of Sambrook *et al.*, (1989) along with lysis in MPBio Lysis Matrix E and stored in -20 °C until further analysis. The q-PCR reaction was performed as described in Section 3.2.6.

9.3.4 DGGE and phylogenetic identification

DGGE analysis was performed as described in Section 3.2.7 for samples of Seed, Day 9, Day 18, Day 27, Day 36, Day 45, Day 54, Day 63 and Day 70, with Day 70 being the final day of reactor operation. The sequences obtained were deposited in GenBank database with the accession numbers from **KT190959-KT190991**.

9.3.5 Statistical analysis of DGGE profiles

Statistical analysis of DGGE profiles were performed using the NMDS technique as described in Section 6.3.6.

9.3.6 Biochemical response analysis

Extracellular protein was extracted, purified and analyzed as described in Section 6.3.7. Total lipids was extracted and analyzed as described in Section 6.3.7. Siderophore production analysis was conducted as described in Section 8.4.6. Blue agar test for observing production of glycolipid biosurfactants was performed as described in Section 6.3.7.

9.3.7 Biomass stress index

A biomass stress index test was performed by measuring the ratios of total ATP to dissolved ATP using the LuminUltra Total and Dissolved ATP detection kit (LuminUltra QG21STM). Protocol followed was as per the manufacturer's instructions.

9.4 RESULTS AND DISCUSSION

9.4.1 Reactor performance during startup

The CSTR system was operated for a total period of 70 days after which the system was stopped due to unavailability of feedstock WW1. The reactor performance is summarized in **Table 9.1**. An average TCOD reduction of 84% was observed within the system over the period of reactor operation. As it can be observed, phase separation was achieved with AR producing negligible amount of methane. The bulk volume of methane was produced in MR (~99%). However the rate of methane generation was quite low with mean generation of 1.4 Lday^{-1} . The maximum observed methane content in the methane reactor was 65.6% of the total biogas volume. A pH drop in AR was observed wherein the pH dropped from 7.2 on Day 0 to 5.6 on Day 36. This was in concurrence with the increase of VFAs production within AR. In spite of a HRT of 15days in AR, there was no observable methanogenesis. This was likely due to certain biosynthetic pathways being affected within the aceticlastic *Methanosarcinaceae* in the face of wastewater compounds (Shin *et al.*, 2011). The acid reactor in a phased system faces the full brunt of wastewater compounds and the acidogenic bacteria adapt better than their methanogenic counterparts due to wide range of substrate utilization coupled with high growth rates and low doubling times.

Figure 9.1 highlights the VFAs profile of AR and MR. From the figure it may be noted that there was no initial lag phase within the AR system. VFAs level reached a maximum of 5.5g/L on Day 3 after which the total VFA levels were maintained around 4.5g/L. Acetate was the major VFA intermediate produced ($3.17 \pm 0.77 \text{ g/L}$) followed by propionate ($0.66 \pm 0.17 \text{ g/L}$) and butyrate ($0.31 \pm 0.11 \text{ g/L}$). In MR, VFAs level reached the maximum of 1.43g/L on Day 6 after which there was degradation phase. Acetate was the major VFA intermediate ($0.63 \pm 0.30 \text{ g/L}$) followed by butyrate ($0.08 \pm 0.03 \text{ g/L}$) and

propionate. Towards the end of the reactor run, MR had very little propionate within the system, indicating almost complete degradation. This may be due to the longer 30day HRT for the MR which could have aided in optimal growth conditions for the propionate oxidizing bacteria (POB). Gavala *et al.*, (2003) postulated that POBs are slow growers and the propionate oxidation pathways are easily susceptible to disruption in the presence of high strength organics. This might have also been a cause for propionic acid retention within the AR system. The presence of high levels of acetate within AR might have also caused propionate accumulation. Fukuzaki *et al.*, (1990) demonstrated that at pH 6.5, when acetate levels increase from 0.6g/L to 3g/L, the q_s of propionic acid decreased from 0.5 to almost 0. Within AR, the mean acetate concentration was 3.17g/L which in turn would have definitely impaired the propionate utilization within the system. Concurrently, the level of acetate within MR was only 0.63g/L which would have aided optimal propionate utilization by POB in MR.

Table 9.1 - Summary of two phase reactor operations during startup

Parameters	Acid Reactor (AR)	Methane Reactor (MR)
HRT (days)	15	30
pH	Day 0 - 7.2 Day 70 - 5.5	Day 0 - 7.2 Day 70 - 6.97
OLR TCOD ($\text{kg m}^{-3} \text{d}^{-1}$)	10.1	4.84
SCOD ($\text{kg m}^{-3} \text{d}^{-1}$)	9.01	3.93
TCOD reduction (%)		84.9±2.6
Total VFA (g L^{-1})	4.3±1.03	0.75±0.31
Methane production (L d^{-1})		1.4
Methane yield ($\text{L gm TCOD added}^{-1}$)		0.28
Biogas composition (%)	Methane	
	41.32 (Day 12)	61.23±0.03

Carbon-di-oxide	51.15 (Day 12)	34.78±0.02
Nitrogen	7.31 (Day 12)	3.35±0.03

Figure 9.1 - VFAs profile of A) AR and B) MR over the period of CSTR startup run

9.4.2 Quantitative profiles of Eubacteria and Methanogens

Changes in the 16s rRNA gene concentration of the target groups over the period of reactor run are elucidated in **Figure 9.2**. The 16s rRNA concentration profiles of methanogens exhibited variance in AR with respect to process performance. There was a continuous decline in the population numbers of

methanogenic groups within AR which was in concomitance with cessation of methane production. This could have also contributed to phase separation. As seen in the figure, Eubacteria were dominant over the methanogens in both reactors while *Methanomicrobiales* dominated the methanogenic group. *Methanococcales* were not detected in the system probably due to their high salt requirements for optimal growth (Boone and Castenholtz, 2001). In AR, there was a gradual decrease in total methanogenic populations from Day 0 (4.79×10^9) till Day 27 (7.91×10^8), a decrease of one log concentration. This was also in concomitance with the decrease in pH as well as increased VFAs production within AR. Another reason which could have contributed to the non production of methane within AR would be the difference in population numbers of Eubacteria and Methanogens. Eubacteria had a population number 100 times more than the total population of methanogens and this could have contributed to lack of methane production, in spite of high acetate concentrations as well as a 15 day HRT period. A similar finding was reported by Kim *et al.*, (2014) when treating a food industry wastewater wherein the Eubacteria had 1000 times more the population of total methanogens in the acid reactor.

The MR exhibited a relatively better profile of methanogenic populations with *Methanosarcinaceae* increasing in copy numbers from Day 12 (7.96×10^5) to Day 42 (5.11×10^6). The other groups did not evidence a population increase but did not decrease drastically. The increase of *Methanosarcinaceae* could have contributed to an increase in the methane production within MR since there was considerable acetate degradation from Day 12 (**Figure 9.1**). There was also no corresponding increase in the concentrations of *Methanosaetaceae* levels which would further point out that *Methanosarcinaceae* were responsible for methanation of acetate. This is in concordance with the findings of Yu *et al.*, (2006) who postulated that *Methanosarcinaceae* have a higher growth rate and outcompete *Methanosaetaceae* at acetate concentrations ≥ 0.06 g/L. The active presence of

hydrogenotrophic methanogens would have also contributed to the production of methane but at a lesser rate. Hydrogenotrophic methanogenesis would have occurred when the eubacteria transferred hydrogen via syntrophic pathways to the hydrogenotrophic methanogens while performing acidogenic reactions (Stams and Plugge, 2009). Most likely, the hydrogenotrophic pathway for methane production in MR would have occurred during the earlier phases of reactor operation when the rate of methane generation was slower as compared to rate of methane generation once acetoclastic started.

Figure 9.2 - Quantitative PCR profile of A) AR and B) MR reactor vessels over the reaction period. The error bars represent standard deviation of duplicate analysis

9.4.3 Phylogenetic analysis of bacterial and archaeal DGGE profiles

DGGE analysis was performed on the DNA extracted from both AR and MR reactor vessels along with the DNA extracted from the seed. DGGE profile is illustrated in **Figure 9.3**. A total of 33 bands were excised from the gels and sequenced for comparison to nearest similar members. **Tables 9.2** and **9.3** highlight the similarity percentages for sequenced bands with known bacteria and archaea respectively. All the bands had high sequence similarity to known bacterial and archaeal species/strains. The bacterial species of both AR and MR fell primarily under the obligate anaerobic order, *Clostridiales*, which are highly ubiquitous in anaerobic systems treating organic wastes.

Figure 9.3 - DGGE band patterns of A) AR and B) MR. Time periods of analysis and target groups are indicated on the top.

Table 9.2 - Phylogenetic affiliation of Bacterial 16S rRNA DGGE bands

DGGE number sequence similarity)	band (%)	Closest species (accession number)	Phylum/class
AR-1, (98%)	MR-1	<i>Clostridium acidisoli</i> strain CK74 (AJ237756)	<i>Firmicutes/Clostridia</i>
AR-2, (98%)	MR-2	<i>Sulfurospirillum barnesii</i> SES-3 strain (CP00333.1)	<i>Proteobacteria/ε-Proteobacteria</i>
AR-3, (96%)	MR-3	<i>Treponema zuelzeri</i> strain DSM 1903 (FR749928)	<i>Spirochaetes/Spirochaetales</i>
AR-4, (94%)	MR-4	<i>Acetobacterium wieringae</i> strain DSM 1911 (X96955)	<i>Firmicutes/Clostridia</i>
AR-5 (97%)		<i>Sedimentibacter saalensis</i> strain ZF2 (AJ404680)	<i>Firmicutes/Clostridia</i>
AR-6, (95%)	MR-5	<i>Syntrophomonas wolfei</i> subsp. <i>saponavida</i> strain DSM4212 (CP000448)	<i>Firmicutes/Clostridia</i>
AR-7, (96%)	MR-6	<i>Syntrophomonas cellicola</i> strain 19J-3 (DQ288691)	<i>Firmicutes/Clostridia</i>
AR-8, (98%)	MR-7	<i>Syntrophomonas sapovorans</i> strain OM (AF022249)	<i>Firmicutes/Clostridia</i>
AR-9, (95%)	MR-8	<i>Pelospora glutarica</i> strain WoGl3 (AJ251214)	<i>Firmicutes/Clostridia</i>
AR-10, (94%)	MR9	<i>Desulfobulbus propionicus</i> strain DSM 2032 (CP002364)	<i>Proteobacteria/δ-Proteobacteria</i>
AR-11, (99%)	MR-10	<i>Ottowia shaoguanensis</i> strain J5-66 (KC222642)	<i>Proteobacteria/β-Proteobacteria</i>

Table 9.3 - Phylogenetic affiliation of archaeal 16s rRNA DGGE bands

DGGE number sequence similarity)	band (%)	Closest species (accession number)				Order/family
AR-12, (99%)	MR-11	<i>Methanospirillum</i> (CP000254)	<i>hungatei</i>	JF-1	strain	<i>Methanomicrobiales/Methanospirillaceae</i>
AR-13, (99%)	MR-12	<i>Methanoculleus</i> (AY196674)	<i>bourgensis</i>	strain	MS2	<i>Methanomicrobiales/Methanomicrobiaceae</i>
AR-14, (97%)	MR-13	<i>Methanoculleus</i> (DQ787476)	<i>receptaculi</i>	strain	ZC-2	<i>Methanomicrobiales/Methanomicrobiaceae</i>
AR-15, (100%)	MR-14	<i>Methanosaeta</i> (AY817738)	<i>harundinacea</i>	strain	8Ac	<i>Methanosarcinales/Methanosaetaceae</i>
AR-16, (99%)	MR-15	<i>Methanosarcina</i> (AE010299.1)	<i>acetivorans</i>	C2A	strain	<i>Methanosarcinales/Methanosarcinaceae</i>
AR-17, (99%)	MR-16	<i>Methanosarcina</i> OCM 26 (AJ012095)	<i>mazei</i>	strain	DSM 2053,	<i>Methanosarcinales/Methanosarcinaceae</i>

In both the reactors, organisms resembling *Syntrophomonas sapovorans* (AR-8, MR-7) were responsible for acidogenic reactions by hydrolyzing long chain fatty acids into shorter VFA intermediates like acetate and butyrate (Roy *et al.*, 1986). This particular organism increased in band intensity in AR which would indicate that it would have successfully adapted to the wastewater compounds. In MR, this organism did not reduce in intensity or disappear during any stage of operation. Other *Syntrophomonads* detected within the systems were *Syntrophomonas wolfei* (AR-6, MR-5) which converted long chain VFA into acetate (McInerney *et al.*, 1981) and *Syntrophomonas cellicola* (AR-7, MR-6) which performed oxidizing reactions to produce acetate, propionate and butyrate as intermediates (Wu *et al.*, 2006). However, band AR-6, resembling *Syntrophomonas wolfei* disappeared after Day 18 which would indicate that this particular organism would not have contributed much to the acidogenic reactions within AR. But it can be said that the *Syntrophomonads* were the major acidogenic community within the AR system. Band AR-5 resembling

Sedimentibacter saalensis would have been responsible for decarboxylation reactions to produce acetate and butyrate end products (Breitenstein *et al.*, 2002). This organism was present in low intensities in AR and was not detected in MR. Bands AR-1, MR-1, AR-4, MR-4, AR-9 and MR-8 all belonged to the *Clostridiales* but followed entirely different reaction patterns. AR-4, MR-4 closely resembled *Acetobacterium wieringae* which performed acidogenic reactions either chemoorganotrophically using complex carbohydrates or chemolithotrophically using H₂ and CO₂ as substrate to produce acetate as end product of fermentation reactions (Braun *et al.*, 1981). AR-1 and MR-1 resembled *Clostridium acidisoli* which most likely performed acidogenic reactions forming butyrate, acetate and formate as end products (Kuhner *et al.*, 2000) while AR-9 and MR-9 closely resembled *Pelospira glutarica* which performed the acidogenic reaction with butyrate as its main byproduct (Matthies *et al.*, 2000). This would also explain the continued presence of butyrate in the system throughout the experimental period. Bands AR-4, MR-4, AR-8, MR-9 disappear after some days of reaction suggesting that microbial community, especially acidogens, undergo a drastic shift during the process in order to establish a tolerant and robust microflora to better perform under adverse conditions of high strength wastewater treatment. Also the diverse nature of *Clostridiales* within the system could contribute to process stability especially for robust acidogenesis. Other organisms detected were AR-3, MR-3 which resembled *Treponema zuelzeriae*, a major acetogenic bacterial species (Veldkamp., 1960) and AR-11, MR-10 resembling *Ottawia shaoguanensis*, responsible for metabolizing various amino acids via a chemolithotrophic pathway (Geng *et al.*, 2014). A member of propionate oxidizing bacteria (POB) was also detected in bands AR-10 and MR-9, resembling *Desulfobulbus propionicus*, which ferments complex carbohydrates into ethanol and uses the same pathway to convert propionate into acetate as well as using sulfate as an electron donor (Stams *et al.*, 1984). AR-10 had variable intensities which would explain the continued accumulation of propionic acid within the AR system.

The POB have slow growth and substrate utilization rates coupled with a high sensitivity to high strength organics (Gavala *et al.*, 2003). The organics present in the wastewater could have contributed to inhibition of certain biosynthetic pathways within the POB, thereby further curtailing the utilization rate of propionate within AR. There was virtually no propionate accumulation within MR, probably due to optimal growth conditions for the POB via longer HRT as well as dilution of high strength organics within the system coupled with lower acetate levels, thereby increasing q_s of propionate for increased degradation.

DGGE bands patterns of both AR and MR were similar in terms of organisms detected. However, archaeal DGGE profile of MR showed simple banding patterns with little changes in profile over the experimental period while there were marked absences of certain bands in AR. Majority of the Archaeal members were found to resemble the order *Methanomicrobiales* indicating that these were the dominant methanogenic populations within the total methanogenic population. This was in concordance with the real time PCR results (**Figure 9.2**). Recent studies have reported the presence of *Methanospirillum* and *Methanoculleus* as the dominant methanogenic population within test systems (Cardinali-Rezende *et al.*, 2012). It is to be noted that there was disappearance of detectable *Methanosarcina acetivorans* in the DGGE profile of AR after Day 18. This may be due to *Methanosarcinaceae* being extremely sensitive to the presence of inhibitors like high strength organics (Shin *et al.*, 2011). This is also in accordance with the real time PCR results (**Figure 9.2**) wherein there was a decrease in the total copy numbers of *Methanosarcinaceae* in AR from Day 3 onwards. This particular member of the *Methanosarcinaceae* family uses acetate as its sole substrate and consumes it at a better utilization rate compared to other members of the family (Sowers *et al.*, 1984). A better utilization rate in conjunction with a higher population levels in MR might have caused the difference in methanogenesis profile as well as the increased cumulative production of methane. No archaeal band related to *Methanobacteriales* was detected during the phylogenetic analysis, even though

successfully quantified in the real-time PCR technique. Shin *et al.*, (2008) postulated that the relatively low abundance of this order within anaerobic systems (<13% of total sum of methanogenic species) in combination with a slow and low growth rate of population could be responsible for the lack of visible bands in the domain-level analysis.

9.4.4 Statistical analysis of DGGE profiles

NMS was used to visualize the changes in microbial community structure of the three test groups. To avoid PCR based errors and multi copy rRNA genes (Fromin *et al.*, 2002), presence or absence of bands was used to measure species abundance rather than DGGE band intensities. The NMS plots shown in **Figure 9.4** explained more than 90% of the variability of communities in the reactors (cumulative $r^2 > 0.9$ for the ordination axes) with a sufficiently low instability, which indicated that the resultant plots provided a meaningful picture of the community variance within AR and MR (McCune and Grace, 2002).

Figure 9.4 - NMS plots for A) AR bacterial migration, B) AR archaeal migration and C) MR bacterial migration. Arrows indicate spatial movement of communities and proportion of variance for each axis indicated in parenthesis

AR exhibited significant bacterial community development with a shift of communities from the Seed to Day 70 from quadrant 3 to quadrant 4 via quadrant 2. Maximum shift of communities occurred between Day 9 to Day 45 with very little shift observed afterwards. There was also clustering of data points observed, indicating time points of no community development. This would explain the 47% variance on axis 1 and 17% variance on axis 2 as well as being in accordance with the VFA profile which was stable after Day 45. Surprisingly, there was considerable shifting of archaeal communities within AR with the communities moving away from Seed to Day 70 via quadrants 4, 3, 2 and 1. Again significant clustering of points was observed. Such a significant movement would also indicate that the archaeal communities within AR try to constantly adapt towards the environment, though no production of methane was observed. The bacterial community shifts in AR suggest that migration occurs distinctly away from the seed profile during the reactor operation. The DGGE profile highlights that certain bacterial species decayed during the process (reducing in band intensities or disappearing completely) and might have acted as "noise bands", causing this particular pattern of migration. The noising of these weak bands was likely caused by some form of inhibition to the biosynthetic pathways of the bacterial acidogenic communities. In contrast, the MR bacterial profile exhibited very little dynamism, indicating that the longer HRT was beneficial for optimal growth conditions and substrate degradation. Also, the archaeal communities in MR exhibited no pattern of migration, indicating that they were almost, if not completely, unaffected by the presence of wastewater compounds. Although the interpretation of the results may vary, it can be said that the change in bacterial community structure with changes in population size may have contributed to increased/decreased acidogenesis rates within AR. From a qualitative aspect, these results also imply that methanogenic community members in MR evolve in a pattern similar to the seed inoculum along with the production of methane. It can also be concluded

that within MR, the evolution of bacterial communities had little effect on dynamism of methanogenic communities which evolve separately.

9.4.5 Biochemical response analysis

A biochemical response study was performed on the mixed liquor samples extracted periodically from both AR and MR and was tested for the production of increased extracellular protein (ECP), increase in total lipids, production of siderophores as well as production of glycolipid biosurfactants. There was increased production of ECP (**Figure 9.5a**) in both AR and MR but the levels were highly elevated in AR (846.6 ± 1.66 mg/L; Day 24) in comparison to MR (624.46 ± 2.52 mg/L; Day 24). In the presence of high strength organics, microbes tend to secrete proteins into solution in the form of compatible osmotic solutes to prevent any form of osmotic shock (Vyrides and Stuckey, 2009). AR had higher concentrations of ECP mainly due to bearing the brunt of wastewater loading, thereby preventing MR from shock conditions. Also the acidogenic communities in AR must be robust enough to prevent shock conditions which were the case in AR of this system. This would provide a greater insight as to why two phase systems fare better in methane production. An increase in the total lipids was also observed in AR and MR (**Figure 9.5b**), again with AR (5.46 g/L; Day 45) exhibiting higher total lipid production in comparison to MR (2.37 g/L; Day 45). This could have resulted from presence of lipophilic compounds in the substrate which could have altered the membrane structure of cells and thereby triggering increased production of membrane lipids to maintain rigidity and fluidity of cell membranes (Sikkema *et al.*, 1994). Again, AR would have had to produce more of lipids in order to better adapt to presence of higher strength of wastewater compounds in comparison to MR. This would also hold the key to process stability of a two phase system.

Figure 9.5 - Biochemical response profile for B) ECP concentration and B) Total Lipid concentration

One other form of stress which is encountered by the microbes are from metal deficiency/toxicity. Liu *et al.*, (2001) postulated that certain extra cellular substances play a decisive role in metal uptake by the cell. Hence a test for confirming production of biological metal chelators; "siderophores", was performed on the cell separated mixed liquor samples of all the test groups. The results are tabulated in **Table 9.4**. The confirmatory CAS assay results elucidate that AR produced siderophores from Day 3 while MR produced siderophores only from Day 15 onwards. Qualitative tests indicated that the siderophores produced were of "catecholate" type of siderophore as confirmed by the Arnow assay. Siderophores possess maximum affinity towards Fe (III) but also have a tendency to bind to other transition elements like Zn, Ni, Co and Sn (Neilands, 1995). Iron acts as an important cofactor in many biosynthetic pathways along with Zn, Ni and Co. Absence or deficiency of these metal ions reduce and in some cases lead to spontaneous collapse of anaerobic process. Low amounts of

these essential trace metal ions in seed biomass or from wastewater substrate could have contributed to a slow process performance leading to lesser methane production in comparison to the TCOD reduced.

Table 9.4 - Siderophore analysis of AR and MR

Reactor	Confirmatory CAS assay	Catecholate	Phenolate	Hydroxamate
Acid Reactor (AR)	+ Day 3 onwards	+	-	-
Methane Reactor (MR)	+ Day 15 onwards	+	-	-

Figure 9.6 highlights the blue agar confirmatory test for production of extracellular glycolipid biosurfactants. Both AR and MR produced glycolipid biosurfactants with AR exhibiting production from Day 3 and MR exhibiting production from Day 9 onwards. Since the biosurfactant was detected even on Day 66, the analysis was performed only till Day 66 as biosurfactants tend to be recalcitrant within the system. The blue agar plate consists of a cationic detergent, hexa-cetyltrimethylammoniumbromide (CTAB) and methylene blue dye in a bound complex in agar-agar solid medium. Glycolipid biosurfactants are anionic in nature and when a liquid sample containing it is incubated in the blue agar medium, it would react with CTAB and release methylene blue from the complex, thereby creating a dark blue precipitate (Siegmund and Wagner, 1991). It may be noted that the size of precipitates surrounding the incubation wells reduced in size towards the end of the experimental period in both reactors. It could be hypothesized that the organisms produce lesser biosurfactants once they acclimated to the wastewater compounds and based on the size of precipitates, AR produced more biosurfactant than MR. However further studies are needed to validate this hypothesis.

Figure 9.6 - Blue agar confirmatory test for glycolipid biosurfactant production for A) AR and B) MR. NOTE: presence of dark blue precipitate around wells of incubation indicate positive for biosurfactant production

9.4.6 Biomass stress index test

A biomass stress index test was performed for the samples drawn on Day 42 and Day 51, when pH in AR and MR were at their lowest recorded and on Day 70, the end of the reactor run. The results are illustrated in **Figure 9.7**. It can be observed that AR recorded more stress levels of $30.53\% \pm 0.50\%$ as compared to $21.52\% \pm 0.14\%$ in MR. This may be due to AR being exposed to more of the wastewater compounds than MR leading to some of the microbial population dying out, unable to cope with stress of high strength organics. Also, the increased stress levels in AR may have occurred due to a reduction in the population numbers of methanogenic communities within the AR reactor vessel as evidenced from the q-PCR profile. However, the interpretation of results may vary since there is no acceptable benchmark stress level as of yet for any individual reactor system. Nevertheless, it does provide an insight into the stability of the process, especially within a two phase system.

Figure 9.7 - Biomass stress index test for the acid reactor and methane reactor. Days indicate lowest pH within system and end point of reactor run

9.5 CONCLUSIONS

During the start-up of a two phase anaerobic CSTR system for treating an optimized mixture of high strength industrial wastewaters, complete phase separation was possible with acid reactor generating high levels of VFAs and the methane reactor degrading the VFAs and producing methane. *Clostridiales* formed the bulk of acidogenic species in both AR and MR while *Methanomicrobiales* formed the majority of the methanogenic population in AR and MR. Statistical analysis of microbial community profiles indicate that within the acid reactor, both acidogenic and methanogenic communities evolve distinctly away from seed profile and the evolution of methanogenic communities would be interlinked with the efficacy of acidogenesis. Within the methane reactor, the acidogenic and methanogenic communities evolve independent of each other with evolution of methanogenic communities not dependent or affected by efficacy of acidogenesis or evolution of acidogenic communities. Phase separation also led to increased stress levels in the acid

reactor as evidenced by an increased production of ECP content and increased total lipid content. Siderophore production and glycolipid biosurfactant production was observed in both reactors. Increased ECP, total lipid, biosurfactants as well as increased stress in the biomass stress index test would indicate that AR absorbs bulk of stress from wastewater compounds, thereby increasing stability of system and reducing chances of failure of MR. The continuous monitoring of microbial communities and their biochemical responses during start-up would help in developing a robust process for wastewater treatment by overcoming process failure and aiding in optimal recovery.

CHAPTER 10 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

10.1 SUMMARY

10.1.1 Wastewater composition influences anaerobic process

In Chapter 4, the BMP tests using INDWW proved that the composition of wastewater substrate heavily influences the type and amount of intermediates generated. The wastewater substrate INDWW was rich in valerate and the presence of valerate or any odd numbered carbon atom VFA or compound would lead to generation and accumulation of propionate as an intermediate. Propionate is not readily degraded within anaerobic systems due to an energetically unfavourable reaction pathway. Its build-up is detrimental and severely slows down the anaerobic process.

10.1.2 One step temperature increase may not suitably acclimate mesophilic seed

In Chapter 5, a one step temperature increase from 35°C-55°C was experimented to acclimate a mesophilic anaerobic seed to high temperatures for subsequent usage in a thermophilic process. However, a one step increase may not be suitable for all types of seed biomass, especially those harboring eurythermal organisms (those which can operate at mesophilic and thermophilic temperatures). These types of homologues can cause process imbalance and lead to build-up of undesired intermediates, especially when fed with certain type of organics as well as high organic content.

10.1.3 Acidogenic and methanogenic communities evolve independently in a single stage batch process

Chapter 6 dealt with the treating of INDWW wastewater in a single stage batch process, the conventional mode for treating high strength wastewaters. However, the excess acidogenesis led to buildup of propionate within the system. Statistical analysis of phylogeny revealed that the acidogenic and methanogenic communities evolve independent of each other and acidogenesis would be the rate limiting step in a single stage process. More importantly, to combat stress, the microbial communities would produce extracellular proteins and increase its total lipid content to maintain cellular membrane stability.

10.1.4 Methanosarcinaceae diversity key to stability of aceticlastic methanogenesis

When anaerobic process is performed in three separate batch systems seeded with different biomass, the diversity of microbial communities would hold key to stability of the process as a whole. Experiments performed in Chapter 8 exclusively revealed how the diversity of seed biomass played a key role in maintaining the stability of acidogenesis and more importantly how the difference in diversity of *Methanosarcinaceae* held the key to effective and stable aceticlastic methanogenesis and subsequently overall process stability.

10.1.5 Acid reactor in a two phase system combats stress effectively and helps in stable methane reactor function

The microbial community dynamics and biochemical responses during the startup of a two phase anaerobic CSTR revealed certain mechanisms of how the acid reactor effectively coped with stress imparted by wastewater organics and at the same time paved way for stable methane reactor performance. Increased ECP production, total lipid content, glycolipid biosurfactant production as well

as increased stress levels in the biomass stress index test would point in the general direction of acid reactor facing more stress than the methane reactor. Phylogenetic analysis revealed that development of microbial communities was interdependent within the acid reactor but evolve independently in the methane reactor.

10.2 Conclusions and implications

This study suggests the probable impact of wastewater compounds on the efficacy and stability of anaerobic degradation via generation and accumulation of undesired VFAs intermediates. It also proposed that a one-step temperature increase to acclimate a mesophilic seed to operate at thermophilic conditions would prove unfeasible if the seed contains eurythermal homologues. Molecular microbial techniques help in monitoring the microbial community dynamics within an anaerobic system along with studying the biochemical responses of communities. Within a single stage system, the independent evolution of acidogens and methanogens would hold key as to how the process can be stabilized with minimal impact on efficacy. The study also proposes that the diversity of seed biomass would hold key to stability of acidogenic process and the diversity of *Methanosarcinacea* would have an impact on stability and efficacy of acetoclastic methanogenesis. Studying the microbial community dynamics and biochemical responses within a two phase anaerobic system significantly bridged the knowledge gap of how the acid reactor holds key to stability of process and maintaining efficacy of methane reactor. The study also implicated limited methanogenic community divergence in the face of wastewater substrate, indicating that stability and adaptability of acidogenic communities was the key and would be the rate limiting step, irrespective of anaerobic process configuration. At the same time, the study could only partially define the role of microbial communities in sustaining anaerobic degradation of wastewater compounds. Further studies are needed to completely

define the role of microflora in the degradation of wastewater substrate in order to develop a robust process and avoid system failure even under high loading rates of wastewaters.

10.3 Recommendations

In this study, use of molecular techniques was found to be feasible to observe the reaction patterns of microbial communities within anaerobic systems. While this study suggests the use of culture independent microbial tracking using techniques like q-PCR, DGGE, it is imperative to track current changes and developments of methodologies. While q-PCR and DGGE nevertheless prove to be an effective tool in highlighting the changes occurring within major microbial communities involved in the acidogenic process, it would bode well to track the niche community members which may play an important role in substrate degradation. These niche community members cannot be easily identified using the q-PCR, DGGE techniques and it could very well be that their stability, adaptability and substrate degradation efficacy may hold the key to a stable anaerobic process. Recent developments in sequencing technologies incorporating the Next Generation Sequencing methods (pyrosequencing) may prove effective in tracking changes of the niche communities. However, like all DNA based methods, these techniques are subjected to a form of PCR bias as well. Moreover, due to the large number of sequences generated, it may prove difficult in interpreting results in a time bound study where large number of sampling points is involved. These techniques provide answers till the genus level identification only in comparison to DGGE where the identification is done to strain level but with a limited number of sequences. Lastly, one major question remains unanswered in using these techniques. While the identification of microbial communities was successfully performed, the organisms responsible for assimilating the substrate and their metabolic pathways for substrate degradation were not completely identified. The answer may lie with RNA

dependent methods such as stable isotope probing methods. In this technique, certain compounds of the wastewater substrate are labelled with radio-isotopes and with the help of biomarkers, could help in tracing the organisms assimilating the substrate at cellular levels (Manfield et al, 2002). The exact roles of acidogenic and methanogenic organisms in the anaerobic process could then be elucidated with firm evidence.

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