Diverting electron fluxes in anaerobic microbial communities using long chain fatty acids (LCFAs)

Mamata Sharma

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DIVERTING ELECTRON FLUXES IN ANAEROBIC MICROBIAL COMMUNITIES USING LONG CHAIN FATTY ACIDS (LCFAs)

by

Mamata Sharma

A Dissertation
Submitted to the Faculty of Graduate Studies through the Department of Civil & Environmental Engineering in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada
2008
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ABSTRACT

Long chain fatty acids (LCFAs) possessing 18 carbons were used to inhibit methanogenesis, a terminal metabolic step in the anaerobic degradation of glucose. The LCFA inhibitors diverted the electron flux towards hydrogen production in the absence of sulfate, and in the presence of sulfate (SO$_4^{2-}$) the electron flux was diverted towards SO$_4^{2-}$ reduction.

The experiments were performed in batch (160 ml) reactors containing a mixed anaerobic culture which was acclimated to glucose or glucose plus sulfate at 37°C. In the case of SO$_4^{2-}$ reduction, the chemical oxygen demand (COD)/SO$_4^{2-}$ ratio was 1.25 and the LCFAs used were linoleic acid (LA; C18:2), oleic acid (OA; C18:1), and stearic acid (SA; C18:0). Diversion of electron fluxes to sulfate reducing bacteria (SRBs) was observed in cultures fed LA (> 30 % electron flow as compared to the glucose plus SO$_4^{2-}$ controls) and OA (> 20 % electron flow as compared to the glucose plus SO$_4^{2-}$ controls) plus glucose, while SA had no significant effect on sulfate removal. Sulfate reduction increased with LCFA concentration and a maximum of approximately 90 % and 70 % sulfate removal was achieved in cultures receiving LA and OA, respectively, plus glucose.

Prior to performing the hydrogen production studies, experiments were conducted to assess the synergistic effect of low pH (pH 5 or 6) and OA or LA (individual and mixtures) on hydrogenotrophic methanogens. Data from the latter work provided optimum conditions for conducting the hydrogen production studies (pH 5 and 2000 mg l$^{-1}$ LCFA). In the hydrogen production studies, hydrogen was produced and accumulated in all the LA or OA incubated cultures (ranging from 0 - 25 days in 5 days consecutive
increments). In the LA or OA incubated cultures, the maximum hydrogen yield was 2.80 ± 0.20 and 2.44 ± 0.12 mole H₂ mole⁻¹ glucose, respectively. In the LCFA incubated cultures, the hydrogen yield was a function of the predominant LCFA β-oxidation byproduct prevalent during the time of analysis. All three β-oxidation byproducts, plamitic, myristic and lauric acid inhibited hydrogen consumption; however, the greatest inhibition was detected in cultures containing lauric acid.
THESIS ORGANIZATION

Chapter 1 outlines a brief introduction on how the technological concepts on diversion of electron equivalents using LCFAs can be efficiently and economically used for resolving issues associated with the treatment of industrial effluents with high levels of sulfate and long chain fatty acids. This chapter also details the research and experimental objectives pertaining to each phase. The literature review covered in Chapter 2 describes concepts, chemistry and microbiology associated with the anaerobic treatment of organic matter. This chapter mainly focuses on providing sufficient details on hydrogen production, consumption and its management in anaerobic communities. The aim of this section is to present sufficient information describing the manipulation of electron fluxes. Section 2.1 provides an overview of the literature review. Chapter 3 lists all the materials and methods used in this study. The results of this research work are reported in the form of five manuscripts (Chapter 4 to Chapter 8). Sulfate reduction and inhibition of methanogenesis using linoleic acid (LA: C18:2) as an inhibitor is discussed in Chapter 4. Chapter 5 focuses on the inhibition of methanogenesis and diverting electron fluxes towards sulfate reduction using two LCFAs, oleic acid (OA: C18:1) and stearic acid (SA: C18:0). Chapter 6 describes a study to understand the effects of LCFAs and low pH on hydrogen degradation in mixed anaerobic communities. This study provided the optimum conditions for the hydrogen production experiments. Chapter 7 describes the effect of varying LA incubation periods on diverting electron fluxes away from methanogens in the absence of sulfate to produce hydrogen. Hydrogen production studies using incubated cultures (0 - 25 days at the increment of 5 days) to OA are explained in Chapter 8. Chapter 9 provides summary and general conclusions relating the
manuscript chapters to each other and to the discipline or field of study. Chapter 10 provides the engineering significance and significance of the thesis research to the field of study; discussions of any potential applications of the research findings, and comments on future research that should be conducted.
DEDICATION

I dedicate this thesis to my loving and proud parents, Mr. K.B.P. Sharma and Mrs. Lalmani Sharma.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my Advisors, Dr. Jerald Lalman and Dr. Nihar Biswas, for their continued help, support, inspiration and friendship throughout my research work. Your suggestions and guidance were very valuable and this research would have been incomplete without your input. I extend my earnest appreciation to my other committee members, Dr. Wayne Parker, Dr. Andrew Hubberstey, Dr. Rajesh Seth and Dr. Paul Henshaw for taking the time to review and providing comments and suggestions to improve this thesis.

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I would like to thank the Department of Civil and Environmental Engineering at the University of Windsor for providing the financial assistance to make this work possible.

I would like to thank my family members for being there for me throughout my research work. Special thanks to my parents, who always encouraged, motivated and instilled confidence and faith in me for completing this research work. I would also like to thank my brothers, Rajeev Sharma and Mukul Sharma for supporting and encouraging me during this time. Finally, I would like to express my deepest thanks to my husband, Ashutosh Sharma, for the suggestions, support, understanding and love during the entire process of this research work. I admire your patience and the encouragement you provided at times of difficulty. On several occasions, your participation in the discussions
on many of the chemical engineering concepts made the entire learning process fun.

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## NOMENCLATURE

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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPB</td>
<td>aceticlastic MPB (methane producing bacteria)</td>
</tr>
<tr>
<td>APS</td>
<td>adenosine phosphosulfate</td>
</tr>
<tr>
<td>ASRB</td>
<td>acetate degrading SRB (sulfate reducing bacteria)</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BES</td>
<td>bromoethanesulphonate</td>
</tr>
<tr>
<td>CMTP</td>
<td>conventional mesophilic two-phased</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A (3'-phosphate group)</td>
</tr>
<tr>
<td>COD</td>
<td>chemical oxygen demand</td>
</tr>
<tr>
<td>CoM</td>
<td>coenzyme M (2-mercaptoethanesulfonate)</td>
</tr>
<tr>
<td>CSTR</td>
<td>continuous stirred tank reactor</td>
</tr>
<tr>
<td>FADH</td>
<td>flavin adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>FB</td>
<td>Facultative bacteria</td>
</tr>
<tr>
<td>Fd(ox)</td>
<td>ferredoxin (oxidized form)</td>
</tr>
<tr>
<td>Fd(red)</td>
<td>ferredoxin (reduced form)</td>
</tr>
<tr>
<td>HT</td>
<td>heat-treated</td>
</tr>
<tr>
<td>HRT</td>
<td>hydraulic retention time</td>
</tr>
<tr>
<td>LA</td>
<td>linoleic acid</td>
</tr>
<tr>
<td>LCFAs</td>
<td>long chain fatty acids</td>
</tr>
<tr>
<td>MPB</td>
<td>methane producing bacteria</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NHT</td>
<td>non heat-treated</td>
</tr>
<tr>
<td>OA</td>
<td>oleic acid</td>
</tr>
<tr>
<td>PBR</td>
<td>packed-bed reactor</td>
</tr>
<tr>
<td>PFL</td>
<td>pyruvate:formate lyase</td>
</tr>
<tr>
<td>PFOR</td>
<td>pyruvate:ferredoxin oxidoreductase</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
</tbody>
</table>
SA  stearic acid  
SRB  sulfate reducing bacteria  
TP  temperature-phased  
TS  total sulfide  
TSS  total suspended solids  
UASB  upflow anaerobic sludge blanket  
VFAs  volatile fatty acids  
VSS  volatile suspended solids
CHAPTER 1: INTRODUCTION

1.1 Context

Liquid effluents discharged from various industrial facilities have varying chemical composition, which is characteristic of the industry type. For example, effluents from metallurgical, petroleum and mining and mineral industries are rich in sulfate [Lens et al., 1998] while those from diaries, food manufacturing and vegetable oil contain elevated levels of long chain fatty acids (LCFAs) [Kramer, 1971]. Process streams containing sulfate and LCFAs are a major pollution concern and treatment presents several challenges because of their toxic effects on variety of microbial populations.

LCFAs are derived from the enzymatic hydrolysis of oils and fats [Hwu et al., 1998, Hanaki et al., 1981]. Although these acids degrade to acetate and hydrogen via a β-oxidation reaction scheme [Weng and Jeris, 1976], LCFAs and LCFA byproducts are inhibitory to hydrogenotrophic and aceticlastic microorganisms [Lalman and Bagley, 2002; Lalman and Bagley, 2001; Angelidaki and Ahring, 1992; Koster and Cramer, 1987; Hanaki et al., 1981]. Methanogenic inhibition causes an electron flow imbalance between the acidogenic and methanogenic populations and this eventually increases the production of reduced volatile fatty acids (VFAs) with a simultaneous lowering of the hydrogen partial pressure.

An efficient anaerobic treatment of industrial effluents containing elevated levels of sulfate is a technological challenge due to a competition between the sulfate reducing bacteria (SRB) (sulfate reducers) and methane producing bacteria (MPB) (methane producers) for electrons derived from donor compounds such as hydrogen and acetate. The role of SRBs in anaerobic communities is analogous to that of other terminal
degraders such as methanogenic bacteria. In the case of SRBs, the terminal electron acceptor is \( \text{SO}_4^{2-} \) while in methanogens, \( \text{CO}_2 \) is the analogous compound. [McCartney and Oleszkiewicz, 1993; Lens et al., 1998]. A competition between these populations for simple electron donors renders a fraction towards methanogenesis instead of sulfate reduction causing the process to become less efficient [Weijma et al., 2002].

No commercially available bioprocessing treatment technology on the market is designed to effectively treat industrial effluents containing \( \text{SO}_4^{2-} \) or LCFAs; hence, there exist opportunities to advance knowledge in this area. The significance of this work is to gain a better understanding of the diversion of electron fluxes in anaerobic bioprocesses. The proposed work is especially concerned with understating the final step of anaerobic degradation in which methane is produced from \( \text{CO}_2 \) reduction or from splitting acetate into \( \text{CH}_4 \) and \( \text{CO}_2 \). Acetilastic methanogens and hydrogenotrophic methanogens mediate the conversion of acetate and the \( \text{CO}_2 \) reduction reaction, respectively. This study focuses on diverting electron fluxes to desired terminal metabolic pathways, such as sulfate reduction and hydrogen accumulation, inhibiting hydrogenotrophic methanogens selectively through the use of C18 LCFAs. Inhibiting hydrogenotrophic methanogens with C18 LCFAs is a unique and novel process to divert electron fluxes away from \( \text{CH}_4 \) production. This research was conducted to address an issue such as can electron equivalents be utilized by electron acceptors such as protons or \( \text{SO}_4^{2-} \) resulting in \( \text{H}_2 \) accumulation and sulfate reduction to sulfide, respectively, while methanogens are inhibited by LCFAs? Note that sulfides released in this process are potent toxins and can be removed by precipitation using heavy metals [El Bayoumy et al. 1999].
1.2 Objectives

In this study, experiments were designed to assess hydrogen production by inhibiting hydrogenotrophic methanogens using LCFAs and to understand the competition for electron donors between SRBs and hydrogenotrophic methanogens using LCFAs. The work was conducted using mixed anaerobic mesophilic microbial cultures, which were maintained at 37°C. The specific objectives were as follows:

Objective 1: Assess sulfate reduction by SRBs in the presence of three C18 LCFAs (linoleic acid (LA, C18:2), oleic acid (OA, C18:1, and stearic acid (SA, C18:0) under mesophilic conditions.

Because of the issues to be resolved in the main research objective, the following sub-objectives were implemented to address as much as possible the complex scope of work:

i. Determine the inhibitory effect of LCFAs on glucose degradation and examine LCFAs degradation over a specified period.

ii. Assess the contribution of electron fluxes from LCFA degradation towards sulfate reduction.

iii. Examine the diversion of electron fluxes from glucose degradation to sulfate reduction in the presence of LCFAs.

iv. Examine the production and removal of volatile fatty acids (VFAs) by the acetogenic bacteria in the presence of LCFAs and SRBs.

v. Determine the removal of sulfate by the SRBs in the presence of varying concentrations of LCFAs.

vi. Access the inhibitory effect of LCFAs and sulfate on methane production by the MPBs.
Objective 2: To evaluate the combined inhibitory effect of low pH and individual LCFA levels or low pH and mixtures of C18 LCFAs (OA and LA) on hydrogenotrophic methanogens. In this objective, the following sub-objectives were implemented to completely address the scope of work:

i. Examine hydrogen consumption in the absence of LCFAs at pH 5 and 6.

ii. Assess hydrogen metabolism in the presence of individual and mixtures of LCFAs at pH 5 and 6.

iii. Determine the inhibitory threshold levels of C18 LCFA on hydrogenotrophic methanogens at low pH.

Objective 3: To assess the effect of varying LCFAs incubations on hydrogen production by incubated anaerobic mixed microbial mesophilic cultures to LCFAs at pH 5. The sub-objectives in this study were as follows:

i. Examine hydrogen production using cultures incubated with LCFAs for varying periods (ranging between 0 to 30 days at an increment of 5).

ii. Establish the effect of LCFAs on glucose degradation.

iii. Determine the production and removal profiles of VFAs and alcohols in the presence of LCFAs.

iv. Examine LCFA β-oxidation byproducts and its effects on hydrogen yields.

v. Determine the methane production profiles in the presence of LCFAs and LCFA β-oxidation byproducts and examine their effects on hydrogenotrophic inhibition.
1.3 References


CHAPTER 2: LITERATURE REVIEW

2.1 Overview

The focus of this chapter is to provide sufficient details on hydrogen production, consumption and its management in anaerobic communities. Of special importance in this section is to present sufficient information describing the manipulation of electron fluxes.

In anaerobic bioprocessing, the management of hydrogen production and consumption is important because it is an intermediate from processes involved with acidogenesis and methanogenesis. A description of anaerobic hydrogen fermentation, which includes formation pathways, important reactions, microbiology, hydrogen yields using pure and mixed microbial communities and operational factors affecting anaerobic microbial hydrogen production such as, pH, temperature and hydraulic retention time (HRT) is presented in this section. An understanding of previous work describing the inhibition of methanogens using several techniques such as, heat treatment and chemical inhibition, and the mechanisms involved is also described. Use of LCFAs as a methanogenic inhibitor is a novel technique adopted for methanogenic inhibition. This section describes the sources, degradation mechanisms and inhibition patterns of various LCFAs.

Sulfate reduction in anaerobic biodegradation is a metabolic pathway for disposing of excess electron equivalences. This section also covers pathways, microbiology, and electron donors used for sulfate reduction. A discussion on competition between sulfidogens and methanogens for electrons from acetate and hydrogen, factors affecting this competition and sulfide inhibition of methanogens and sulfidogens is presented.
2.2 Concepts in Anaerobic wastewater treatment

Anaerobic decomposition of organic chemicals is an important operation in wastewater treatment processes. The process has become a practical requirement in many full-scale facilities because of its cost effectiveness and energy saving [Lettinga, 1995]. The conversion of complex organic substrates to methane is anaerobically mediated by a consortium of different microbial populations, which includes hydrolytic microorganisms, acidogens, acetogens, and methanogens [Veeken et al., 2000; Bagley and Brodkorb, 1999]. Individual enzyme systems of these distinct bacterial populations catalyze all essential reactions involving dissolved organic matter to methane production. These processes include hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2.1) [Kaspar and Wuhrmann, 1978]. In this four-step process, byproducts from one reaction serve as substrate for other reactions and the major end products along with methane are biomass, water and carbon dioxide (CO₂). However, methane is not the terminal product in the presence of alternative terminal electron acceptors such as sulfate. Several reactions for each of these microbial steps are provided in Table 2.1. Since both methanogens, also known as MPB, and SRBs are terminal electron acceptors there exists a competition between the two for electron donors such as hydrogen and acetate [Lens et al., 1998]. A competition pathway between acetogenic (Facultative bacteria (FB)), MPB and SRB is shown in Figure 2.2.

2.2.1 Hydrolysis

Hydrolysis, the first step in anaerobic biodegradation, is the degradation of large complex organic polymers to simple monomers by extra cellular enzymes excreted by hydrolytic microorganisms [Veeken et al., 2000; Annachhatre, 1996]. Hydrolysis is
regarded as the rate-limiting step for the overall degradation process [Noike, 1985; Eastman and Ferguson, 1981] and its rate a function of factors such as pH, temperature, composition and particle size of the substrate, and high concentrations of intermediate products [Veeken and Hamelers, 1999; Gujer and Zehnder, 1983].

Figure 2.1: Pathway of anaerobic biodegradation [Adapted from Gujer and Zehnder, 1983].

2.2.2 Acidogenesis

The next step in the sequence, acidogenesis, is fermentation of hydrolysis products, carbohydrates, amino acids and LCFA to volatile fatty acids (VFA) (e.g., acetate, propionate, and butyrate), alcohols, hydrogen and carbon dioxide [Veeken et al., 2000;
Boone, 1985]. Fast growing fermentative bacteria such as for example, *Enterobacter aerogenes* and *Escherichia coli* mediate these reactions [Malina and Pohland, 1992].

### 2.2.3 Acetogenesis

Acetogenesis, the third step in this process, converts higher VFAs and alcohols into acetate, hydrogen and carbon dioxide. Acetogenesis bacteria require low hydrogen partial pressure to degrade long chain VFAs into smaller carbon chain molecules. At relatively high hydrogen partial pressures, the formation of acetate is reduced and the products are diverted from methane production into reduced compounds such as propionate, butyrate, and ethanol. [Kaspar and Wuhrmann, 1978; Bitton, 1994].

### 2.2.4 Methanogenesis

Methanogenesis, the terminal step in the absence of any other electron acceptor, is conversion of acetate and hydrogen to methane and CO₂. A group of strictly anaerobic archaea also referred to as MPB carries out this step [Madigan *et al.*, 2000].

The methanogenic group consists of both gram-positive and gram-negative bacteria in wide variety of forms [Bitton, 1994]. Structurally methanogens are prokaryotic cells that show a diversity of cell wall chemistries. Many methanogens such as for example, *Methanospirillum hungatti*, *Methanobrevibacter ruminantium*, *Methanobacterium strain AZ* and *Methanosarcina barkeri* are mesophilic. However, extremophilic species growing optimally at very high or very low temperature or at very high salt concentration also exist. *Methanococcus jannaschii* (optimum temperature, 85°C), *Methanococcus igneus* (optimum temperature, 88°C), and *Methanothrix thermophila* (optimum temperature 60°C) are examples of extremophiles.
Many studies have shown evidence that two major pathways for methane formation exist in the terminal anaerobic reaction (Eqs. 2.13 - 2.17, Table 2.1). In one pathway, carbon dioxide reduction by hydrogen to form methane and water is mediated by hydrogenotrophic methanogens and in the second, acetate conversion by the reduction of the methyl group to methane and oxidation of the carboxyl group to carbon dioxide is mediated by aceticlastic methanogens [Baresi et al., 1978; Smith and Mah, 1965].

The aceticlastic methanogenic population grows much more slowly (doubling time of a few days) when compared to acid-forming bacteria (doubling time of a few hours) [Bitton, 1994]. However, aceticlastic methanogens grow approximately 5 to 10 times slower than hydrogenotrophic methanogens because the free energy of reaction for
Acetate conversion to methane and carbon dioxide is less than that for the reduction of carbon dioxide to methane and water. The biomass yield per unit of chemical oxygen demand (COD) substrate consumed is less when compared to hydrogenotrophic methanogens.

Table 2.1: Anaerobic degradation reactions and free energies.

<table>
<thead>
<tr>
<th>Example Hydrolytic Reactions</th>
<th>ΔGo° (kJ·mole⁻¹)</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactose + H₂O → α-D-galactose + α-D-glucose</td>
<td>-106.5</td>
<td>2.1</td>
</tr>
<tr>
<td>β-Maltose + H₂O → 2α-D-glucose</td>
<td>-45.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Sucrose + H₂O → D-fructose + α-D-glucose</td>
<td>-43.6</td>
<td>2.3</td>
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</table>

<table>
<thead>
<tr>
<th>Example Acidogenic Reactions</th>
<th>ΔGo° (kJ·mole⁻¹)</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₆H₁₂O₆ + 4 H₂O → 2 CH₃COO⁻ + 2 HCO₃⁻ + 4 H₂ + 4 H⁺</td>
<td>-206.0</td>
<td>2.4</td>
</tr>
<tr>
<td>C₆H₁₂O₁₀ + 5 H₂O → CH₃CH₂COO⁻ + 3 HCO₃⁻ + 5 H₂ + 4 H⁺</td>
<td>-177.9</td>
<td>2.5</td>
</tr>
<tr>
<td>C₆H₁₂O₆ → CH₃CH(OH)COO⁻ + 2 H⁺</td>
<td>-198.5</td>
<td>2.6</td>
</tr>
<tr>
<td>C₆H₁₂O₆ + 2 H₂O → CH₃(CH₂)₂COO⁻ + 2 HCO₃⁻ + 2 H₂ + 3 H⁺</td>
<td>-253.8</td>
<td>2.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Example Acetogenic Reactions</th>
<th>ΔGo° (kJ·mole⁻¹)</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃CH₂COO⁻ + 3 H₂O → CH₃COO⁻ + HCO₃⁻ + H⁺ + 3 H₂</td>
<td>357.6</td>
<td>2.8</td>
</tr>
<tr>
<td>CH₃CH(OH)COO⁻ + 2 H₂O → CH₃COO⁻ + HCO₃⁻ + H⁺ + 2 H₂</td>
<td>277.2</td>
<td>2.9</td>
</tr>
<tr>
<td>CH₃(CH₂)₂COO⁻ + 2 H₂O → CH₃COO⁻ + H⁺ + 2 H₂</td>
<td>48.3</td>
<td>2.10</td>
</tr>
<tr>
<td>CH₃CH₂OH + H₂O → CH₃COO⁻ + H⁺ + 2 H₂</td>
<td>9.6</td>
<td>2.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Example Methanogenic Reactions</th>
<th>ΔGo° (kJ·mole⁻¹)</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃COO⁻ + H⁺ → CO₂ + CH₄</td>
<td>-27.5</td>
<td>2.13</td>
</tr>
<tr>
<td>CO₂ + 4 H₂ → CH₄ + 2 H₂O</td>
<td>-139.1</td>
<td>2.14</td>
</tr>
<tr>
<td>4 CH₃OH → CH₄ + CO₂ + 2 H₂O</td>
<td>-544.8</td>
<td>2.15</td>
</tr>
<tr>
<td>CH₂OH + H₂ → CH₄ + H₂O</td>
<td>-149.8</td>
<td>2.16</td>
</tr>
<tr>
<td>4 CHOOCO⁻ + 2H⁺ → CH₄ + CO₂ + 2 HCO₃⁻</td>
<td>-302.6</td>
<td>2.17</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Example Sulfidogenic Reactions</th>
<th>ΔGo° (kJ·mole⁻¹)</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃COO⁻ + SO₄²⁻ → HS⁻ + 2HCO₃⁻</td>
<td>-47.6</td>
<td>2.18</td>
</tr>
<tr>
<td>CH₃CH₂COO⁻ + ½ SO₄²⁻ → CH₃COO⁻ + HCO₃⁻ + ½ HS⁻ + ½ H⁺</td>
<td>-37.7</td>
<td>2.19</td>
</tr>
<tr>
<td>CH₂CH₂CH₂COO⁻ + ½ SO₄²⁻ → 2 CH₃COO⁻ + ½ HS⁻ + ½ H⁺</td>
<td>-27.8</td>
<td>2.20</td>
</tr>
<tr>
<td>SO₄²⁻ + 4H₂ + H⁺ → HS⁻ + 4H₂O</td>
<td>-151.9</td>
<td>2.21</td>
</tr>
</tbody>
</table>

ΔGo° is the free energy for the reactor under standard conditions (temperature, 237ºK; pressure, 1.0 atm, pH, 7.0 and products at 1 M [Thauer, 1977])

Approximately 2/3 of the methane produced in an efficiently operating anaerobic reactor is derived from acetate, whereas the remainder is derived from hydrogen and carbon dioxide [Smith and Mah, 1965]. Equilibrium between the acid production rate (hydrolysis and acidogenesis) and acid consumption rate (acetogenesis and methanogenesis) is necessary to maintain approximately neutral conditions. In addition,
a balance between hydrogen production and consumption is necessary to maintain low hydrogen levels. Hydrogenotrophic methanogens play a major role in this regard by maintaining low partial pressures for the conversion of VFAs and alcohols to acetate [Speece, 1983]. A major operational issue is the lack of a proper balance between the acidogenic and methanogenic populations, which eventually causes VFAs, and hydrogen accumulation to inhibitory levels.

2.2.5 Sulfidogenesis
Sulfidogenesis occurs when SRBs and SO$_4^{2-}$ are present in an anaerobic treatment system. A coexistence between SRBs and MPBs causes them to compete for several electron donors [McCartney and Oleszkiewicz, 1993]. Typical terminal reactions mediated by SRB (Eq. 2.18 - 2.21) and MPB (Eq. 2.13 - 2.17), respectively, are shown in Table 2.1. Sulfides released in this process are potent toxins and can be removed from the wastewater by precipitation using heavy metals [El Bayoumy et al., 1999]. Metal sulfide precipitation is widely used by many mining industries for heavy metals removal [Maree et al., 2004].

2.2.6 Anaerobic Reactor Technologies
Anaerobic reactor systems are classified with respect to their organic loading rates. In low rate systems, the load ranges from 0.1 to 2 kg COD m$^{-3}$ d$^{-1}$ and in the high rate systems, the range is from 5 to 25 kg COD m$^{-3}$ d$^{-1}$ [Hall, 1992]. The different types of reactor technologies currently used in the low and high rate reactors are shown in Figure 2.3. Low rate treatment processes are designed to handle influent COD concentrations from 1,000 to 12,000 mg l$^{-1}$ with long HRTs of up to 30 days [Hall, 1992]. High rate systems are operated with higher COD levels and HRTs ranging from 0.1 to 3 days in
comparison to low rate systems. High rate systems are advantageous when land cost is expensive and odors are a nuisance to neighbors.

Various reactor technologies used for hydrogen production, LCFA and sulfate treatment are described in this section. Reactor technologies used for continuous hydrogen production include the following: completely mixed, packed-bed, fluidized-bed, sequencing-continuous reactor, trickling biofilter, and membrane bioreactors [Li and Fang, 2007]. Continuous stirred tank reactor (CSTR) is the most commonly used experimental reactor for hydrogen production [Lin and Chang, 1999; Mizuno et al., 2000; Iyer et al., 2004; Hussy et al., 2005]. In the conventional CSTR, the HRT and solids retention time (SRT) are not decoupled because the microbial culture and liquid are not separated and hence, the SRT is same as the HRT. Suspended microbial microorganisms are a potential limiting factor towards hydrogen production because reactors using these cultures have low suspended solids compared to those with granulated cultures. Recent work by several research groups have demonstrated that high VSS granulated cultures are able to produce hydrogen [Li and Fang, 2007; Chang and Lin, 2004]. Bioreactors operating in an upflow mode and containing granulated cultures are typically categorized as upflow anaerobic sludge blanket (UASB). Packed bed reactors have also been designed to produce hydrogen. In the latter case, the biomass is immobilized onto a stationary support such as in the case of biofilms in a trickling biofilter [Oh et al., 2004a] or entrapped in packed media [Chang et al., 2002].

Another treatment configuration involves the use of membrane to support biological growth [Vallero et al., 2005]. In these reactors, a membrane is used to retain the microbial culture. The latter configuration permits the reactor to operate at high biomass
concentrations and low culture yield. However, attempts at hydrogen production using membrane bioreactors have not gained much success [Oh et al., 2004b].

![Diagram of Anaerobic Treatment Technologies](image)

Legend: 
- Low: 0.1 - 2 kg COD m$^{-3}$ d$^{-1}$
- High: 5 - 25 kg COD m$^{-3}$ d$^{-1}$
- Low/High: 0.1 - 25 kg COD m$^{-3}$ d$^{-1}$

Figure 2.3: Anaerobic treatment process classification [modified after Hall, 1992].

Effluents containing LCFAs have been reported to be treated in UASBs containing suspended growth cultures [Sayed and Zeeuw, 1988] as well as in UASBs containing granulated microorganisms [Sayed et al., 1987]. Other treatment configurations include anaerobic filters [Metzner and Temper, 1990] and anaerobic sequencing batch reactors [Dague, 1992]. For sulfate treatment, reactor technologies which have been used include
the following: a packed bed system [Silva et al., 2002], a UASB [Omil et al., 1998; Omil et al., 1997; Omil et al., 1996; Harada et al., 1994], hybrid reactors (UASB plus packed bed) [Flaherty and Colleran, 1999], upflow anaerobic fixed-film (UAFF) [El Bayoumy et al., 1999] and recently, a membrane bioreactor [Vallero et al., 2005].

2.3 Product Formation and Distribution

VFAs byproducts from carbohydrate degradation generally include acetic, propionic and butyric acids [Zheng and Yu, 2004; Rittman and McCarty, 2001] (Table 2.2). Acetic acid is the most common product from the fermentation of carbohydrates and proteins; propionic acid is mostly formed from carbohydrates; and butyric acid is mainly generated in fermentation carbohydrates [Elefsiniotis and Oldham, 1994a]. Li and Fang [2007] reported acetate and butyrate to be the most common products in the fermentation of carbohydrates. Glucose and other carbohydrates are easy to convert into hydrogen and organic acids [Mizuno et al., 2000]. Several acidogenic byproducts from glucose fermentation are shown in Table 2.2. Notice during glucose degradation, 4.0 and 2.0 mole H₂ mole⁻¹ glucose are produced when acetate (Eq. 2.22) and butyrate (Eq. 2.23) are the byproducts, respectively [Nandi and Sengupta, 1998].

In addition to these reactions, another hydrogen producing reaction (Eq. 2.28) corresponding to 2.0 mole H₂ mole⁻¹ glucose exists [Gaudy and Gaudy, 1980; Hwang et al., 2004]. The formation of lactate, propionate and ethanol byproducts does not lead to hydrogen production (Eq. 2.24, 2.25, and 2.27). Formation of the latter products is undesirable since the carbon structure is reduced and contains electrons that could be used to increase the hydrogen yields [Hawkes et al., 2002; Ueno et al., 2001]. Ethanol is produced (Eq. 2.27) at a low pH (5.0) as an alternative route to acetic acid formation;
however, it is converted to acetic acid and hydrogen (Table 2.3, Eq. 2.33) at low hydrogen partial pressure levels.

Table 2.2: Products from glucose acidification [adapted from IWA, 2002].

<table>
<thead>
<tr>
<th>Product</th>
<th>Reaction</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>C₆H₁₂O₆ + 2H₂O → 2CH₃COOH + 4H₂ + 2CO₂</td>
<td>2.22</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>C₆H₁₂O₆ → CH₃CH₂CH₂COOH + 2H₂ + 2CO₂</td>
<td>2.23</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>C₆H₁₂O₆ + 2H₂ → 2CH₃CH₂COOH + 2H₂O</td>
<td>2.24</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>C₆H₁₂O₆ → 2CH₃CHOHCOOH</td>
<td>2.25</td>
</tr>
<tr>
<td>Propionic and Acetic acid</td>
<td>3C₆H₁₂O₆ → 4CH₃CH₂COOH + 2CH₃COOH + 2H₂O + 2CO₂</td>
<td>2.26</td>
</tr>
<tr>
<td>Ethanol</td>
<td>C₆H₁₂O₆ → 2CH₃CH₂OH + 2CO₂</td>
<td>2.27</td>
</tr>
<tr>
<td>Ethanol and Acetic Acid</td>
<td>C₆H₁₂O₆ + 2H₂O → CH₃CH₂OH + CH₃COOH + 2H₂ + 2CO₂</td>
<td>2.28</td>
</tr>
</tbody>
</table>

Butyric and propionic acid are converted into acetate plus hydrogen under favorable hydrogen levels. Carbon dioxide is produced during the degradation of lactic and propionic acids (Eqs. 2.30 and 2.32). Elevated levels of carbon dioxide are undesirable with respect to the overall hydrogen production process [Tanisho et al., 1998] because they aid in the formation of formate, which consumes an NADH.

Table 2.3: Fermentation reactions of byproducts from glucose acidification [Bagley and Brodkorb, 1999].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Reaction</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid</td>
<td>Propionic acid</td>
<td>CH₃CHOHCOOH + H₂ → CH₃CH₂COOH + H₂O</td>
<td>2.29</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>Acetic acid</td>
<td>CH₃CHOHCOOH + H₂O → CH₃COOH + 2H₂ + CO₂</td>
<td>2.30</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>Acetic acid</td>
<td>CH₃CH₂CH₂COOH + 2H₂O → 2CH₃COOH + 2H₂</td>
<td>2.31</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>Acetic acid</td>
<td>CH₃CH₂COOH + 2H₂O → CH₃COOH + 3H₂ + CO₂</td>
<td>2.32</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Acetic acid</td>
<td>CH₃CH₂OH + H₂O → CH₃COOH + 2H₂</td>
<td>2.33</td>
</tr>
</tbody>
</table>
Alcohols generally formed during acidification include ethanol, propanol, butanol, n-propanol and n-butanol [Elefsiniotis and Oldham, 1994b]. The distribution of acids and alcohols is determined by the dissolved hydrogen levels. Low hydrogen partial pressure favors the formation of less reduced acids such as acetic acid and high levels produces a shift towards more reduced organic products such as propionic and lactic acid [Levin et al., 2004; Dabrock et al., 1992]. Evidence from some studies have shown propionic and butyric acids are fermented only when the hydrogen partial pressure is less than $10^{-3.5}$ to $10^{-4.4}$ atm, respectively, and compounds such as ethanol and lactic acid are readily fermented at hydrogen partial pressures 2 to 3 of orders magnitude higher [Fennell et al., 1997]. Hence, the hydrogen partial pressure is an important factor that determines the type of the intermediate products and thus, the final hydrogen production in the anaerobic hydrogen fermentation.

High VFAs concentrations are also inhibitory to hydrogen production. Evidence from some studies has shown that increasing butyrate concentrations lower the hydrogen yield during glucose fermentation [Zheng and Yu, 2005]. Hydrogen yields were decreased significantly in the presence of elevated VFAs levels (25 - 60mM), which was added to cultures [Van Ginkel and Logan, 2005]. These researchers noted that the inhibition caused by VFAs which were produced from a high initial glucose concentration was greater than when the VFAs were added externally. When they examined the selective inhibitory properties of only butyrate, they observed more inhibition to hydrogen production in comparison to acetate.

The actual hydrogen yield in mixed microbial communities is less than the stoichiometry yield because a fraction of the electron equivalence is lost in the formation
of byproducts as well as microbial growth. Hallenbeck and Benemann, [2002] suggest that the stoichiometric yield is achievable only under near equilibrium condition, which implies a slow production rate and a low hydrogen partial pressure. A fraction of the hydrogen is eventually utilized by the other reactions (Eqs. 2.24 and 2.29) [Vavilin et al., 1995].

2.4 Anaerobic Hydrogen Fermentation

In the previous section of the literature review, the anaerobic conversion of organic compounds was described as a continuous breakdown of organic compounds into simple byproducts [Smith and Mah, 1965]. The terminal reaction is controlled by the presence of a terminal electron acceptor. Hydrogen is produced by several intermediate steps and consumed by electron acceptors such as carbon dioxide or sulfate. The microbes involved in the terminal reactions dispose of excess electron equivalences through the activity of hydrogenase enzymes. Several pathways of hydrogen production by dark fermentation are shown in Figure 2.4. Glucose degradation to pyruvate generates adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). Pyruvate further produces acetyl CoA, carbon dioxide and hydrogen with the activities of pyruvate-ferredoxin-oxidoreductase (PFOR) and hydrogenase [Khanal et al., 2004; Hallenbeck and Benemann, 2002; Das and Veziroglu, 2001; Nandi and Sengupta, 1998; Tanisho et al., 1998]. Obligate anaerobes such as Clostridia mediate this conversion (Eq., 2.34) to produce reduced ferredoxin, which is oxidized releasing electrons. In facultative anaerobes such as Escherichia coli, pyruvate can also convert to acetyl-CoA and formate and the enzyme involved is pyruvate:formate lyase (PFL) [Levin et al., 2004; Hallenbeck and Benemann, 2002; Tanisho et al., 1998; Stickland, 1929]. The
formate species is readily converted into carbon dioxide and hydrogen according to Eq., 2.39.

\[ \text{Pyruvate} + \text{CoA} + 2\text{Fd (ox)} \xrightarrow{\text{PFOR}} \text{acetyl-CoA} + \text{CO}_2 + 2\text{Fd(red)} \]  
(2.34)

\[ \text{Acetyl-CoA} \rightarrow \text{Acetyl-phosphate} \]  
(2.35)

\[ \text{Acetyl-phosphate} + \text{ADP} \rightarrow \text{Acetate} + \text{ATP} \]  
(2.36)

\[ \text{Fd(red)} \rightarrow \text{Fd(ox)} + \text{H}_2 \]  
(2.37)

\[ \text{Pyruvate} + \text{CoA} \xrightarrow{\text{PFL}} \text{acetyl-CoA} + \text{Formate} \]  
(2.38)

\[ \text{Formic acid} \rightarrow \text{H}_2 + \text{CO}_2 \]  
(2.39)

During dark fermentation, hydrogen is also generated through NADH oxidation. Residual NADH from the metabolic reactions is re-oxidized, producing \( \text{H}_2 \) and \( \text{NAD}^+ \) (Equation 2.40) [Tanisho et al., 1998]. Therefore, an increase in residual NADH leads to an improvement in the hydrogen yield.

\[ \text{NADH} + \text{H}^+ \rightarrow \text{H}_2 + \text{NAD}^+ \]  
(2.40)

### 2.4.1 Hydrogen fermentation microbiology

In addition to providing a description between the formation of hydrogen and use of hydrogen in the anaerobic bio-degradation systems, accounting for the different types of microorganisms associated with these processes is also important. There are several microbes, which are capable of utilizing electron fluxes. Nandi and Sengupta [1998] and Gray and Gest [1965] categorized anaerobic hydrogen producing microorganisms into two groups: (i) strict anaerobes without cytochrome system (\textit{Clostridia} and \textit{Micrococcini}) (ii) facultative anaerobes (\textit{Escherichia coli}, \textit{Enterobacter}, \textit{Citrobacter}). Amongst the hydrogen-producing bacteria, \textit{Clostridia} and \textit{Enterobacter} have been examined in
numerous studies. *Clostridia* belongs to the genus Clostridium, a group of gram-positive spore forming rod shaped bacteria [Madigan *et al.*, 2000; Nandi and Sengupta, 1998], whereas *Enterobacter* are gram-negative rod shaped bacteria [Li and Fang, 2007].

Several gram-positive bacteria such as *Clostridium pasteurianum* can produce yields up to 1.5 mole H$_2$ mole$^{-1}$ glucose [Brosseau and Zajic, 1982] while a *Clostridium beijerincki* AM21B strain has shown yields to 1.8 to 2.0 mole H$_2$ mole$^{-1}$ glucose [Taguchi *et al.*, 1992]. In studies with another gram-positive microbe, several authors have reported

![Figure 2.4: Pathway of hydrogen production from fermentation of glucose [Adapted from Li and Fang, 2007; Nath and Das, 2004].](image)
a continuous hydrogen yield of 1.3 - 2.0 mole H$_2$ mole$^{-1}$ glucose at pH 6.7, HRT 8 h and 30°C with *Clostridium butyricum* [Kataoka et al., 1997; Suzuki et al., 1980].

Several gram-negative bacteria are also known hydrogen producers. Tanisho et al. [1983] and Tanisho et al. [1998] reported hydrogen yields in an *Enterobacter Aerogenes* E82005 culture maintained at 38°C with values of 0.20 - 0.21 l H$_2$ h$^{-1}$ l$^{-1}$ culture medium at pH 7.0 and 1.58 mole H$_2$ mole$^{-1}$ glucose at pH 6.0. In another culture consisting of a *Aciduric Enterobacter aerogenes* strain HO-39, a yield of 1.0 mole H$_2$ mole$^{-1}$ glucose at a pH 6.5 and 38°C was attained [Yokoi et al., 1995]. Additional studies with *Enterobacter cloacae* IIT-BT 08 have shown higher yields of 2.2 mole H$_2$ mole$^{-1}$ glucose [Kumar and Das, 2000]. Several studies have been also conducted with *Enterobacter aerogenes* HU 101 [Rachman et al., 1997]; *Escherichia coli* [San et al., 2002]; *Rhodopseudomonas palustris* [Oh et al., 2002]; *Enterobacter* sp. BY-29 [Yokoi et al., 2001]. Anaerobic decomposition of formate to hydrogen and carbon dioxide by *Escherichia coli* was extensively researched and the pathway for hydrogen production from formate decomposition by pyruvate: formate lyase (PFL) was confirmed [Stickland, 1929; Stephenson and Stickland, 1932]. Oh et al. [2003] examined hydrogen production from *Citrobacter* sp. Y19 at a temperature range of 25 - 36°C and pH 4.0 - 9.0. These authors reported a maximum yield of 2.49 mole H$_2$ mole$^{-1}$ glucose at 36°C and pH 7.0.

### 2.4.2 Hydrogen production yields

Studies reported on hydrogen yield in different operational conditions using mixed microbial cultures are summarized in Table 2.4. Hydrogen yields in mole H$_2$ mole$^{-1}$ glucose range from 0.7 - 2.7. Many of the reported yields are less than or equal to 2.0 mole H$_2$ mole$^{-1}$ glucose based on the theoretical yield 4.0 mole H$_2$ mole$^{-1}$ glucose [Hussy
et al., 2005; Iyer et al., 2004; Chang and Lin, 2004; Lin and Jo, 2003; Sung et al., 2002; Mizuno et al., 2000, Lin and Chang, 1999]. If acetate is the only volatile fatty acid byproduct from glucose degradation a theoretical yield 4.0 mole $H_2$ mole$^{-1}$ glucose is attainable.

Chowdhury [2005] reported a yield of 2.37 mole $H_2$ mole$^{-1}$ glucose using a 37°C mixed anaerobic culture with an initial pH of 5.0 and fed with 2000 mg l$^{-1}$ linoleic acid (LA). Chowdhury observed a lower yield of 1.55 mole $H_2$ mole$^{-1}$ glucose at a pH of approximately 7.0. In addition, 1.44 and 2.14 mole $H_2$ mole$^{-1}$ glucose were observed when oleic acid (OA) was used as an inhibitor at a pH 7.0 and 5.0, respectively.

Table 2.4: Hydrogen yields from carbohydrates using mixed microbial cultures.

<table>
<thead>
<tr>
<th>Mixed Culture Source</th>
<th>Feedstock</th>
<th>$H_2$ yield (mole $H_2$.mole$^{-1}$ glucose)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSTR that produced $H_2$ from sucrose</td>
<td>Glucose</td>
<td>2.1</td>
<td>Fang &amp; Liu, 2002</td>
</tr>
<tr>
<td>Soybean meal sludge</td>
<td>Glucose</td>
<td>1.4</td>
<td>Mizuno et al., 2000</td>
</tr>
<tr>
<td>Sewage sludge</td>
<td>Glucose</td>
<td>1.7</td>
<td>Lin &amp; Chang, 1999</td>
</tr>
<tr>
<td>Sewage sludge</td>
<td>Glucose</td>
<td>2.0</td>
<td>Park et al., 2005</td>
</tr>
<tr>
<td>Soil culture</td>
<td>Glucose</td>
<td>1.61</td>
<td>Iyer et al., 2004</td>
</tr>
<tr>
<td>Mixed anaerobic culture</td>
<td>Glucose</td>
<td>2.7</td>
<td>Gurukar, 2005</td>
</tr>
<tr>
<td>Mixed anaerobic culture</td>
<td>Glucose</td>
<td>2.37</td>
<td>Chowdhury, 2005</td>
</tr>
<tr>
<td>Digester sludge</td>
<td>Sucrose</td>
<td>0.77</td>
<td>Sung et al., 2002</td>
</tr>
<tr>
<td>Secondary sedimentation tank sludge</td>
<td>Sucrose</td>
<td>1.2</td>
<td>Chang &amp; Lin, 2004</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>Sucrose</td>
<td>1.3</td>
<td>Lin &amp; Jo, 2003</td>
</tr>
<tr>
<td>Sewage sludge</td>
<td>Sucrose</td>
<td>1.7</td>
<td>Lin &amp; Chang, 1999</td>
</tr>
<tr>
<td>Digester sewage</td>
<td>Sucrose</td>
<td>1.9</td>
<td>Hussy et al., 2005</td>
</tr>
<tr>
<td>Sludge compost</td>
<td>Wastewater sugar factory</td>
<td>2.5</td>
<td>Ueno et al., 1996</td>
</tr>
<tr>
<td>Thermophilic anaerobic digester/ compost sludge</td>
<td>Cellulose / sugarbeet water extract</td>
<td>2.4</td>
<td>Ueno, et al., 1995</td>
</tr>
<tr>
<td>Digester sewage</td>
<td>Sugarbeet water extract</td>
<td>1.7</td>
<td>Hussy et al., 2005</td>
</tr>
</tbody>
</table>
In comparison, at 25°C and neutral pH, a lower yield was observed: 1.27 and 1.11 mole H₂ mole⁻¹ glucose with OA and LA, respectively. Concurrently, at pH 5.0 the yields at 25°C were 2.7 and 2.2 mole H₂ mole⁻¹ glucose respectively, with LA and OA as inhibitors [Gurukar, 2005].

2.5 Operational Factors affecting Microbial Hydrogen Production

The product mixture formed by mixed microbial communities is very complex and is influenced by operational factors such as pH, temperature and HRT.

2.5.1 Effects of pH on hydrogen production

In microbial hydrogen production, pH is an important parameter because it affects the enzymatic activities in many metabolic pathways [Hwang et al., 2004; Zheng and Yu., 2004; Lay, 2000]. In glucose fermentation, pH is a parameter that determines whether the anaerobic pathway follows the butyrate or propionate fermentation route [Kim et al., 2004]. A competition for substrate (glucose), by microorganisms and a change in the main fermentation pathway in the various pH ranges was observed [Hwang et al., 2004]. pH is also important parameter, which affects the formation of many acidogenic products that determines hydrogen production. Fermentative bacteria produce hydrogen gas in the acidogenic phase of anaerobic degradation of organic substrates producing organic acids, and the accumulation of the organic acids causes a decrease in pH [Yokoi et al., 1995]. For hydrogen production, and to prevent hydrogen removal by methanogenesis, pH needs to be controlled at an optimal value. Very low pH can inhibit hydrogen production, and at pH values lower than 6.3 and higher than 7.8, methanogenesis decreases or stops [Yokoi et al., 1995].
Researchers have found that the optimum pH for hydrogen production and formation of appropriate VFAs are quite low, ranging between 5.0 - 5.7 [Hwang et al., 2004; Kim et al., 2004; Khanal et al., 2004; Fang and Liu, 2002; Van Ginkel and Sung, 2001]. Several hydrogen production studies at varying pH values are described in this section.

In a pH study conducted by Yokoi et al. [1995] at 30°C, the hydrogen yield increased with a rise in pH and remained constant at pH more than 4.5. Also, an increase in the hydrogen evolution rate was observed with an increase in pH and the rate was highest between 6.0 - 7.0. The researchers noted the culture was able to produce hydrogen at pH 4.0 but the yields were extremely low. According to Hwang et al. [2004], a pH at 4.0 is regarded as the operational limit for hydrogen production. Hwang et al. [2004] reported butyrate, ethanol and propionate byproducts in the pH ranges 4.0 - 4.5, 4.5 - 5.0 and 5.0 - 6.0, respectively from glucose as the carbon source. They also suggested that the hydrogen utilizing methanogens (hydrogenotrophic methanogens) are more tolerant than the other methanogens at pH 5.0. The maximum hydrogen yield was 200 ml H\textsubscript{2} g\textsuperscript{-1} glucose at pH 5.0 and the hydrogen production decreased with an increase or decrease in pH of 5.0.

Evidence by Kim et al. [2004] supported the conclusions by Hwang et al. [2004]. They also concluded that hydrogen production reactors must be operated at pH 5.0 or below to control the formation of propionate and promote butyrate type fermentation, because unlike propionate, butyrate type fermentation can generate hydrogen. The studies conducted by Kim et al. [2004] were performed at pH 4.5 and a HRT of 9 days in a semi-continuous reactor. At the 9 day HRT, the hydrogen yield was low (35 ± 5 ml H\textsubscript{2} g\textsuperscript{-1} glucose) and the primary product was butyrate. However, at pH 4.3, hydrogen
production ceased and the culture produced butanol. They also reported that the aceticlastic methanogens were inhibited at pH 4.5 but the hydrogenotrophic methanogens were still active. Kim et al. [2004] suggest that pH is considered more effective in affecting the anaerobic pathway compared to the inhibition of methanogens.

At an optimal pH of 5.5, a hydrogen yield of 2.1 mole H₂ mole⁻¹ glucose (64 % of hydrogen in biogas) was reported by Fang and Liu [2002]. They investigated a pH range of 4.0 - 7.0 at 0.5 pH increments and the hydrogen content increased from 40 % at pH 4.0 to 64 % at pH 5.5. A further increase in pH drastically lowered the hydrogen content and at pH 7.0 the hydrogen level was 35 %. No methane was observed at pH 5.5; however, the methane content increased from 3 % at pH 6.0 to 9 % at pH 7.0, accompanied by the decrease of hydrogen content.

The effects of two pH levels (6.2 and 7.5) on a heat-treated (HT) and a non heat-treated (NHT) inoculum were examined by Oh et al. [2003]. They reported a high percentage hydrogen yield at 6.2 pH for the heat-treated culture and the conversion efficiencies based on the theoretical yield of 4.0 mole H₂ mole⁻¹ glucose were as follows: 1) 24.2 % (HT, pH = 6.2); 2) 18.5 % (HT, pH = 7.5); 3) 14.9 % (NHT, pH = 6.2) and 4) 12.1% (NHT, pH 7.5).

Khanal et al. [2004] reported the effect of pH and intermediate products on hydrogen production from starch and sucrose in batch tests. After varying the initial pH from 4.5 to 6.5, they determined that the optimal operating pH range was between 5.5 - 5.7. However, at a higher initial pH value of 6.0, rapid hydrogen production was accompanied with a rapid VFA production to inhibitory levels. These authors observed that acidic conditions inhibited the hydrogen-producing microorganisms.
The effect of initial pH (from 4.0 - 9.0 with 1.0 increments) at 55°C with a sucrose
containing wastewater was examined by Zhang et al. [2003]. This study demonstrated
that no hydrogen was produced at pH 4.0. The hydrogen yield increased with initial pH
from 5.0 to 6.0 and then decreased as pH further increased from 6.0 to 9.0. A maximum
of 17 % (92 ml H₂ g⁻¹ cellulose) of theoretical yield was achieved at pH 6.0. The final pH
values ranged between 4.0 - 4.6 regardless of the initial pH, and acidified products
mainly consisted of acetate (50.0 - 53.4 %) and butyrate (26.0 - 31.6 %). Hence, based on
the above reports, researchers have found that the pH range to optimize hydrogen
production is quite low, from 5.0 - 6.0.

2.5.2 Effects of temperature on hydrogen production

Temperature is a crucial parameter in the optimization of hydrogen production using
microbial cultures. Optimum microbial growth is accomplished at temperature ranges
within the mesophilic (30 - 37°C) and thermophilic (40 - 85°C) regions [Rittmann and
McCarty, 2001]. The temperature also affects the rate of enzyme catalyzed reactions,
whether the temperature is suited for mesophilic or thermophilic growth [Rittman and
McCarty, 2001], as well as the dissolution of the gaseous products in the liquid medium.

In studies describing the effects of temperature on hydrogen production, increasing
yields are observed as the temperature increases. Extreme thermophilic conditions were
examined in the production of hydrogen from a dairy cow waste slurry (13.4 g VSS l⁻¹)
by batch cultures in a temperature range from 37 to 85°C (37, 50, 55, 60, 67, 75 and
85°C), using microflora naturally present within the slurry [Yokoyama et al., 2007].
They reported two peaks of fermentation temperatures, 60°C (392 ml H₂ l⁻¹ on day 4) and
75°C (248 ml H₂ l⁻¹ on day 6). No hydrogen consumption was detected in the microbial
cultures at 75°C for 24 days. The authors noted the total quantity of hydrogen produced was low between 37°C to 50°C; however, the levels increased substantially above 50°C. Butyrate and acetate were the two dominant VFAs under all the temperature conditions examined.

Youn and Shin [2005] examined the effect of temperature in a two-phased reactor system. These researchers compared a conventional mesophilic two-phased (CMTP) process at 35°C to a temperature-phased (TP) process, which consisted of a thermophilic-acidogenic fermenter at 55°C followed by a mesophilic-methanogenic fermenter at 35°C. The TP at 55°C process was free of methane with the biogas comprising mostly of hydrogen and carbon dioxide, and acetate and n-butyrate were the main VFAs produced. The CMTP process at 35°C resulted in a low percent of hydrogen in the biogas stream compared to methane. Likely, the nature of the organic substrate and pulping chemicals affected the hydrogen yield. In studies reported by Shin et al. [2004], the quantity of hydrogen produced from a thermophilic acidogenic culture at 55°C was significantly greater than that from the mesophilic culture at 35°C at several pH conditions. Evidence from these authors showed no methane was detected in the biogas stream and negligible amounts of propionate were observed in the thermophilic cultures. They noted the low hydrogen production from the mesophilic cultures, which was attributed to the higher production of propionate and methanogenesis. For heat-treated inocula at 105°C for 2.0 h, the hydrogen yield was greater for high temperature conditions at pH 5.5 and 10-h HRT [Iyer et al., 2004]. Iyer et al. [2004] reported a yield of 1.8 mole H₂ mole⁻¹ glucose and 1.6 mole H₂ mole⁻¹ glucose at 30°C and 37°C, respectively.

2.5.3 Effects of HRT on hydrogen production
Besides pH and temperature, HRT also affects the growth of microorganisms and the distribution pattern of byproducts in hydrogen producing reactors. Hence, HRT is a parameter, which must be controlled in a bioreactor fermenting a particular substrate type. A longer HRT may result in methane generation, which is not desirable in the hydrogen reactor [Iyer et al., 2004] and a shorter HRT may wash out cells and result in less substrate degradation and therefore low hydrogen yield [Li and Fang, 2007]. Several studies have reported the effects of HRT on hydrogen production. Many researchers suggest different optimum HRTs because of its dependence on several factors such as substrate used and type of the reactor. Most reported optimal HRT values for glucose and sucrose range between 3-8 h [Li and Fang, 2007].

According to the evidence by Iyer et al. [2004], greater hydrogen yields were observed in continuous flow reactors operating under a 10 h HRT condition and inoculated with a heat-treated soil culture. The hydrogen production rate and yield were 436 ml h\(^{-1}\) and 1.61 mole H\(_2\) mole\(^{-1}\) glucose, respectively for the 10 h HRT condition. In the latter study, the hydrogen production rate increased approximately 5-fold when the HRT was decreased 3-fold from 30-h to 10 h. The rRNA gene sequence diversity at 30 h HRT was more diverse than at 10 h. Bacillaceae, Clostridiaceae, and Enterobacteriaceae were detected at 30 h HRT, while only Clostridiaceae were detected at 10 h HRT.

In addition to this, there are several studies reported in Table 2.5 on optimization of HRT using CSTR and packed-bed reactors (PBRs). The optimum HRT varies between 5.4 h and 6.0 h for the 3 studies reported on hydrogen production using CSTR and glucose as feedstock at mesophilic conditions.
Two optimal HRT values for studies using starch effluents were conducted at 5 h [Hussy et al., 2003] and 17 h [Lay, 2000]. Longer HRT operating conditions are required for starch degradation due to its slow hydrolysis. In comparison, a 12 h optimum HRT (examined in the range of 7-72 h) was reported for a sugar (non-starch) factory wastewater in a CSTR [Ueno et al., 1996].

Table 2.5: Optimum HRT from hydrogen production studies [Li ad Fang, 2007]

<table>
<thead>
<tr>
<th>Feedstock</th>
<th>pH</th>
<th>Temp. (°C)</th>
<th>HRT Range (h)</th>
<th>Opt. (h)</th>
<th>Reactor Type</th>
<th>Yield (ml H₂ g⁻¹ hexose)</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Glucose</td>
<td>5.5</td>
<td>30</td>
<td>10, 30</td>
<td>10</td>
<td>CSTR</td>
<td>245</td>
<td>Iyer et al., 2004</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.7</td>
<td>35</td>
<td>6-48</td>
<td>6</td>
<td>CSTR</td>
<td>231</td>
<td>Lin and Chang, 1999</td>
</tr>
<tr>
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<td>6.0</td>
<td>37</td>
<td>3.0-12.5</td>
<td>5.4</td>
<td>CSTR</td>
<td>-</td>
<td>Horiuchi et al., 2002</td>
</tr>
<tr>
<td>Glucose</td>
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<td>37</td>
<td>20-50</td>
<td>20</td>
<td>PBR</td>
<td>-</td>
<td>Kim et al., 2003</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.2</td>
<td>15-34</td>
<td>6-60</td>
<td>6</td>
<td>CSTR</td>
<td>193</td>
<td>Lin and Chang, 2004</td>
</tr>
<tr>
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<td>26</td>
<td>4.6-28.6</td>
<td>13.7</td>
<td>CSTR</td>
<td>256</td>
<td>Fang and Liu, 2004</td>
</tr>
<tr>
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<td>39</td>
<td>2-30</td>
<td>3</td>
<td>PBR</td>
<td>186</td>
<td>Mu and Yu, 2004</td>
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<td>17</td>
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<td>Lay, 2000</td>
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<td>Wheat starch</td>
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<td>CSTR</td>
<td>254</td>
<td>Hussy et al., 2003</td>
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<tr>
<td>Sugar factory waste-water</td>
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<td>60</td>
<td>12-72</td>
<td>12</td>
<td>CSTR</td>
<td>343</td>
<td>Ueno et al., 1996</td>
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</table>

2.6 Methanogenic Inhibition

Hydrogen is an intermediate product in anaerobic communities and is produced by acidogenic and acetogenic microorganisms. Hydrogen is readily consumed by hydrogenotrophic methanogens to produce methane (Figure 2.1). Hence, a mechanism to prevent the electron flow towards hydrogenotrophic methanogenesis is desirable when
hydrogen is the required end product or when electron equivalents from hydrogen need to be used by the other processes such as sulfate reduction.

Using physical (heat treatment and pH effects) and chemical methods to control the electron flow in mixed anaerobic communities has been described in several studies. Inhibiting the growth and activity of hydrogenotrophic methanogens by changing pH conditions has demonstrated compelling evidence of methanogenic growth control in the short-term. However, to date no research has demonstrated an effect on long-term sustained growth control. Over the long-term, after the stress is relieved, microbial growth is expected to resume, as conditions become favorable [Oh et al., 2003; Sung et al. 2002; Fang and Liu, 2002].

2.6.1 Heat Treatment

The coexistence of hydrogen consuming microorganisms is one difficulty associated with hydrogen production using mixed microbial communities. Heat treatment of seed inoculum as a method to inactivate or eliminate these microorganisms is one technique adopted by many researchers. Many of the hydrogen producing bacteria for example, *Clostridium* and *Bacillus* species form endospores when unfavorable environmental conditions (high temperature, lack of carbon or nitrogen source and, chemical toxicity) are encountered and with the return of favorable conditions, the spores germinate and become active [Sung et al., 2002]. Many studies examining the effect of heat treatment of seed inoculum have indicated that hydrogen production is promoted by eliminating non-spore forming hydrogen consuming microorganism and by selecting for hydrogen producing spore forming bacteria [Van Ginkel et al., 2001; Van Ginkel and Sung, 2001; Sung et al., 2002]. Sung et al. [2002] heat treated an inoculum at 100°C for 15 minutes,
repeated the heat treatments during the course of experimentation, and reported a yield of 1.5 mole H₂ mole⁻¹ sucrose at pH 5.5 and 37°C. Van Ginkel et al. [2001] used dry heat treatment of compost and soil cultures by baking at 104°C for 2.0 h, whereas Lay [2000] and Okamoto [2000] used a wet heat treatment of anaerobic digester sludge by boiling for 15 minutes. Oh et al. [2003] reported a maximum yield of 0.97 mole H₂ mole⁻¹ glucose for a heat-treated culture and 0.6 mole H₂ mole⁻¹ glucose for the non heat-treated cultures, which were maintained at pH 6.2.

2.6.2 Chemical Inhibitors

Microbial inhibition of selective anaerobic populations and subsequent prevention of electron consumption by hydrogenotrophic methanogens has been reported using a variety of chemicals. Inhibition of methane production has been shown using 2-bromoethanesulphonate (BES) [Oremland and Capone, 1988; Scholten et al., 2000]; acetylene [Sparling et al., 1997; Ahring and Westermann, 1987; Bomar et al., 1985; Sprott et al., 1982]; chlorinated methane analogues, such as chloroform, carbon tetrachloride, and methylene chloride [Prins et al., 1972; Bauchop, 1967] and ethylene [Oremland and Taylor, 1975].

Compounds such as BES are selective inhibitors whose interaction is restricted to an enzyme or cofactor unique to a particular class of organism. The specific inhibitor, BES, is an analogue of a cofactor (mercaptoethanesulfonic acid, known as HS-coenzyme M) unique to methanogens and is highly effective in blocking methanogenesis [Gunsalus and Wolfe, 1978]. Coenzyme M (CoM) is associated with the terminal methylation reactions involved in methanogenesis, including the methyl-CoM reductase enzyme complex from which methane is evolved. [Gunsalus and Wolfe, 1978; Gunsalus and Wolfe, 1980]. The
effect of BES was examined as an inhibitor to study the inhibition of methyl-CoM reductase activity in cell-free extracts of *Methanobacterium thermoautotrophicum* [Gunsalus *et al.*, 1978]. Fifty percent inhibition of methane production occurred at levels reaching $5 \times 10^{-4}$ M and total inhibition occurred at approximately $10^{-5}$ M. Smith and Mah [1978] reported that the growth of *Methanosarcina* strain 227 on acetate was blocked by 70 μM BES. BES is known to function as a competitive inhibitor of the methyl-CoM reductase complex.

The quantity of inhibitor added is dependent on a particular culture and this is a major application problem. The cost of BES and its discharge to the environment could pose major disadvantages in full-scale applications. Smith and Mah [1981] reported that the spontaneous mutants of *Methanosarcina* strain 227 were resistant to 0.24 mM BES when previously exposed to levels of 0.024 mM, and a strain of *Methanobacterium formicicum* was found to be resistant to 0.2 mM BES without any prior exposure. Resistance to BES was conferred to impermeability to the compound because the cell-free extracts of resistant mutants were susceptible to BES inhibition of methyl-CoM-reductase [Smith, 1983]. While, Bouwer and McCarty [1983] speculated that the partial inhibition may have been due to the resistant mutants or degradation of BES in their work. Fixed film acetate digesters were partially inhibited (41%) when exposed to 0.6 mM BES.

Acetylene is not a specific inhibitor as it inhibits a number of different microbes (such as those containing nitrogenase activities). Oremland and Taylor [1975] reported inhibition of methanogenesis by acetylene and the inhibition could not be reversed after gassing with nitrogen. Low levels of acetylene (8 μM) inhibited growth of *Methanospirillum hungatei* [Sprott *et al.*, 1982]. However, in the latter study, they could
not detect any adverse effect of acetylene on any of the methanogenic enzymes (hydrogenase, methyl-CoM reductase, NADP reductase, or ATP hydrolase); however, a drastic drop in ATP and nickel (Ni) uptake by *Methanospirillum hungatei*, when exposed to acetylene, was observed. Sprott *et al.* [1982] concluded that acetylene disrupted the transmembrane pH gradient, thereby blocking methanogenesis, the formation of a proton flux and nickel uptake.

One major problem associated with acetylene is that it also affects several microorganisms. However, few studies have reported its effects on sulfidogenesis. Growth and respiration by *Desulfovibrio desulfuricans* were strongly inhibited by an atmosphere of 20% acetylene, though lower amounts caused only partial inhibition [Payne and Grant, 1982]. No significant disruption occurred for *Desulfotomaculum ruminis* thus, implying that there is a difference among genera of sulfate respiring bacteria with respect to their susceptibility to acetylene. Also, Oremland and Capone [1988] reported that sulfate linked uptake of molecular hydrogen by marine sediments was uninfluenced by the presence of acetylene. Thus, the general functions of the sulfate reducing biota may be unaffected by acetylene even though growth of some individual species may be impeded.

Chlorinated methane analogues, such as chloroform and carbon tetrachloride have demonstrated inhibition of pure cultures at micromolar levels [Prins *et al.*, 1972]. According to Gunsalus and Wolfe [1978], chloroform inhibits methyl-Coenzyme M reductase.
Ethylene inhibitory effects on methanogenesis have also been reported in marine sediments [Oremland and Taylor, 1975] and pure cultures [Schink, 1985]. Inhibition by ethylene is reversible and hence, methanogenesis can resume upon removal of the gas.

Unlike other eubacteria, methanogens differ in their cellular wall composition as their cell walls are composed of pseudomurein, protein subunits [Kandler and Hippe, 1977], or heteropolysaccharides [Kandler and Hippe, 1977]. Thus, methanogens are not susceptible to a variety of antibiotics that disrupt cell wall synthesis in eubacteria, such as penicillin [Oremland and Capone, 1988].

2.6.3 Long chain fatty acids

2.6.3.1 Fate and transport of LCFAs in microbial cells

LCFA are degraded by hydrogen producing acetogens to acetate via a β-oxidation mechanism [Weng and Jeris, 1976]. Prior to β-oxidation, LCFAs must enter the cell via uptake systems, which translocate them across the membrane [Maloy, 1981]. Nunn [1986] examined the uptake of LCFAs in E. coli, a gram-positive bacterium, and proposed a model for LCFA transport across the membrane. LCFAs traverse the cell membrane, and are activated by acyl-CoA synthetase prior to undergoing β-oxidation.

LCFAs are important substrates for mammalian cell growth, and hence, cell physiologists and biochemists [Mangroo et al., 1995] have researched LCFA transport mechanism extensively. LCFAs are used for energy storage (triglyceride synthesis in mammalian cells), lipid synthesis (in prokaryotic and eukaryotic cells) and energy production via β-oxidation (in prokaryotic and eukaryotic cells). Mangroo et al. [1995] used E. coli as a model to investigate LCFA uptake mechanism across the cell membrane.
The uptake of LCFA by *E. coli* occurs through four steps [Mangroo et al., 1995]. A schematic membrane transport mechanism is shown in Figure 2.5. FadL, a transversal outer membrane protein mediates LCFA movement across the membrane. Initially, ionized LCFA bind to FadL and are after that transported across the peptidoglycan layer into the periplasm. The transport mechanism across the peptidoglycan layer has not been revealed, but a protein may be involved in mediating LCFA transfer across the layer.

Medium-chain fatty acids (C7-C11)

Long-chain fatty acids (C12-C18)

**Figure 2.5:** Proposed model of fatty acid transport in *E coli*. LCFAs (C12 - C18) traverse the outer membrane via a membrane protein (FadL). MCFAs (C7 - C11) traverse the outer membrane via FadL and via a diffusion process. Fatty acid becomes activated by acyl-CoA synthetase (ACS) protein to form long chain fatty acid-CoA. OM = outer membrane; PG = peptidoglycan; PS = periplasmic space; IM = inner membrane; FadL = membrane protein; ACS = acyl synthase CoA. [Adapted from Nunn, 1986].

Tsp, a protein facilitate binding and releasing of LCFAs across the periplasmic space [Azizan and Black, 1994]. After passing through the peptidoglycan layer, LCFAs are protonated in the periplasmic space and diffuse to the inner membrane. Further, the protonated LCFA molecule anchors to another proposed transversal inner membrane protein on the outer part of the inner membrane adjacent to the periplasmic space for
activation by ATP. Finally, on the inside of the inner membrane, acyl-CoA synthetase activates free LCFAs into long chain acyl-CoA complexes using ATP.

Before entering the cells, LCFAs may exert antibacterial effects in gram-negative bacteria (not capable of oxidizing LCFAs) by disrupting several membrane components and inactivating many energy-linked reactions. For example, they interfere with K⁺, Na⁺ regulator proteins and other cell proteins involved, responsible for maintaining cell homeostasis [Cherrington et al., 1991]. LCFAs also act as membrane disrupting agents causing protein or ion leakage in gram-positive bacteria [Greenway and Dyke, 1979; Galbraith and Miller, 1973]. Also, unsaturated LCFAs adhere to the walls of the bacterial cell by adsorbing on the cell wall surface, which alters the cell’s permeability and limits or impedes the passage and transport of important nutrients [Hwu et al., 1998; Rinzema et al., 1994; Sayed et al., 1987; Henderson, 1973; Demeyer and Henderickx, 1967]. Evidence from Pires et al. [2001] has shown that LCFAs adsorbs onto cellular surfaces of active as well as inactive (dead) anaerobic cultures. The VSS concentration is determined using a method which measures the mass of both the active and dead microorganisms and hence, using the LCFA/VSS ratio as a measure to quantify the inhibitor levels in these studies would be misleading [Pires et al., 2001].

After entering the cytoplasm, carboxylic acids dissociate causing acidification of the cytoplasm, and the overall impact is a reduction in the net pH across the membrane, reducing the electro-motive potential. In the cell, most enzymes become inactivated and lose their activity at a pH value less than 7 [Madigan et al., 2000]. The presence of organic acids also affects the synthesis of macromolecules such as DNA, RNA, proteins and lipids [Cherrington et al., 1991]. The sensitivity of individual biosynthetic pathway
depends upon the bacterium and the acid. No information is available on the impact of LCFAs on macromolecular synthesis. Although, shorter chain fatty acids such as formic and propionic cause substantial disruption of DNA, RNA and lipid synthesis in *E. coli* [Cherrington *et al.*, 1991].

### 2.6.3.2 Effects on anaerobic microorganisms

LCFAs such as, LA (C18:2), OA (C18:1) and SA (C18:0) are inhibitory to several anaerobic populations. These chemicals are known inhibitors of gram-positive bacteria and yeast, while they exert no effect on the gram-negative bacteria [Nieman, 1954]. Methanogens have cell wall similar to gram-positive bacteria [Madigan *et al.*, 2000] and hence, are subjected to LCFA inhibition.

Several researchers have studied the anaerobic degradation of LCFA and the effects of LCFAs on different microbial degradation processes [Lalman and Bagley, 2002; Lalman and Bagley, 2001; Alves *et al.*, 2001; Weng and Jeris, 1976; Novak and Carlson, 1970]. LCFAs are derived from lipids and fats, which in turn are produced from edible oil refineries, slaughterhouse, and dairy products industries [Kramer, 1971]. Lipids are readily hydrolyzed to LCFAs and glycerol (Eq. 2.41: R₁, R₂ and R₃ are the alkyl groups in this Eq.). As mentioned earlier, LCFAs are degraded by hydrogen producing acetogens to acetate via a β-oxidation mechanism [Weng and Jeris, 1976].

\[
\begin{align*}
\text{Neutral fat} & \rightarrow \text{long chain fatty acids} + \text{glycerol} \\
\text{CH}_2\text{OCOR}_1 + 3\text{H}_2\text{O} & \rightarrow \text{R}_1\text{COOH} + \text{CH}_2\text{OH} \\
\text{CHOCOR}_2 & \rightarrow \text{R}_2\text{COOH} + \text{CHOH} \\
\text{CH}_2\text{OCOR}_3 & \rightarrow \text{R}_3\text{COOH} + \text{CH}_2\text{OH}
\end{align*}
\]
The degradation products from each step are acetate, hydrogen and a LCFA with a reduction of two carbons in the alkyl group (Eq. 2.42). The β-oxidation reactions proceed until all the LCFA is converted to acetate. Acetate is utilized by the aceticlastic methanogens to produce methane (if methanogens are uninhibited by LCFA) or other microorganisms such as sulfate reducers in the absence of methanogens for their terminal product reduction [Weng and Jeris, 1976; Novak and Carlson, 1970]. As an example β-oxidation of LA is shown in Eq. 2.43.

\[
\text{CH}_3\text{(CH}_2\text{)}_n\text{COOH} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{(CH}_2\text{)}_{n-2}\text{COOH} + \text{CH}_3\text{COOH} + 2\text{H}_2 \quad (2.42)
\]

\[
\begin{align*}
\text{C}_{18}\text{H}_{31}\text{O}_2^- + \text{H}_2 & \rightarrow \text{C}_{18}\text{H}_{33}\text{O}_2^- + \text{H}_2 \\
\text{C}_{18}\text{H}_{33}\text{O}_2^- + 2\text{H}_2 & \rightarrow \text{C}_{16}\text{H}_{31}\text{O}_2^- + \text{C}_2\text{H}_3\text{O}_2^- + 2\text{H}_2 + \text{H}^+ \\
\text{C}_{14}\text{H}_{27}\text{O}_2^- + \text{C}_2\text{H}_3\text{O}_2^- + 2\text{H}_2 + \text{H}^+ & \rightarrow 2\text{H}_2 + \text{H}^+ \\
\text{Several β-oxidation steps} & \\
\text{C}_2\text{H}_3\text{O}_2^- + 2\text{H}_2 + \text{H}^+ & \quad (2.43)
\end{align*}
\]

The number of acetate formed at the end depends upon the pathway adopted for degradation of the LCFA [Roy et al., 1986]. Biodegradation of LCFAs proceed via several steps including adsorption onto the cell surface, movement across the cell membrane and LCFA conversion to lower molecular weight components. Two steps involved in degradation are hydrogenation and oxidation as shown in Eq. 2.43. Researchers have reported that complete saturation of LCFAs is not necessary before β-oxidation [Lalman and Bagley, 2000; Canovas-Diaz et al., 1991]. Canovas-Diaz et al. [1991] reported unsaturated LCFA byproducts during OA degradation, which was supported by Lalman and Bagley [2001], who reported that the production of shorter chain LCFAs from OA and LA is more energetically favorable than from SA, and hence
supporting the hypothesis that β-oxidation of unsaturated C18 LCFAs can occur directly. However, according to Novak and Carlson [1970] only saturated LCFAs can enter the β-oxidation pathway. If complete LCFA saturation is required prior to β-oxidation, SA may go to β-oxidation directly and unsaturated acids such as LA (C18:2) and OA (C18:1) would require hydrogenation prior to β-oxidation [Novak and Carlson]. Hence, it is unclear whether complete LCFA double bond saturation is necessary before β-oxidation. Lalman and Bagley [2001] reported palmitic (C16:0) and mysiric (C14:0) acid byproducts from anaerobic degradation of OA at 21°C using a culture unacclimated to LCFAs. However, no LCFA by-products were detected when SA was fed to anaerobic cultures. Their findings are similar to the findings of previous studies on the anaerobic degradation of OA and SA [Novak and Carlson, 1970; Angelidaki and Ahring, 1995].

Although degradable, LCFAs are inhibitory to the activity of many anaerobic microorganisms. Koster and Cramer [1987] observed significant aceticlastic inhibition with OA concentrations ranging from 300 - 1500 mg l⁻¹ at 30°C; however, the effect on hydrogenotrophic methanogens was not examined. At 55°C and in cultures receiving 100 -1000 mg l⁻¹ of OA and greater than 300 mg l⁻¹ of SA, aceticlastic methanogens are inhibited [Angelidaki and Ahring, 1995]. Angelidaki and Ahring [1995] reported, the inhibitory effect is concentration dependent, and which is supported by several researchers [Lalman and Bagley, 2001; Hwu et al., 1998; Koster and Cramer, 1987]. In studies using greater than 30 mg l⁻¹ OA, aceticlastic methanogenic inhibition was detected [Lalman and Bagley, 2001]; however, SA (10 - 100 mg l⁻¹) inhibition was negligible.
The effect of LCFA on hydrogenotrophic methanogens was accessed by many researchers [Lalman and Bagley, 2000; Lalman and Bagley, 2001; Hanaki et al., 1981; Demeyer and Henderickx, 1967]. At 21°C, hydrogenotrophic methanogens were inhibited by OA and SA; however, the extent of inhibition was less than that observed for aceticlastic methanogens [Lalman and Bagley, 2001]. Additionally, linolenic acid (C_{18:3}) also inhibited hydrogenotrophic methanogens at 39°C [Demeyer and Henderickx, 1967]. Lalman and Bagley [2000] also reported the effect of LA on aceticlastic and hydrogenotrophic methanogens and found more than 30 mg l^{-1} LA to be completely inhibitory to acetogenic activity and hydrogenotrophic methanogens were slightly inhibited by LA (10 - 100 mg l^{-1}).

The extent of inhibition due to LCFAs is known to increase with the carbon chain length as well as an increase in the number of double bonds. For example, C18 LCFAs, LA and OA bearing carbon double bond are more inhibitory to aceticlastic and hydrogenotrophic methanogens when compared to SA bearing no carbon double bond [Lalman and Bagley, 2002; Lalman and Bagley 2001]. LA acts as a surfactant (surface-active agents) by reducing the interfacial tension between the bacterial membrane and the bulk aqueous phase of the growth medium [Greenway and Dyke, 1979]. Gilby and Few [1960] have demonstrated the lysis of protoplasts by surface-active agents and correlated the lytic activity with antibacterial activity on whole bacterial cells. The ability of a compound such as LA (C_{18:2}) to migrate to cellular surfaces and lower the interfacial tension is related to its surface tension. In comparison to LA, SA (C_{18:0}) was reported not to inhibit growth at 30°C since it is a much poorer surfactant [Greenway and Dyke, 1979]. The soap solubilities of the C18 LCFAs (LA( C_{18:2}), OA (C_{18:1}) and
SA(C18:0)) are approximately equal [Irani and Callis, 1960] and hence, the surfactant property due to the difference in the chemical structure is responsible for high methanogenic inhibition in LCFAs bearing double bond such as LA when compared to SA [Lalman, 2000].

The advantages of using LCFA as an inhibitor and diverting electron fluxes away from methanogens (methanogenic consumption of electron equivalents) are two fold: (i) they are cost effective and readily available and (ii) they are degradable and the electron equivalents from the degradation of LCFAs can be used by other terminal electron acceptors such as sulfate reducers or in the absence of inorganic electron acceptors such nitrate or sulfate hydrogen will accumulate, which is a potential energy carrier.

A hydrogen fuel based economy would likely be less polluting than a fossil fuel based economy since hydrogen is clean, efficient, and renewable [Hansel and Lindblad, 1998]. In addition, hydrogen has a high-energy yield of 122 kJ g\(^{-1}\), which is much greater than that compared to any hydrocarbon fuel [Mizuno et al., 2000; Lay et al., 1999].

2.7 Sulfate reduction in anaerobic bio-degradation

Two types of biological sulfate reduction are possible, assimilatory and dissimilatory. In assimilatory sulfate (SO\(_4^{2-}\)) reduction, hydrogen sulfide (H\(_2\)S) results from the anaerobic decomposition of organic matter containing sulfur amino acids such as methionine, cystein, and cystine, by proteolytic bacteria ( e.g., Clostridia, Vellionella) [Bitton, 1994]. In addition to prokaryotes, sulfate is used as a sulfur source for the biosynthesis of many organisms, including higher plant, and algae. The H\(_2\)S formed is converted into organic sulfur in the form of amino acids [Madigan et al., 2000]. In the case of dissimilatory sulfate reduction, H\(_2\)S is generated, or in other words excreted,
because of sulfate reduction by SRB. The ability to use sulfate as an electron acceptor for an energy generating process involves large-scale reduction of sulfate (e.g., in anaerobic wastewater treatment processes) and is unique to SRBs. Our interest here is dissimilatory sulfate reduction and is discussed below. A few of the sulfate reduction reactions taking place in anaerobic communities are provided in Table 2.1 (Eqs. 2.18 - 2.21).

The reduction of \( \text{SO}_4^{2-} \) to \( \text{H}_2\text{S} \), an eight-electron reduction, proceeds through a number of intermediate stages. The \( \text{SO}_4^{2-} \) ion is stable and cannot be reduced without first being activated. \( \text{SO}_4^{2-} \) is activated by means of ATP and the enzyme ATP sulfurylase catalyzes the attachment of the \( \text{SO}_4^{2-} \) ion to a phosphate of ATP. The latter leads to the formation of adenosine phosphosulfate (APS). The \( \text{SO}_4^{2-} \) moiety of APS is reduced directly to sulfite (\( \text{SO}_3^{2-} \)) by the enzyme APS reductase with the release of adenosine monophosphate (AMP). Once \( \text{SO}_3^{2-} \) is formed, sulfide is formed by the enzyme sulfite reductase as shown in Figure 2.6.

2.7.1 Microbiology of SRB

SRB are a large group of anaerobic bacteria that respire anaerobically with sulfate as electron acceptor, producing hydrogen sulfide (\( \text{H}_2\text{S} \)) [Madigan et al., 2000]. Sulfate is a much less favorable electron acceptor than either \( \text{O}_2 \) or \( \text{NO}_3^- \) (reduction potentials for redox pairs, \( \text{O}_2/\text{H}_2\text{O}: 0.82 \text{ V}; \text{NO}_3^-/\text{NO}_2^-: 0.43 \text{ V}; \text{SO}_4^{2-}/\text{HS}^-: -0.217 \text{ V} \)). Because of the less favorable energetics, growth yields of SRB are lower than those growing on \( \text{O}_2 \) or \( \text{NO}_3^- \). However, sufficient energy to make ATP is available when an electron donor that yields NADH or FADH is used [Madigan et al., 2000].
The different types of electron donors used by SRBs are discussed in section 2.7.2. SRBs constitute a diverse group of prokaryotes that contribute to a variety of essential functions in many anaerobic environments [Castro et al., 2000]. In addition to their importance to the sulfur cycle, SRBs are important regulators for a variety of processes, in wetland soils, biodegradation of chlorinated aromatic pollutants in anaerobic soils and sediments, and sulfate reduction in anaerobic wastewater treatment. SRBs can be organized into the following four distinct groups based on the analysis of ribosomal ribonucleic acid (rRNA) sequences: 1) Gram-negative mesophilic SRB; 2) Gram-positive spore forming SRB; 3) thermophilic bacterial SRB; and 4) thermophilic archaeal SRB. All of these groups are characterized by their use of sulfate as a terminal electron acceptor during anaerobic respiration [Castro et al., 2000].
Over twenty genera of these organisms are known and SRBs belonging to the following genera have since been isolated from environmental samples (anaerobic sludge digesters, aquatic sediments, gastrointestinal tract): *Desulfovibrio* (gram-negative), *Desulfotomaculum* (gram-negative), *Desulfobulbus* (gram-negative), *Desulfomonas* (gram-negative), *Desulfobacter* (gram-negative), *Desulfooccus* (gram-negative), *Desulfonema* (gram-positive), *Desulfosarcina* (gram-negative), *Desulfobacterium*, and *Thermodesulfobacterium* (gram-negative) [Madigan et al., 2000; Bitton, 1994]. Hence, most of the reported anaerobic SRBs are gram-negative bacteria. *Desulfotomaculum* is the only spore-forming genus among SRBs [Madigan et al., 2000; Widdel, 1988].

### 2.7.2 Electron Sources for SRBs

The type of the carbon source utilized for the reduction of an electron acceptor (SO$_4^{2-}$) varies according to genus and the range of electron donors used by SRBs is broad. The preferred carbon source for SRBs are always low-molecular-weight compounds such as organic acids (e.g. lactate, pyruvate, formate, and malate), VFAs (e.g. acetate), and alcohols (e.g., ethanol, propanol, methanol, and butanol) and occasionally sugars and longer chain fatty acids [Hao et al., 1996; Gibson, 1990]. Nearly all of these are fermentation products from the anaerobic degradation of carbohydrates, proteins and lipids. Thus, SRBs are terminal degraders, and their role is analogous to that of methanogenic bacteria that produce methane and carbon dioxide as final products.

Based on their substrate utilization, SRBs can be broadly categorized into, non-acetate oxidizers and acetate oxidizers. The non-acetate oxidizers such as *Desulfovibrio*, *Desulfomonas*, *Desulfotomaculum*, *Desulfobulbus*, *Thermodesulfobacterium*, utilize lactate, pyruvate, ethanol, propanol and butanol or certain fatty acids as electron donors,
reducing $\text{SO}_4^{2-}$ to $\text{H}_2\text{S}$ [Hao et al., 1996]. Some strains of Desulfotomaculum utilize glucose, but this is rather rare among sulfate reducers in general. These non-acetate utilizers oxidize their energy source to the level of acetate and excrete this fatty acid as an end product.

The genera utilizing acetate include, Desulfococcus, Desulfosarcina, and Desulfonema, they specialize in the oxidation of fatty acids. These SRBs oxidize fatty acids, lactate, succinate and even benzoate in some cases to $\text{CO}_2$ (Eq. 2.44).

\[
\text{CH}_3\text{COO}^- + \text{SO}_4^{2-} + 3\text{H}^+ \rightarrow 2 \text{CO}_2 + \text{H}_2\text{S} + 2\text{H}_2\text{O} \quad \Delta G_0' = -57.5 \text{ kJ mole}^{-1} \quad (2.44)
\]

Therefore, in sulfidogenic breakdown of VFAs, two oxidation patterns can be distinguished. Some SRBs are able to completely oxidize VFAs to $\text{CO}_2$ and sulfide as end products. Other SRBs lack the tricarboxylic acid cycle and carry out an incomplete oxidation of VFAs with acetate and sulfide as end products [Lens et al., 1998].

Desulfosarcina, Desulfonema, Desulfococcus, Desulfobacterium, Desulfotomaculum and certain species of Desulfovibrio are unique among sulfate-reducers in their ability to grow chemolithotrophically and autotrophically with hydrogen as electron donor, sulfate as electron acceptor and carbon dioxide as sole carbon source [Madigan et al., 2000].

In the absence of an electron-acceptor, SRBs are able to grow through a fermentative or acetogenic reaction. Pyruvate, lactate, and ethanol are easily fermented by many SRBs [Widdel, 1988]. SRBs are able to perform acetogenic oxidation in syntrophy with hydrogenotrophic MPBs. Syntrophic oxidation has been described for co-cultures of MPB with Desulfovibrio sp. using lactate and ethanol [Widdel, 1988; Oude et al., 1994] and with Desulfobulbus-like bacteria using propionate [Wu et al., 1991]. Acetogenic oxidation of propionate by Desulfobulbus sp. has also been reported in UASB [Wu et al.,
1992] and fluidized bed [Heppner et al., 1992] reactors. In the presence of sulfate; however, these bacteria behave as true SRB and use propionate as electron-donor for the reduction of sulfate. Of the total microbial population, approximately 15 % SRBs were present in a methanogenic reactor fed with glucose, even though sulfate was not present in the influent of the reactor. The presence of SRBs (primarily *Desulfovibrio* spp. and *Desulfobacterium* spp.) in the absence of sulfate may be explained by their ability to function as proton-reducing acetogens and/or fermenters [Raskin et al., 1996]. Hence, in the absence of an electron acceptor, SRB are able to grow through a fermentative or acetogenic reaction. Pyruvate, lactate, and ethanol are easily fermented by many SRBs [Widdel, 1988].

In addition to using sulfate as an electron acceptor, many SRBs can grow using nitrate (NO$_3^-$) as an electron acceptor, reducing NO$_3^-$ to NH$_3$, or sulfonates, such as isothionate (HO-CH$_2$-CH$_2$-SO$_3^-$), or elemental sulfur (S$_0^-$) both of which are reduced to H$_2$S, or can use certain organic compounds for energy generation by fermentative pathways in the complete absence of terminal electron acceptors. The most common fermentable compound is pyruvate, which is converted via the phosphoroclastic reaction to acetate, carbon dioxide and hydrogen [Madigan et al., 2000]. Nevertheless, in the anaerobic treatment of wastewater, both SO$_4^{2-}$ reduction and methanogenesis can take place; hence, there is a competition for the organic substrate. The following section highlights the competition for the organic substrate between SRBs and MPBs.

2.7.3 Competition for Organic Substrate between SRBs and MPBs

SRB and MPB coexist in mixed anaerobic communities and typically, both these microorganisms are terminal degraders, since they compete for electron donors such as
hydrogen and acetate [Lens et al., 1998]. Compared to MPB, SRBs are much more versatile in terms of substrate utilization. Compounds such as propionate and butyrate, which require syntrophic consortia in methanogenic environments, are degraded directly by a single species of SRB (Desulfovibrio and Desulfomicrobium) in environments where sufficient sulfate is present [Stams et al., 2005]. Also, SRBs have a greater affinity for substrates such as acetate, formate and hydrogen [McCartney and Oleszkiewicz, 1993; Widdel, 1988; Isa et al., 1986]. Based on kinetic and thermodynamic properties, SRBs are believed to out-compete methanogens in the presence of non-limiting sulfate concentrations. The hydrogen saturation constant ($K_m$) is 0.002 mg l$^{-1}$ for SRBs and 0.012 mg l$^{-1}$ for MPBs. In case of acetate as an electron donor, the $K_m$ values for SRBs and MPBs are, respectively, 12 mg l$^{-1}$ and 180 mg l$^{-1}$ [Isa et al., 1986]. The free energy values for SRBs and MPBs, when hydrogen is the electron donor are, -38.1 kJ mole$^{-1}$ and -33.9 kJ mole$^{-1}$, respectively; and when acetate is the electron donor, they are -47 kJ mole$^{-1}$ and -31 kJ mole$^{-1}$ for MPBs [Thauer et al., 1977]. Hence, SRBs are the favored because of their lower free energy and saturation constant values.

### 2.7.3.1 Competition for Hydrogen

SRBs can out-compete methanogens and homoacetogens for hydrogen easily. Thermodynamically, using hydrogen homoacetogenesis is less favorable than methanogenesis, while sulfate reduction is more favorable than methanogenesis [Stams et al., 2005]. Homoacetogens are very poor hydrogen utilizing bacteria (Eq. 2.45).

$$4\text{H}_2 + 2\text{HCO}_3^- + \text{H}^+ \rightarrow \text{CH}_3\text{CHOO}^- + 4\text{H}_2\text{O} \ (\Delta G^\circ = -104.6 \text{ kJ/reaction})$$ (2.45)

Studies with cultures from bioreactors have indicated that hydrogen is mainly consumed by SRBs when sufficient sulfate is present [Isa et al., 1968]. However, in
reactors with immobilized biomass, the activity of hydrogenotrophic methanogens is immediately suppressed when sulfate is added [Visser et al., 1993a]. This can be explained by the fact that methanogens can reach threshold hydrogen values at about 1.1 Pa, while SRBs can reach values of about 0.2 Pa [Lovley et al., 1982]. Hence, SRBs inhibit methane production by lowering the hydrogen partial pressure below a threshold level necessary for hydrogen utilization by MPBs because of their low saturation constant (low hydrogen threshold) for hydrogen.

2.7.3.2 Competition for Acetate

In marine sediments, acetate is mainly consumed by sulfate reducers. However, for anaerobic digesters the fate of acetate is less clear [Stams et al., 2005]. The expected predominance of acetate degrading SRB (ASRB) over aceticlastic MPB (AMPB) in excess of sulfate has been confirmed in CSTRs and in the contact activated sludge process [Middleton and Lawrence, 1977; Gupta et al., 1994]. However, the outcome of the competition is less predictable in modern high rate anaerobic reactors based on sludge immobilization [Lens et al., 1998]. Several studies reported that acetate is completely converted into methane, even in excess of sulfate [Mulder, 1984; Polprasert and Haas, 1995], while others report a predominance of ASRB [Choi and Rim, 1991; Stucki et al., 1993; Visser et al., 1993b; Omil et al., 1996, Omil et al., 1997].

In general, there is no agreement on the factors affecting the competition between ASRBs and AMPBs. Several authors have reported different factors such as organic and sulfate loading rates [Yoda et al., 1987], COD/SO$_4^{2-}$ ratio [Choi and Rim, 1991; Bhattacharya et al., 1996; Isa et al., 1986], pH [Isa et al., 1986], reactor design [Isa et al., 1986], temperature [Shin et al., 1996] and HRT [Omil et al., 1998; Isa et al., 1986].
However, from a thermodynamic and kinetic point of view, ASRBs have an advantage over AMPBs in their competition for acetate because of their low $K_m$ and free energy values. ASRBs gain more energy from the consumption of acetate than AMPBs and they tend to have higher growth rates than AMPBs, especially at low acetate concentrations [Colleran et al., 1995; Oude et al., 1994].

**COD/SO$_4^{2-}$ Ratio:** The COD/SO$_4^{2-}$ ratio is a major variable controlling the diversion of electron flux to SRBs. Lowering the COD/SO$_4^{2-}$ ratio is favorable for SO$_4^{2-}$ reduction over methanogenesis. Theoretically, for a COD/ SO$_4^{2-}$ ratio of 0.67, there is enough SO$_4^{2-}$ available for complete removal of organic matter (COD) by SRBs only [Omil et al., 1998; Choi and Rim, 1991]. The latter suggest that stoichiometrically, 1.0 g SO$_4^{2-}$ can be reduced for 0.67 g COD oxidized.

In systems where the COD/SO$_4^{2-}$ ratio exceeds a value of 2.7, aceticlastic methanogens predominate, whereas at COD/SO$_4^{2-}$ ratios lower than 1.7 acetate consuming SRBs predominant [Choi and Rim, 1991]. At COD/ SO$_4^{2-}$ ratios between 1.7 and 2.7 there is an active competition between the MPBs and SRBs and methane production is almost terminated at 1.0, suggesting that MPBs are inhibited by sulfide production. However, when this ratio reaches a value of 0.4 only SRBs survived. Hence, the importance of this competition increases with a decrease in the COD/ SO$_4^{2-}$ ratio [Choi and Rim, 1991]. Isa et al. [1986] observed an increase in percent electron flow (in their work defined as the ratio of electron flow to SRB to the summation of electron flow to SRB and MPB) from 11 to 34 % by decreasing the COD/ SO$_4^{2-}$ ratio from 10 to 1.0.

**HRT:** As the HRT increases, the competition between SRB and MPB decreases and this leads to increase in sulfate reduction. In high rate anaerobic reactors fed with
acetate, an increase in HRT from 0.5 to 10 days increased the percent electron flow to the SRBs by 5.2 % and also the percent sulfate reduced by 7.6 %. [Isa et al., 1986]. However, the methanogenic activities increased after the reactor was reinoculated with anaerobic liquor and by 7 weeks the methanogenic populations out-compete the SRBs though the reactor was initially predominantly populated by the SRBs. Competition between acetate utilizing MPBs and SRBs in mesophilic (30°C) UASB reactors fed with VFA mixture (with an acetate: propionate: butyrate ratio of 5:3:2 on COD basis) or acetate as the sole substrate at different COD/SO₄²⁻ ratios was examined by Omil et al. [1998]. They observed that when there was an excess of sulfate (COD/ SO₄²⁻ ratio lower than 0.67), SRBs became predominant over MPBs after a prolonged reactor operational period. Approximately 250 and 400 days were required to increase the amount of acetate used by SRBs from 50 to 90 % in the reactor treating, the VFA mixture or acetate as the sole substrate, respectively. Hence, the time required for a population shift between ASRB and AMPB depends also on the type of substrate, as mixotrophic ASRB use only acetate when higher VFAs are depleted [Laanbroek et al., 1984]. Omil et al. [1998] findings were in agreement with Harada et al. [1994] who also reported that acetate became predominant only after long-term operation (more than 100 days) of UASB reactors.

**pH:** The optimal pH values for the acetate consuming SRB and MPB are in the same range. The optimal, minimal and maximum pH for the growth of pre-dominant acetophic methanogen (*Methanothrix soehngenii*) are about 7.1 - 7.8, 6.8 and 8.3, respectively. For another species, *Methanosarcina* (acetophic methanogen) the optimal, minimal and maximum pH are 6.5 - 7.5, 5.5 and 8.0, respectively [Visser et al., 1996]. In
the case of SRBs (acetate degrading), an optimal pH in the range of 7.3 - 7.6 is required. The minimal and maximal pH values for their growth are about 6.0 and 9.0 [Widdel, 1988]. Hence, the optimal pH values for the acetate degrading MPB and SRB are in the same range. Even though SRBs can tolerate higher pH values than MPB, Visser et al. [1996] observed that in a USAB at pH levels below 6.9 acetate degrading MPB out-compete SRB. Whereas above a pH of 7.7, acetate degrading SRB were able to out-compete MPB and at pH values between 6.9 - 7.7 both groups were very competitive. In addition to a direct pH-effect, an indirect influence of pH on the competition between ASRB and AMPB is possible, due to the pH dependence of sulfide toxicity. The effect of sulfide toxicity on ASRB and MPB is discussed in section 2.7.4.

**Temperature:** Mesophilic ASRB and AMPB have similar temperature ranges and optima. Consequently, both populations respond to temperature changes similarly in the range of 10 - 50°C [Visser et al., 1992]. Compared to methanogens, increasing temperatures are more favorable for SRBs growth. In mixed culture treatment systems operating at elevated temperatures, methanogens are strongly suppressed with a large fraction of the electron flow distributed to the SRBs [Shin et al., 1996]. Evidence from batch studies using mesophilic (30°C) granular sludge have shown that SRB are less sensitive to high temperature shocks (65°C for 8 - 9 hrs). In continuous reactors, a decrease in the reactor temperature from 35 to 25°C for a prolonged duration (30 days) increased the fraction of electron flow used by SRBs from 43 to 80 % [Shin et al., 1996]. The latter could be attributed to the spore forming abilities of few SRB species when exposed to adverse conditions. Hence, temperature shocks can be instrumental in steering the competition between ASRB and AMPB.
2.7.4 Sulfide Inhibition

Only a small amount of hydrogen sulfide formed by dissimilatory sulfate reduction is assimilated by the organism for cell synthesis, as these bacteria have relatively low cell yields; thus, almost all the H₂S formed is released in to the environment [Widdel, 1988]. H₂S produced from microbial reduction of SO₄²⁻ is inhibitory to SRBs and MPBs. The inhibition may be the result of an intrinsic toxicity of H₂S [Reis et al., 1992]. The toxic effect may be attributed to the non-ionized state of H₂S, which allows it to cross the cell membrane more readily. In the cell, the non-ionized H₂S species may interfere with the sulfur metabolism or affect the intracellular pH which denatures native protein or metabolic enzymes [Oude et al., 1994]. Alternatively, it might react with iron causing it to form an insoluble ferrous sulfide (FeS) salt. Iron is needed for cell constituents such as ferrodoxin and cytochrome [Reis et al., 1992]. In addition, the metal sulfides formed appear to act as barriers that prevent the access of reactants to the necessary enzymes [Utgikar et al., 2002]. However, complete inhibition of SRB observed for a H₂S concentration of 547 mg l⁻¹ was found to be direct i.e., not due to unavailability of iron. The inhibition was reversible i.e., after H₂S stripping from the inhibited SRB culture, an increase in activity was observed [Reis et al., 1992].

Sulfide toxicity is strongly dependent on pH, because the chemical equilibrium of sulfide species is pH dependent [Okabe et al., 1995]. According to Hao et al. [1996], the distribution of different sulfide species, H₂S, HS⁻ and S²⁻ (Total sulfide = H₂S + HS⁻ + S²⁻), is a function of pH. Therefore, pH control is an important parameter to drive the equilibrium to the species of interest. The variation of sulfide species with pH is shown
in Figure 2.7. At pH 8.0 most of the total sulfide (TS) is in the HS⁻ form, while at pH 6 most is in the H₂S form.

The sulfide inhibition on MPB depends on the culture characteristics [Visser et al., 1996]. In suspended sludges, inhibition is determined by the H₂S concentration both at low and high pH values [McCartney and Oleszkiewicz, 1993] and 50 % inhibition was found at H₂S concentrations ranging from 50 - 130 mg l⁻¹ in studies reported by different authors [Lens et al., 1998]. In sludge granules, inhibition is dictated by the total sulfide (TS) concentration [Visser et al., 1996] and 50 % inhibition was reported with H₂S concentrations of 250 at pH of 6.4 to 7.2 and 90 mg l⁻¹ at a pH of 7.8 to 8.0. The inhibition of MPB is significantly higher at higher pH values compared with the lower pH range. Thus, studies under mesophilic and thermophilic conditions have shown that granular sludge is less inhibited by H₂S than suspended sludges at low and neutral pH, whereas the inhibition is very similar at high pH values in both the cases [Lens et al., 1998]. These effects can be attributed to internal pH gradients in granules [Koster et al., 1986], although these are very unlikely in well-buffered wastewaters [Lens et al., 1993]. However, local growth conditions can still be different from the bulk liquid due to mass transfer limitation [Visser et al., 1996], thus explaining why higher sulfide concentrations can be tolerated in biofilm reactors operating at neutral pH values [Maillacheruvu et al., 1993].

MPBs are more sensitive than acidogens to H₂S inhibition, both in suspended [Oleszkiewicz et al., 1989] and granular [Shin et al., 1995] cultures and SRBs [Isa et al., 1986]. Inhibitory concentrations of sulfide were 160 - 200 mg l⁻¹ and 120-140 mg l⁻¹ for SRBs and MPBs, respectively [Choi and Rim, 1991]. A good correlation between the
Figure 2.7: Sulfide speciation as a function of pH [Adapted from Hao et al., 1996].

decrease in the specific methanogenic activity and in the growth rates with increasing sulfide concentration at pH values 7.0 - 7.5 was observed [Visser et al., 1996]. At pH values in this range, the inhibition of both free H₂S and TS on acetate degrading MPBs and SRBs were about same. This suggests that at pH 7.0 - 7.5, the presence of sulfide has no important additional effect on the competition between acetate degrading SRBs and MPBs. Visser et al. [1996] also reported that the inhibitory effect of un-dissociated H₂S on MPB as well as SRB was stronger at higher pH values and the growth is more strongly inhibited when compared to the metabolic activity.
2.8 References


Lalman, J.A. (2000). “Anaerobic Degradation of Linoleic (C_{18:2}), Oleic (C_{18:1}) and Stearic (C_{18:0}) Acids and their Inhibitory Effects on Acidogens, Acetogens and Methanogens.” Ph.D. Dissertation, Department of Civil Engineering, University of Toronto, ON, Canada.


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CHAPTER 3: MATERIALS AND METHODS

3.1 Inoculum Source and Startup

The inoculum cultures were obtained from the Little River Wastewater Treatment Facility in Windsor, Ontario and also from an ethanol manufacturing facility in Chatham, Ontario. For the sulfate reduction studies, the ethanol cultures were blended with the primary municipal sludge in a 80:20 ratio (Designated as mother reactor A) in order to ensure sufficient sulfidogenic population. All microbial studies to assess hydrogen consumption and production were conducted with cultures derived from the ethanol production facility (Designated as mother reactor B).

In reactor A, increments of 250 mg l\(^{-1}\) glucose and 100 mg l\(^{-1}\) sulfate were added on a weekly basis to enrich the methanogenic and SRB populations. Eventually, glucose and sulfate concentrations were fixed at 2000 mg l\(^{-1}\) and 1500 mg l\(^{-1}\), respectively. Reactor A (8 L semi-continuous) containing 30,000 mg l\(^{-1}\) VSS was maintained at 37°C ± 2°C. Inoculum from Reactor A was diluted with basal media into a 4 L semi-continuous reactor (Reactor A1) to obtain 5,000 mg l\(^{-1}\) VSS. The performance of the culture in both reactors (A and A1) was assessed by monitoring the VFA levels, gas production, alkalinity (as CaCO\(_3\) mg l\(^{-1}\)) and VSS/TSS ratio [APHA et al., 1998]. The reactor temperatures (37°C ± 2°C) were maintained using a thermal tape (TP FG STD, Omegalux, USA) wrapped around the reactors wall and a variable transformer was used for temperature control (Staco, Inc. Ohio). Reactors B and B1 were maintained and operated in the same manner as A and A1, except in the former case, glucose was added in increments to approximately 5,000 mg l\(^{-1}\). The basal media for the sulfate reduction studies (at a pH of 7.0-7.2) [McCartney and Oleszkiewicz, 1991] consisted the following
constituents (mg l⁻¹): K₂HPO₄, 14; (NH₄)₂SO₄, 10, NaHCO₃, 6000; NH₄HCO₃, 70; MgCl₂.4H₂O, 9; KCl, 25; H₃BO₃, 0.05, FeCl₂.4H₂O, 2.0; ZnCl₂, 0.05; MnCl₂.4H₂O, 0.5; CuCl₂.2H₂O, 0.03; (NH₄)₆MoO₇.4H₂O, 0.09; CoCl₂.6H₂O, 0.15; NiCl₂.6H₂O, 0.05; Na₂SeO₃, 0.1; EDTA, 1.0; Resazurin, 1.0; Yeast extract, 10. On the other the basal medium used to dilute the cultures from reactor B₁ for hydrogen production studies contained the following constituents (mg l⁻¹): NaHCO₃, 6000; NH₄HCO₃, 70; KCl, 25; K₂HPO₄, 14; (NH₄)₂SO₄, 10; yeast extract, 10; MgCl₂.4H₂O, 9; FeCl₂.4H₂O, 2; resazurin, 1.0 ; EDTA, 1.0; MnCl₂.4H₂O, 0.5; CoCl₂.6H₂O, 0.15; Na₂SeO₃, 0.1; (NH₄)₆MoO₇.4H₂O, 0.09; ZnCl₂, 0.05; H₃BO₃, 0.05; NiCl₂.6H₂O, 0.05; and CuCl₂.2H₂O, 0.03. The pH of the basal media was between 7.0 - 7.2.

After feeding glucose (on a weekly basis), the quantity of gas produced was measured over 7 days. Inocula characterization experiments were also performed in order to monitor selected microbial activities. In the characterization experiments glucose degradation and removal was analyzed, subsequently terminating in to methane or methane and sulfide (in the presence of sulfate). Acidogenesis was characterized based on the glucose degradation profiles; acetogenesis was monitored by the pattern of VFAs formation and removal; methanogenesis was monitored by the methane levels in cultures; and sulfidogenesis was monitored by analyzing sulfate degradation patterns. The characterization experiments were conducted in batch reactors containing 2000 mg l⁻¹ VSS at 37°C. All the characterization batch reactors were prepared in triplicates in a similar manner as per the procedure described in section, 3.2.1. Example results of characterization experiments using inocula from Reactor A₁ and B₁ are shown in Appendix A and B, respectively.
3.2 Experimental Design and Details

Experiments were designed in accordance with the research objectives. The experimental phase was divided into the following three phases: Phase I: Diversion of electron equivalents to SRB using LCFAs as an inhibitor; Phase II: Hydrogen consumption in the presence of LCFA and; Phase III: Diversion of electron equivalents away from MPB for accumulation of hydrogen.

3.2.1 Phase I: Diversion of electron equivalents to SRB

In phase I, experiments were designed to examine the effect of C18 LCFAs on sulfate reduction using culture from Reactor A1 at pH 7.0 - 7.2. Three LCFAs bearing 18 carbons (SA, OA and LA) were used as methanogenic inhibitors [Lalman and Bagley, 2000; Lalman and Bagley, 2001]. The experimental design in this phase consisted of (Table 3.1): 1) Control experiments with LCFA (SA, OA and LA), 2) Control experiments with LCFA (SA, OA and LA) plus sulfate and 3) Experiments with glucose, sulfate and LCFA (SA, OA and LA). All experimental conditions were examined in triplicates in 160 ml batch reactors with a liquid volume of 100 ml.

Prior to conducting the experiments with glucose, sulfate and LCFA (SA, OA and LA) (Stage III), control studies with LCFA (SA, OA and LA) (Stage I) and LCFA (SA, OA and LA) plus sulfate (Stage II) were conducted to access the degradation of the LCFAs (SA, OA and LA) and also the effect of LCFAs (SA, OA and LA) on sulfate removal. In addition, controls containing culture plus sulfate and culture plus glucose were examined in these studies. The main objectives of the control experiments were to determine the extent of LCFA degradation over the duration of the experiments (Stage I) and to assess if the LCFAs could serve as an electron donor (Stage II). The objective of
the experiments with glucose, sulfate and LCFA (SA, OA and LA) (Stage III) was to access the effect of LCFAs (SA, OA and LA) on the pattern of electron flow in sulfate reduction by a mixed anaerobic culture.

Table 3.1: Experimental design matrix for Phase I

<table>
<thead>
<tr>
<th>Substrates or Inhibitors (mg l(^{-1}))</th>
<th>Analysis Parameters</th>
<th>LCFA Control Experiments</th>
<th>LCFA plus Sulfate Control Experiments</th>
<th>Glucose, Sulfate and LCFA Experiments</th>
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<tbody>
<tr>
<td>LCFA</td>
<td>Glucose (1,870)</td>
<td>Sulfate (1,500)</td>
<td>Sulfate (mg l(^{-1}))</td>
<td>Glucose (mg l(^{-1}))</td>
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<tr>
<td>SA</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td>OA</td>
<td>x</td>
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<td>LA</td>
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<td>LCFA plus Sulfate Control Experiments</td>
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<td>SA</td>
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<td>LA</td>
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<td>√</td>
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<td>x</td>
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<tr>
<td>Glucose, Sulfate and LCFA Experiments</td>
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<td>SA</td>
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<td>LA</td>
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Note: LCFAs concentrations examined were 0, 100, 300, 500, 700 and 1000 (mg l\(^{-1}\))

An excess quantity of glucose (electron donor) oxidation was added to ensure that the reduction of sulfate (electron acceptor) was not limited. In all SA, OA and LA batch experiments, 1,870 mg l\(^{-1}\) of glucose was added to 1500 mg l\(^{-1}\) sulfate.

As mentioned earlier inocula from Reactor A1 (5000 mg l\(^{-1}\) VSS at 37°C ± 2°C) were characterized for glucose degradation and sulfate removal prior to the batch experiments. The inhibition and characterization experiments were conducted with a total liquid volume of 100 ml (inoculum, basal medium and substrates). The batch reactors were prepared with inoculum (2000 mg l\(^{-1}\) VSS) from Reactor A1 in an anaerobic glove box (COY® Laboratory Products Inc., Michigan, USA). The inoculum was used for preparing
the batch reactors after 7 - 8 days of feeding Reactor A1 with glucose and sulfate, when all the substrates were removed from the culture. The culture was monitored for residual VFAs and sulfate to ensure that it was free of these substrates before preparing the batch reactors. The bottles were sealed with Teflon®-lined rubber septa and aluminum crimp caps under an atmosphere consisting of 70 - 75 % N₂, 20 - 25 % CO₂ and less than 1 % H₂ (Praxair, ON, Canada). To avoid the formation of a negative pressure during sampling, 20 ml of gas from the anaerobic glove box was injected into the headspace of each serum bottle. The serum bottle reactors were agitated using an orbital shaker (Lab Line Instruments, USA, Model HHKE 4000) to convert any trace hydrogen (from the anaerobic chamber) and CO₂ into methane. After agitation for 24 h at 37°C ± 2°C in an environmental chamber, no detectable levels of hydrogen were observed in the headspace.

Glucose and sulfate (as NaSO₄) were injected into the serum bottles from stock solutions. The glucose and sulfate stock solutions were both 100,000 mg l⁻¹ (ACP Chemicals, Montreal, Quebec). LCFA was injected into the reactors from a 50,000 mg l⁻¹ saponified stock solution. Because LCFAs adhere onto most surfaces and they are relatively insoluble [Kramer, 1971], a reproducible and accurate saponification method to deliver the LCFAs into the serum bottles was adapted from a method developed by Angelidaki and Ahring [1992]. The LCFAs were melted au bain-marie and dissolved into a hot (50°C), vigorously stirred NaOH (EMD Chemicals, USA) solution. The quantities of sodium hydroxide used g NaOH g⁻¹ of LCFA for LA (>99%), OA (>99%), SA (>99%) (TCI, USA) were 0.143, 0.142, and 0.141, respectively. Once, injected with LCFA, sulfate and glucose, samples were analyzed for VFAs, sulfate, glucose and
headspace gases (H₂, CH₄ and CO₂) for seven days based on data from the glucose characterization studies.

3.2.2 Phase II: Hydrogen consumption in the presence of LCFA

In phase II, experiments were designed to access hydrogen degradation in the presence of C18 LCFA (OA, LA and OA plus LA). The experimental design consisted of the following (Table 3.2): 1) LCFA (OA, LA and OA plus LA) plus hydrogen at pH 6.0 and 2. LCFA (OA, LA and OA plus LA) plus hydrogen at pH 5.0. Hence, individual and mixtures of LA and OA at levels of 100, 500, 1000 and 2000 mg l⁻¹ were used in this study. Experiments were conducted at 37 ± 2°C under two pH conditions, 5.0 and 6.0 and 2000 mg l⁻¹ VSS. Controls in these experiments consisted of culture plus hydrogen. All the experiments were conducted in triplicates in 160 ml batch reactors with a liquid volume of 50 ml. The objective of these experiments was to access the effect of LCFA on hydrogenotrophic methanogens. The significance of the study in understanding LCFA inhibition on hydrogen consumption is important because LCFAs can be used to redirect electron fluxes to produce hydrogen from a variety of carbohydrate sources by mixed anaerobic cultures.

Table 3.2: Experimental design matrix for hydrogen consumption studies.

<table>
<thead>
<tr>
<th>LCFA (mg l⁻¹)</th>
<th>Hydrogen (5 ml)</th>
<th>Carbon-dioxide (1.25 ml)</th>
<th>Nitrogen (5 ml)</th>
<th>Analysis Parameters (Headspace gases: H₂, CH₄ and CO₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments at pH 6.0</td>
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<tr>
<td>OA</td>
<td>√</td>
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<tr>
<td>LA</td>
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<tr>
<td>LA-OA*</td>
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</tr>
<tr>
<td>Experiments at pH 5.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>LA</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>LA-OA*</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

√: yes

NOTE: 1. The LCFA levels were 100, 500, 1000 and 2000 (mg l⁻¹)
2. For LA-OA, the concentrations of an individual LCFA is 50% of the total amount
All the experiments were conducted using inoculum from Reactor Bl and basal medium [Oh et al., 2003] at the desired pH (pH 6.0 or 5.0). The basal medium consisted of the following (g l\(^{-1}\)): \(\text{H}_2\text{HPO}_4\), 0.056; (\(\text{NH}_4\)\(\text{SO}_4\)), 0.040; KCl, 0.1; \(\text{NH}_4\text{Cl}\), 0.126; \(\text{C}_6\text{H}_5\text{N}_2\text{O}_4\), 54.66; yeast extract, 0.04. The pH of the media was adjusted to the desired pH of 5.0 or 6.0 using NaOH (1.0 M). After preparing the batch reactors, ethanesulfonic acid monohydrate (MES) (\(\text{C}_6\text{H}_5\text{N}_2\text{O}_4\), 0.07 M) (13.7 g l\(^{-1}\)) (Fisher Scientific, Toronto, ON) was used as buffer to maintain the desired pH.

Prior to the preparation of the batch reactors, 1.5 L of inoculum from Reactor Bl was washed 2 times with fresh basal media [Lalman and Bagley, 2000]. The culture was washed 2 more times with the basal media [Oh et al., 2003] at selected pH values (pH 6.0 or pH 5.0) to remove any residual substrates from the cultures. The batch reactors were prepared in a similar manner as discussed in section 3.2.1 for the Phase I experiments. After removing the 160 ml batch reactors from the glove box, the cultures were purged with nitrogen, resealed, injected with 5.0 ml of nitrogen (> 99 %) (Praxair, ON) and left inverted on the orbital shaker (Lab Line Instruments, USA, Model HHKE 4000) overnight before hydrogen was injected. Next, LCFA (2000 mg l\(^{-1}\)) (TCI, USA), hydrogen (224.7 µmoles (5.0 ml)) and carbon-dioxide (1.25 ml) (Praxair, ON) were injected to the reactors on the following day and subsequently monitored over approximately 48 h for headspace gases hydrogen, methane and carbon dioxide. LCFA delivery strategy was similar to the method adopted in Phase I experiments (section 3.2.1). After most of the initial hydrogen was consumed, the reactors were purged again with nitrogen and reinjected with 5.0 ml (224.7 µmoles) of hydrogen. The hydrogen consumption was monitored again over another 48 h period.
3.2.3 Phase III: Diversion of electron equivalents away from MPB

In phase III, experiments were designed to examine the effect of LCFA (OA and LA) on the flow of electron fluxes in an anaerobic mixed community in the absence of an inorganic electron acceptor. The experimental design consisted of the following conditions (Table 3.3): 1) Control incubation experiments with LCFA (OA and LA) over a period of 32 days and 2) Incubation experiments with LCFA (OA and LA) and glucose over a period of 32 days. The novelty of this work was to explore hydrogen production using varying incubation periods and to assess the effect of degradation of the parent LCFA on hydrogen production. LCFA are degraded and removed from the anaerobic cultures within 20 - 25 days and hence, maximum incubation duration of 25 days was selected to determine the minimum dosage (quantity of LCFA) that should be added to the cultures for maintaining the high hydrogen yields and also to determine the effect of LCFA β-oxidation byproducts. Inocculum from Reactor B1 with basal media [Lalman and Bagley, 2000] was used to prepare the 160 ml reactors (37 ± 2°C, pH 5.0 and VSS 2000 mg l⁻¹). A pH of 5.0 was maintained using ethanesulfonic acid monohydrate (MES) (C₆H₁₃NO₄S, 0.07 M). Control experiments with LCFA (LA and OA) were conducted for a period of 32 days. In the incubation experiments with LCFA (LA or OA) plus glucose, six triplicate sets (6 incubation periods ranging from 0 to 25 days at an increment of 5 days were selected) of reactors were prepared and injected with LCFA (2000 mg l⁻¹ LA or OA) (TCI, USA) on day 0. Glucose (5000 mg l⁻¹) (ACP Chemicals, Montreal, Quebec) was injected on the 0th, 5th, 10th, 15th, 20th, and 25th days, respectively, in set 1, 2, 3, 4, 5 and 6 and analyzed for headspace gases, glucose, VFAs and LCFA for
7 days following each glucose injection. All the batch reactors were prepared following the procedure described for the phase I experiments (Section 3.2.1).

Table 3.3: Experimental design matrix for Phase III: diversion of electron equivalents away from MPB using OA or LA.

<table>
<thead>
<tr>
<th>Substrates or Inhibitors</th>
<th>Analysis parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OA (2000 mg l⁻¹)</td>
</tr>
<tr>
<td>Control Experiments</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>0-30</td>
</tr>
<tr>
<td>LA</td>
<td>0-30</td>
</tr>
<tr>
<td>OA Incubation Experiments</td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td>0-7</td>
</tr>
<tr>
<td>Set 2</td>
<td>5-12</td>
</tr>
<tr>
<td>Set 3</td>
<td>10-17</td>
</tr>
<tr>
<td>Set 4</td>
<td>15-22</td>
</tr>
<tr>
<td>Set 5</td>
<td>20-27</td>
</tr>
<tr>
<td>Set 6</td>
<td>25-32</td>
</tr>
<tr>
<td>LA Incubation Experiments</td>
<td></td>
</tr>
<tr>
<td>Set 1</td>
<td>0-7</td>
</tr>
<tr>
<td>Set 2</td>
<td>5-12</td>
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<tr>
<td>Set 3</td>
<td>10-17</td>
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<tr>
<td>Set 4</td>
<td>15-22</td>
</tr>
<tr>
<td>Set 5</td>
<td>20-27</td>
</tr>
<tr>
<td>Set 6</td>
<td>25-32</td>
</tr>
</tbody>
</table>

✓: Yes; x: No, in the above table

The objectives of control experiments with LCFAs (OA and LA) were to access the extent of degradation of LCFAs and to determine if hydrogen accumulation resulted from the degradation of LCFAs. Recall the objectives for conducting these experiments with LCFA plus glucose were to: examine the effect LCFAs (OA and LA) on hydrogen production; to establish the effect of different LCFA (OA and LA) incubation periods; to assess the effect of degradation byproducts of OA and LA on the diversion of electron equivalents away from MPB and to determine if hydrogen accumulation is feasible in spite of degradation of the parent LCFAs into LCFA byproducts. In addition to the LCFA (OA and LA) controls, three sets (0 - 10, 10 - 20, and 20 - 30 days) of glucose
controls were prepared and analyzed over the entire experimental duration for glucose degradation and byproduct formation.

3.3 Analytical Methods

Analytical parameters consisted of solutes (VFAs, sulfate, glucose, and LCFAs) and headspace gases (H₂, CH₄ and CO₂). Liquid samples were purified before they could be analyzed. The processing procedure adopted for VFAs, sulfate and glucose was the same in all the experiments. A liquid extraction method was adopted for LCFA recovery using MTBE: Hexane (1:1) [Lalman and Bagley, 2000] as described in section 3.3.4.

A common procedure was adopted for the sampling and processing of samples for VFAs, sulfate and glucose. At selected time intervals, samples were withdrawn using a 2.5 ml Hamilton Gastight® (VWR, Canada) syringe, 0.5 ml samples were transferred into 7.5 ml culture tubes containing 4.5 ml of Milli-Q® (Millipore, Barnstead, USA) grade water. The 10 times diluted samples were centrifuged at 1750 g for 5 minutes to remove biological and inorganic solids. The centrate was filtered using 2 filters connected in series. The first filter consisted of a 25 mm diameter syringe filter holder (PAL Sciences, MI, USA) fitted with a 25 mm diameter 0.45 μm polypropylene membrane (GE Osmonics, MN) and the second filter was a 1 ml polypropylene cartridge tube with a 20 μm polyethylene frit (Spe-ed Accessories, PA) and filled with Chelex® 100 to 200 mesh, sodium resin (Bio-Rad Laboratories, CA). The filtrate was transferred to a 5.0 ml polypropylene vial (Dionex, Oakville, ON) and stored at 4°C prior to analysis.

3.3.1 VFA and Sulfate Measurements

VFAs and sulfate analyses were conducted using a Dionex DX-500 ion chromatograph (IC) (Oakville, ON) equipped with a CD 20 conductivity detector, ASRS
suppressor, a GP 40 gradient pump, an AS 40 automated sampler and a 25-µl-sample loop. The IC was configured with a Dionex IonPac® 24 cm x 4 mm diameter AS11-HC analytical column and a Dionex IonPac® AG11-HC guard column. VFAs in phase III experiments were analyzed using the following three eluents: 1) Eluent A, Milli-Q® grade water (18 MΩ); 2) Eluent B, 100 mM NaOH; and 3) Eluent C, 1 mM NaOH. The total eluent flow rate was 2 ml min⁻¹ and the individual flow rates as % of the total flow were: 0 - 15 mins., 80 % A, 20 % C; 15 - 15.1 mins., 85 % A, 15% B; 0 % C ; 15.1 - 25 mins., 65 % A, 35 % B.

A variation to the above method was adopted for the analysis of VFAs in Phase I experiments. Because sulfate was present in cultures used for phase 1 of the study, a method was developed for simultaneous analysis of VFAs and sulfate. The three eluents were as follows: 1) Eluent A, Milli-Q® grade water (18 MΩ); 2) Eluent B, 100 mM NaOH; and 3) Eluent C, 1 mM NaOH were used in the analysis. The total eluent flow rate was 2 ml min⁻¹ and the individual flow rates as percentage of the total flow were: 0 - 10 mins. 80 % A, 20 % C; 10 - 10.1 mins, 80 % A, 20 % C; 10.1 - 15 mins, 80 % A, 20 % C; 15 - 30 mins., 85 % A, 15 % B; 30 - 45 mins., 65 % A, 35 % B. A 12.6 mM H₂SO₄ regenerant (prepared in a Milli-Q® grade water (18 MΩ)) was used at a flow rate of 4.0 ml min⁻¹ at a pressure of 5 psi.

Instrument calibrations were conducted by preparing standards prepared from 5000 mg l⁻¹ stock solutions. For the calibration graph, 10 standards were prepared and analyzed in triplicate at concentrations of 0 (Milli-Q® water), 0.5, 1, 2, 3, 4, 5, 10, 30, 50, 70 and 100 mg l⁻¹ for acetate, propionate, butyrate and sulfate. All the standards were prepared using basal media. The VFAs and sulfate calibration curve plots are shown in
Appendix C. During sample analysis, intermediate standard were analyzed after every 10 samples. A variation of $\pm 5\%$ from the calibration standards was considered acceptable. The detection limits for the VFAs and sulfate were 0.5 mg l$^{-1}$ and 1 mg l$^{-1}$, respectively.

3.3.2 Glucose and alcohols Measurements

Glucose and alcohols were analyzed using a DX-600 IC (Dionex, Oakville, ON) equipped with a GP 50 gradient pump, an AS 40 automated sampler, an ED 50 electrochemical detector, and a 25-μl-sample loop. The instrument was configured with a Dionex CarboPac™ 25 cm x 4 mm diameter MA1 analytical column and a Dionex CarboPac™ 5 cm x 4 mm diameter MA1 guard column. The isocratic method used a 480 mM NaOH eluent at a flow rate of 0.4 ml min$^{-1}$. A 25 μl sample loop was used to maintain a constant volume injection onto the analytical column. The method used 480 mM NaOH eluent at a flow rate of 0.4 ml min$^{-1}$. Total analysis run time was 35 minutes, glucose was detected at approximately 25 minutes, and ethanol, propanol i-propanol, butanol and i-butanol were detected at 7.38, 7.68, 8.98, 10.46 and 11.53 minutes, respectively.

For the calibration graph, triplicate standards were prepared in basal media and analyzed. The detection limit based on the lowest concentration was 1 mg l$^{-1}$ for glucose and 5 mg l$^{-1}$ for each alcohol. The calibration curve plots for glucose and alcohols are shown in Appendix C.

3.3.3 Headspace Gas Analysis

The gas samples withdrawn were manually injected on the GC (Varian, 3800). The gas samples were analyzed using a gas chromatograph (GC) (Varian, 3800) which was equipped with a thermal conductivity detector (TCD) and a 2 m x 1 mm diameter (ID)
packed Shincarbon ST (Restek) column. The gas samples were withdrawn manually, using a 50 µl Hamilton Gastight® (VWR, Canada) syringe, from the headspace of the reactors and injected manually into the injector. The analysis was isothermal with the oven temperature set at 200°C. The nitrogen (99.999%, Praxair, ON) carrier gas flow was set at 21 ml min⁻¹. The detector and injector temperatures were 200°C and 100°C, respectively. The detection limits for hydrogen, methane and carbon dioxide were 0.1900 kPa (2.5 µL/bottle), 0.1226 kPa (2.5 µL/bottle) and 0.0737 kPa (2.5 µL/bottle), respectively.

Calibration standards for the gas chromatograph were prepared in serum bottles (160 ml). The bottles were purged with nitrogen (99.998 %) for 2.0 minutes and capped with aluminum crimp sealed with Teflon® lined septa. Known quantities of H₂, CH₄ and CO₂ were injected into the capped bottles and analyzed. During each headspace analysis, a standard was prepared within the calibration range and analyzed for the different gases. The gas calibration curves are shown in Appendix C.

### 3.3.4 LCFA Measurements

Since LCFAs are insoluble in water [Kramer, 1971], an extraction method [Lalman and Bagley, 2000] was used to ensure accurate measurement of the aqueous samples removed from the reactors. LCFA measurements were conducted only in Phase III experiments. LCFA samples were analyzed before glucose injection and after 7 days sample (H₂, CH₄, CO₂, VFAs) analysis duration in the respective sets over the experimental duration. 1.0 ml samples were withdrawn from the serum bottles for LCFA analysis and extractions were conducted using centrifuged culture supernatant and uncentrifuged cultures. The centrifuged samples (supernatant) used for extraction gave
low percentage recovery likely due to the deposition of the miscible LCFA along with the cultures as centrate during the process of centrifugation. Hence, 1.0 ml uncentrifuged samples were used for LCFA recovery in all the LCFA analyses. The 1.0 ml sample was placed in a 5 ml vial containing 2 ml of 50:50 Hexane:MTBE (EM Science, USA), 0.05 g NaCl (ACP Chemicals, Montreal, Quebec), and 2 drops of 50% H2SO4 (EMD Chemicals, USA). The vial was sealed with Teflon® lined septa and capped with aluminum crimp seals and placed on an orbital shaker at 37°C ± 2 (Lab Line Instruments, USA, Model HHKE 4000) for 20 min at 200 rpm. Next, the vial was centrifuged for 5 min at 1750 g. The organic phase was removed and 1.0 μL sample was analyzed using a Varian 3800 GC equipped with a flame ionization detector (FID) and a 15 m long analytical column with 0.53 mm inner diameter (ID) (J and W Scientific, USA). The FID and the injector were at 250°C. The oven temperature was programmed to 100°C for 1.0 minute, with a 26°C/min temperature gradient to 230°C and a final hold at 230°C for 9.0 minutes. The analysis time was 15 minutes. Nitrogen was used as a carrier gas at a flow rate of 6.5 ml min⁻¹.

Prior to LCFA sample analysis, LCFA extractions studies were conducted to establish the percent recovery. Recovery studies were conducted with LCFAs ranging from C8 to C18, caprylic acid (C8:0) (>99%), capric acid (C10:0) (>99%), lauric acid (C12:0) (>99%), myristic acid (C14:0) (>99%), palmitic acid (C16:0) (>99%), stearic acid (C18:0) (>99%), oleic acid (C18:1) and linoleic acid (C18:2) (>99%) (TCI, USA). Extraction studies were performed using 5, 10, 30, 50, 100, 500, 700, and 1000 mg l⁻¹ of all the LCFAs. All the extractions were conducted in 160 ml serum bottles containing 2000 mg l⁻¹ VSS of culture at 37°C. The required concentrations of LCFA mixture were
injected from a 10,000 mg l\(^{-1}\) LCFA stock solution. LCFA stock solution was prepared using saponification technique described in Section 3.2.1. Table 3.4 shows the quantities of sodium hydroxide used (expressed as g NaOH g\(^{-1}\) LCFA). The percentage recovery in all the extraction studies at all concentrations ranged between 90 - 100 %. The LCFA extraction percentage recovery plots are provided in Appendix D.

Calibration curves for LCFAs were prepared in hexane from a 10,000 mg l\(^{-1}\) stock solution. LCFA calibration curves are shown in Appendix C. The detection limits were 5 mg l\(^{-1}\) for caprylic (C8:0) to palmitic (C16:0) acids and 15 mg l\(^{-1}\) for stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids.

Table 3.4: Quantity of sodium hydroxide used for LCFA stock solution preparation.

<table>
<thead>
<tr>
<th>LCFA</th>
<th>Sodium Hydroxide used (g g(^{-1}) LCFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caprylic</td>
<td>0.278</td>
</tr>
<tr>
<td>Capric</td>
<td>0.233</td>
</tr>
<tr>
<td>Lauric</td>
<td>0.200</td>
</tr>
<tr>
<td>Myristic</td>
<td>0.175</td>
</tr>
<tr>
<td>Palmitic</td>
<td>0.156</td>
</tr>
<tr>
<td>Palmitic</td>
<td>0.156</td>
</tr>
<tr>
<td>Stearic</td>
<td>0.141</td>
</tr>
<tr>
<td>Oleic</td>
<td>0.142</td>
</tr>
<tr>
<td>Linoleic</td>
<td>0.143</td>
</tr>
</tbody>
</table>

3.3.5 pH and Total Solids Measurement

The pH of each batch reactor was measured at the beginning and at the end of each experiment using a VWR SR40C, Symphony pH meter. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined at the beginning of each experiment. The analysis was conducted using 5 ml of liquid sample and 1-\(\mu\)m pore size glass fiber filter papers (VWR, Canada). TSS, VSS, and pH were determined according to the procedures described in Standard Methods (APHA et al., 1998).
3.4 References


CHAPTER 4: SULFATE REDUCTION AND INHIBITION OF METHANOGENESIS BY LINOLEIC ACID (LA) IN AN ACCLIMATED MIXED ANAEROBIC CULTURE

4.1 Introduction

Dissimilatory sulfate ($\text{SO}_4^{2-}$) reduction is a process that is unique to anaerobic communities containing SRBs. SRB are important in sulfate removal from wastewater streams containing elevated levels of sulfate. Various industrial effluents arising from food, pulp and paper, petroleum, metallurgical, brewing industries and mine tailings contain significant levels of sulfate. Under anaerobic conditions, SRB oxidize organic compounds by utilizing sulfate, as an electron acceptor, and a suitable electron donor compound in an energy generating process, which generates sulfide. Only a small fraction of the sulfide formed by dissimilatory sulfate reduction is assimilated by SRB for cell synthesis. These microorganisms have relatively low cell yields and hence, if not precipitated as a metal salt, most of the sulfide remains in the microbial community [Widdel, 1988]. In solution, the prevalent sulfide species varies among $\text{S}^{2-}$, $\text{HS}^-$ and $\text{H}_2\text{S}$ depending upon the pH of the medium. The presence of sulfides poses a major operational problem with many anaerobic systems treating sulfate-rich wastewaters. Sulfides are toxins and can be removed from the wastewater systems by precipitation [El Bayoumy et al. 1999]. The latter is widely used by mining industries for removing heavy metals from effluents [Maree et al., 2004].

SRBs use electron donors such as hydrogen or carbon sources such as acetate. The range of electron donors used by SRB is wide. The type of the carbon source utilized for the reduction of an electron acceptor (sulfate) varies according to genus [Weijma et al.,
The preferred carbon source for SRBs are low-molecular-weight compounds such as organic acids (e.g. lactate, pyruvate, formate, and malate), VFAs (e.g. acetate), and alcohols (e.g., ethanol, propanol, methanol, and butanol) and occasionally sugars and longer chain fatty acids [Weijma et al., 2002; Hao et al., 1996; Gibson, 1990].

SRBs can be categorized in to non-acetate utilizers (*Desulfovibrio*, *Desulfomonas*, *Desulfotomaculum*, *Desulfobulbus*, *Thermodesulfobacterium*) and acetate utilizers (*Desulfococcus*, *Desulfosarcina*, and *Desulfonema*) based on their substrate utilization [Madigan, 2000]. Hence, two oxidation patterns can be distinguished in SRBs; the non-acetate utilizers oxidize their energy source (organic substrate) to acetate (Eqs. 2.19 and 2.20) while, the acetate utilizers oxidize the substrate to carbon dioxide (Eq. 2.44) [Lens et al., 1998; Reis et al., 1992].

Many electron donors are fermentation products from the anaerobic degradation of carbohydrates, proteins and lipids. SRBs are terminal degraders (produce H₂S and CO₂), and their role is analogous to that of methanogenic bacteria that produce CH₄ and CO₂ as final products in anaerobic microbial systems [McCartney and Oleszkiewicz, 1993; Lens et al., 1998]. In sulfate-containing effluents, MPB compete with SRB for electron equivalencies from common substrates such as hydrogen and acetate. Formation of methane is undesirable because it lowers the process economy by consuming a fraction of the reducing equivalence from the substrates [Weijma et al., 2002].

SRBs are able to out-compete methanogens for hydrogen and acetate [Isa et al., 1968]. SRBs have a lower saturation constant and free energy for hydrogen (Kₘ,SRB = 0.002 mg l⁻¹; Kₘ,MPB = 0.012 mg l⁻¹; ΔG°SRB = -38.1 kJ mole⁻¹; ΔG°MPB = -33.9 kJ mole⁻¹) and acetate (Kₘ,SRB = 12 mg l⁻¹; Kₘ,MPB = 180 mg l⁻¹; ΔG°SRB = - 31 kJ mole⁻¹; ΔG°MPB = -
SRBs gain more energy from the utilization of these donor compounds than MPBs and tend to have higher growth rates than MPBs, especially at low acetate concentrations [Oude et al., 1994; Colleran et al., 1995]. However, the outcome of the competition reported in the high rate anaerobic reactors is less predictable based on sludge immobilization. The findings on this are contradictory; several studies reported complete conversion into methane, even in excess of sulfate [Hoeks et al., 1984; Mulder, 1984; Polprasert and Haas, 1995], while others reported a predominance of SRB and increased sulfate reduction [Choi and Rim, 1991; Stucki et al., 1993; Omil et al., 1996].

From the available literature, the competition between SRBs and MPBs for donor compounds is a function of several factors. In general, there is no agreement so far on what are the determining factors affecting the competition between acetate consuming SRB and MPB. Several researchers have reported different factors such as organic and sulfate loading rates [Yoda et al., 1987], COD/SO$_4^{2-}$ ratio [Choi and Rim, 1991; Bhattacharya et al., 1996; Isa et al., 1986], pH [Isa et al., 1986], reactor design [Isa et al., 1986], temperature [Shin et al., 1996] and HRT [Omil et al., 1998; Isa et al., 1986].

Lowering the COD/SO$_4^{2-}$ ratio is favorable towards sulfate reduction compared to methanogenesis. SRB competitiveness increases as the COD/SO$_4^{2-}$ ratio decreases. Below a COD/SO$_4^{2-}$ ratio of 1.7, acetate consuming SRBs predominant while above a value of 2.7, MPBs predominate. In between 1.7 - 2.7, a tough competition between SRBs and MPBs exists for any available acetate [Choi and Rim, 1991].

An increase in HRT decreases the competition between SRB and MPB and hence, increases the sulfate reduction [Omil et al., 1998; Isa et al., 1986]. In high rate anaerobic reactors fed with acetate, the percent electron flow to the SRBs increased by
approximately 5 % when the HRT was increased from 0.5 to 10 days [Isa et al., 1986]. To increase the amount of acetate used by SRB from 50 to 90 % when using a VFA mixture or acetate as the sole substrate, the HRT was increased from 250 and 400 days [Omil et al., 1998].

Optimal pH values for acetate degrading MPB and SRB activity varies from 7.1 - 7.8 [Widdel, 1988]. However, SRBs are able to tolerate higher pH values compared to MPB [Visser et al., 1996]. Mesophilic acetate consuming SRB and MPB also have similar temperature ranges and optima. Consequently, both populations respond to temperature changes similarly in the range of 10 - 50°C [Lens et al., 1998]. SRBs are able to respond better to temperature shocks than MPBs. According to Shin et al. [1996] increasing the temperature from 30°C to 65°C for 8 - 9 hrs and decreasing the temperature from 35°C to 25°C for 30 days resulted in an increase in electron flow to SRB. The latter could be attributed to the spore forming abilities of a few SRB species when exposed to adverse conditions [Madigan et al., 2000; Widdel, 1988].

Therefore, a complete reduction of sulfate along with removal of organic matter is difficult to achieve, because some percentage of electrons is consumed by the MPBs as the competition between the two is governed by the conditions in the reactors and is dependent on several factors. To reduce this competition, a mechanism to divert electron fluxes to optimize the reduction of sulfate is of a major importance in sulfate reducing anaerobic bioprocesses. Several attempts at physical and chemical methods to control the electron flow in mixed anaerobic systems have been reported in the literature. Diverting the electron flow by controlling the growth of hydrogen consuming microorganisms such as hydrogenotrophic methanogens in anaerobic communities has been demonstrated.
using pH and heat-treatment techniques [Oh et al., 2003; Sung et al., 2002]. However, in these studies only short-term growth control was demonstrated.

Inhibiting methane production has been shown using 2-bromoethanesulphonate (BES) [Oremland and Capone, 1988; Scholten et al., 2000]; acetylene [Sparling et al., 1997; Ahring and Westermann, 1987; Bomar et al., 1985]; chlorinated methane analogues, such as chloroform, carbon tetrachloride, and methylene chloride [Bauchop, 1967] and ethylene [Oremland and Taylor, 1975]. However, these chemicals are toxic and not cost effective in full-scale applications. In addition, chemicals such as ethylene and acetylene are non-specific and found to affect nitrogenase activity in other microorganisms.

Evidence from several reports have demonstrated that redirecting electron fluxes in the presence of LA is possible when methanogenesis is inhibited [Lalman and Bagley, 2001; Lalman and Bagley, 2000]. However, no study to date has described the effects of LA on SRB in the presence of MPBs. Using LA to inhibit hydrogen and acetate consuming methanogens is advantageous because, they are easily available from agriculture sources, relatively inexpensive, and can slowly degrade to hydrogen and acetate [Weng and Jeris, 1976].

The objective of this work was to assess the effects of LA on sulfate reduction in mixed anaerobic batch cultures maintained at 37 ± 2°C and examine the diversion of electron fluxes from glucose degradation to sulfate reduction in the presence of LA. In this study, the COD/SO₄²⁻ ratio was 1.25 and COD (glucose) was kept limiting to ensure excess sulfate in the cultures for optimum SRB activity.
4.2 Materials and Methods

The materials and methods for this phase are described in Chapter 3. The inoculum source and the culture start-up procedure are described in section 3.1. The experimental plan and design are shown section 3.2.1 and analytical methods used are detailed in section 3.3. In this work, initial glucose and sulfate degradation rates were calculated using Graphpad Prism software using a non-linear regression by a one-phase exponential decay model. A sample calculation for initial glucose degradation rate is shown in Appendix E. Initial sulfate degradation rates were calculated in similar manner.

4.3 Results

4.3.1 Control Cultures

One set of LA controls were prepared for Stage I and another set containing LA plus sulfate were prepared for Stage II. In addition, control cultures were prepared with glucose, sulfate and glucose plus sulfate. In controls receiving glucose, the glucose degradation rates were higher when compared to the glucose plus sulfate controls. The initial glucose degradation rates in the control cultures receiving glucose and glucose plus sulfate were 6.56 ± 0.18 and 5.81 ± 0.19 μg mg VSS⁻¹ min⁻¹, respectively. In the control cultures receiving only glucose, the levels reached undetectable levels after 6 h of incubation (Figure 4.5(A)). Noticeable levels of acetate were detected in all the controls except, those receiving LA plus sulfate. Detectable propionate levels were higher in the glucose controls (Figure 4.2(A)) when compared to glucose plus sulfate controls (Figure 4.2(A)). Note, no propionate was detected in the LA and LA plus sulfate controls. In addition, no butyrate was detected in the controls examined.
Acetate levels in the glucose cultures were similar to the glucose plus sulfate cultures in between days 3 and 6 (compare Figure 4.1(A) and Figure 4.2(A)). However, the acetate levels were higher in the glucose controls at the beginning 2 days and at the end of the experiment (Figure 4.1(A)). Propionate was detected in the glucose controls throughout the experimental duration (Figure 4.1(A)), unlike cultures receiving glucose and sulfate, where 430 mg l\(^{-1}\) propionate was detected only on day 1 (Figure 4.2(A)) and thereafter no propionate was detected. The acetate and propionate concentrations varied between 530 to 1000 mg l\(^{-1}\) and 0 to 300 mg l\(^{-1}\), respectively, in the cultures receiving glucose (Figure 4.1(A)); while, the acetate levels were between 220 to 670 mg l\(^{-1}\) in the sulfate plus glucose controls over the duration of the study (Figure 4.2(A)). Low levels of acetate were observed in control cultures receiving varying concentrations of LA (Figure 4.3(A)) when compared to the glucose or glucose plus sulfate control. Less than 100 mg l\(^{-1}\) of acetate was observed in cultures receiving \(\leq 700\) mg l\(^{-1}\) LA and 125 mg l\(^{-1}\) acetate was observed in cultures receiving 1000 mg l\(^{-1}\) LA.

Small amounts (maximum of 11 \(\pm\) 1.2 %) of sulfate reduction were noticed in the control cultures receiving sulfate and varying concentrations of LA (Figure 4.4(A)). Also, the sulfate reduction levels were low (13 % lower) in the LA controls (Figure 4.4(A)) when compared to the cultures receiving glucose (Figure 4.2(C)). The percent sulfate removed in cultures receiving glucose was approximately 24 \(\pm\) 3.7 %. Less than 10 % reduction in sulfate was observed in the controls receiving only sulfate. The initial sulfate degradation rates were 0.01 \(\pm\) 0.0 and 0.05 \(\pm\) 0.01 \(\mu\)g mg VSS\(^{-1}\) min\(^{-1}\) in the controls cultures receiving only sulfate and sulfate plus glucose, respectively.
Figure 4.1: Concentration profiles for control cultures fed with 1,870 mg l⁻¹ glucose. (A = VFAs (Acetate and Propionate) and B = Methane. Values shown are mean and standard deviation for triplicate samples).
The amount of methane produced (Figure 4.2(B)) in cultures receiving glucose and sulfate compared to cultures receiving only glucose was less (Figure 4.1(A)). However, an increasing trend in methane production was observed in all the controls. The methane concentration levels at the end of the experimental duration were 210 ± 9.0 and 310 ± 11 μmoles, respectively, in glucose plus sulfate and glucose controls. Methane levels were low in the control cultures receiving LA (Figure 4.3(B)). Also, the methane levels were lower in the cultures receiving LA when compared to the control with only cultures (Figure 4.3(B)).

**4.3.2 Cultures receiving glucose, sulfate and LA**

Sulfate (1,500 mg l⁻¹) reduction and glucose (1,870 mg l⁻¹) degradation was examined in the presence of varying LA levels. A reduction in the glucose degradation rates was observed when sulfate and LA were added to the cultures (Table 4.1). The Tukey’s paired comparison test procedure at 95 % confidence level was used to statistically compare each data set [Box et al., 1978]. Notice the initial glucose degradation rates decreased with increasing LA concentrations (Table 4.1). The initial glucose degradation rates for the cultures receiving 1000 mg l⁻¹ LA was 2.53 ± 0.33 μg mg VSS⁻¹ min⁻¹ while, the degradation rate for 100 mg l⁻¹ LA was 5.40 ± 0.10 μg mg VSS⁻¹ min⁻¹.

The glucose degradation rates in the glucose control cultures, in comparison to the control cultures receiving sulfate and cultures receiving LA plus sulfate were statistically different. However, cultures receiving ≤ 100 and 500 mg l⁻¹ LA were statistically same as the sulfate plus glucose controls.
Figure 4.2: Concentration profiles for control cultures fed with 1,870 mg l\(^{-1}\) glucose and 1,500 mg l\(^{-1}\) sulfate. (A = VFAs (Acetate and Propionate); B = Methane and C = Sulfate. Values shown are mean and standard deviation for triplicate samples).

Table 4.1: Initial glucose degradation rates determined using non-linear regression analysis.

<table>
<thead>
<tr>
<th>LA Concentration (mg l(^{-1}))</th>
<th>Initial glucose degradation rates ((\mu g) mgVSS(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control (Glucose))</td>
<td>6.56 ± 0.18(^{a})</td>
</tr>
<tr>
<td>0 (Control (Glucose plus Sulfate))</td>
<td>5.81 ± 0.19(^{b})</td>
</tr>
<tr>
<td>100</td>
<td>5.40 ± 0.10(^{c})</td>
</tr>
<tr>
<td>300</td>
<td>4.57 ± 0.21(^{d})</td>
</tr>
<tr>
<td>500</td>
<td>4.29 ± 0.05(^{e})</td>
</tr>
<tr>
<td>700</td>
<td>3.16 ± 0.15(^{e})</td>
</tr>
<tr>
<td>1000</td>
<td>2.53 ± 0.33(^{f})</td>
</tr>
</tbody>
</table>

Note: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations a, b, c, d, e and f are used to indicate the means that are statistically different within the same column.

2. Comparisons are based on adjacent data sets.
Figure 4.3: Concentration profiles for control cultures fed with 100 to 1,000 mg l$^{-1}$ LA. (A = Acetate; B = Methane. Values shown are mean and standard deviation for triplicate samples. C1 = Culture control).
Figure 4.4: Concentration profiles for control cultures fed with 1,500 mg l$^{-1}$ sulfate and 100 to 1,000 mg l$^{-1}$ LA. (A = Sulfate; B = Methane). Values shown are mean and standard deviation for triplicate samples. C1= Culture control; C2= Sulfate control.)
Glucose degradation profiles for cultures receiving LA and sulfate are shown in Figure 4.5. The glucose levels in the cultures receiving 100 to 500 mg l\(^{-1}\) LA reached low levels (up to 50 mg l\(^{-1}\)) before 6 h and below detection after approximately 8 h of glucose injection. However, in cultures receiving 700 and 1000 mg l\(^{-1}\) LA, the glucose levels reached below detection at approximately 10 h. The data on initial glucose degradation rates clearly reveals that LA exerted an inhibitory effect on glucose degradation when compared to adding sulfate. According to the data, the degree of inhibition increased with the increasing concentration of LA.

Acetate and propionate were the major VFAs detected from glucose degradation. Results of acetate formation for all the conditions examined are shown in Figure 4.5(C). These results clearly indicate that acetate profile trends were similar in cultures receiving sulfate and LA and the day 1 acetate levels in these reactors were less than two times the glucose control cultures. In all the cultures fed with sulfate, glucose and LA, acetate levels peaked in between day 2 and 5. The acetate levels ranged between 280 to 750 mg l\(^{-1}\) in the cultures receiving LA and sulfate between days 1 and 7 inclusive (Figure 4.5(C)). No particular trend was noticed in the acetate levels on the day 7 with the increasing concentration of LA. However, cultures receiving \(\geq 500\) mg l\(^{-1}\) showed decreasing acetate levels which ranged between 450 to 510 mg l\(^{-1}\) with increasing concentration of LA. The day 7 acetate concentrations were, 640 and 300 mg l\(^{-1}\) in cultures receiving 100 and 300 mg l\(^{-1}\) LA.

Propionate levels peaked on day 1 in all the reactors receiving sulfate plus LA at all concentrations (Figure 4.5(D)). A decreasing trend in propionate levels was observed with the increasing acetate concentrations. The propionate concentrations ranged
between 320 to 940 mg l\(^{-1}\) on day 1 and were reduced to below 100 mg l\(^{-1}\) concentrations on day 2. However, an increasing trend in propionate was observed in cultures fed with only glucose and undetectable levels were recorded on day 1. Note no propionate was detected in cultures receiving \(>300\) mg l\(^{-1}\) LA on day 7. However, the day 7 propionate level was 140 mg l\(^{-1}\) in the cultures receiving 100 mg l\(^{-1}\) LA.

Sulfate profiles in the cultures receiving glucose, sulfate and LA and glucose plus sulfate controls are shown in Figure 4.5(B). Increasing sulfate reduction and decreasing methane production were observed with increasing LA levels. The methane formation and sulfate reduction data suggest that LA selectively inhibited MPB without affecting the activity of SRB. Higher sulfate reduction was observed in cultures fed with high LA concentrations. In cultures receiving 1000 mg l\(^{-1}\) LA, the large sulfate reduction of 92 ± 0.3 % was 68 % more than that amount removed in the glucose controls. The quantity of sulfate removed in the cultures fed with 100, 300, 500 and 700 mg l\(^{-1}\) LA were 62 ± 2, 66 ± 0.4, 77 ± 10 and 84 ± 9 %, respectively. The sulfate reduction data for cultures receiving glucose plus LA depicted a decreasing trend in acetate levels with an increase in sulfate reduction.

Initial sulfate degradation rates are shown in Table 4.2. A Tukey’s paired comparison test at 95 % confidence level indicated that the initial sulfate degradation rates in all the cultures were statistically similar. An increase in initial sulfate degradation rates was observed as the LA levels increased. The initial sulfate degradation rates increased up to approximately 74 % in the cultures receiving LA when compared to glucose control. The highest initial sulfate degradation rate in the cultures receiving LA was 0.19 ± 0.07 \(\mu g\) mg VSS\(^{-1}\) min\(^{-1}\).
However, sulfate reduction in the cultures receiving only LA and sulfate were negligible (≤ 11 ± 1 %) when compared to the 9 % sulfate removal in the sulfate plus culture controls. This indicates that LA did not contribute to electron flux during sulfate reduction (Figure 4.6).

Table 4.2: Initial sulfate degradation rates determined using non-linear regression analysis.

<table>
<thead>
<tr>
<th>LA Conc. (mg l⁻¹)</th>
<th>Initial sulfate degradation rates (μg mgVSS⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control (Sulfate))</td>
<td>0.01 ± 0.00 a</td>
</tr>
<tr>
<td>0 (Control (Glucose plus Sulfate))</td>
<td>0.05 ± 0.01 a</td>
</tr>
<tr>
<td>100</td>
<td>0.09 ± 0.03 a</td>
</tr>
<tr>
<td>300</td>
<td>0.10 ± 0.02 a</td>
</tr>
<tr>
<td>500</td>
<td>0.14 ± 0.03 a</td>
</tr>
<tr>
<td>700</td>
<td>0.15 ± 0.02 a</td>
</tr>
<tr>
<td>1000</td>
<td>0.19 ± 0.07 a</td>
</tr>
</tbody>
</table>

Note: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations a are used to indicate the means that are statistically similar within the same column.
2. Comparisons are based on adjacent data sets.
Figure 4.5: Concentration profiles for cultures fed with 1,870 mg l\(^{-1}\) glucose, 1,500 mg l\(^{-1}\) sulfate and 100 to 1,000 mg l\(^{-1}\) LA. (A = Glucose, B = sulfate, C =Acetate, D = Propionate, E = Methane. Values shown are mean and standard deviation for triplicate samples. C1= Culture control; C2= Sulfate control; C3= Glucose control; C4= Glucose plus sulfate control).
Methane and carbon-dioxide were observed and no hydrogen was detected in any of the cultures. Methane formation decreased as a result of sulfate and LA addition (Figure 4.5(E)). Methane formation was the least in cultures receiving elevated LA concentrations. Methane formation in the cultures receiving ≥700 mg l⁻¹ showed a decrease by more than 73 % and 60 % respectively, when compared to the cultures receiving only glucose and glucose plus sulfate.

![Graph showing percentage sulfate removed vs. LCFA Concentration](image)

**Figure 4.6:** Percentage sulfate reduced in cultures receiving 1,870 mg l⁻¹ glucose, 1,500 mg l⁻¹ sulfate and 100 to 1,000 mg l⁻¹ LA and in controls receiving 100 to 1,000 mg l⁻¹ LA and sulfate. (Values shown are mean and standard deviation for triplicate samples. C2= Sulfate control; C4= Glucose plus sulfate control).

The total electron mass balance and the percentage electron flow to specific end products are shown in Figure 4.7. Sample electron balance calculations are shown in Appendix F. The data indicate that the percentage electron flow of electron fluxes towards sulfidogenesis increased with the increasing concentration of LA. Small amounts, less than 3 %, of the electrons were used for methane formation (Figure 4.7(C)).
While, 42 % and 45 % electrons were used for sulfate reduction in cultures receiving 700 and 1000 mg l\(^{-1}\) LA, respectively. (Figure 4.7(D)). These values ranged between 30 % to 38 % for cultures receiving \(\leq 500\) mg l\(^{-1}\) LA.
Figure 4.7: Electron mass balance for cultures fed with 1,870 mg l\(^{-1}\) glucose, 1,500 mg l\(^{-1}\) sulfate and 100 to 1,000 mg l\(^{-1}\) LA. (A = Total electron mass balance; B = Percent electrons diverted to VFAs; C = Percent electrons diverted to methane; D = Percent electrons diverted to sulfate reduction; Assume 15% of the electrons from glucose degradation is converted into biomass; Averages for triplicate samples and error bars (represents standard deviation) for the samples are shown. C3= Glucose control; C4= Glucose plus sulfate control).
There was an increase of approximately 34% of the electron flow toward sulfate reduction in cultures receiving 1000 mg l\(^{-1}\) LA when compared to the cultures receiving glucose on day 7. The percentage electron flow to VFA formation was significant throughout the experimental duration and ranged between 16% to 52% on day 7. On day 1, the electron mass balance in some cultures was greater than 100% and reasons for this observation are unclear. Possible causes could be attributed to electrons arising from biomass decay byproducts or to electrons from LA degradation.

4.4 Discussion

In this work, selectively inhibiting MPB using LA and diverting electron equivalents to sulfate reduction by SRB was clearly demonstrated. The low percentage (≤ 0.6% for cultures receiving ≥ 700 mg l\(^{-1}\) LA) flow of electrons to MPBs in the presence of LA confirms the inhibition of hydrogenotrophic and aceticlastic methanogens. Subsequently, the electron equivalents were available to SRBs for sulfate reduction. The electron mass balance and sulfate reduction data clearly demonstrates that the electron equivalents from glucose degradation were used for sulfidogenesis while MPBs were inhibited due to the presence of LA.

Hydrogen and VFAs such as acetate and propionate constituted the electron equivalents to SRBs. Hydrogen is produced by the acidogenic reactions in the anaerobic fermentation of glucose and is used by, terminal electron acceptors for the formation of end products (methane or sulfide), and homoacetogens to form acetate. The terminal gas (methane or sulfide) formation is controlled by the presence or absence of the type of terminal electron acceptor in the system [Smith and Mah, 1965]. Researchers have reported accumulation of hydrogen due to inhibiting MPB by LA [Lalman and Bagley,
SRBs can out-compete methanogens and homoacetogens for hydrogen easily [Stams et al., 2005]. Hence, the absence of hydrogen in the headspace gases detected while inhibiting hydrogenotrophic methanogens suggest that all the hydrogen was mainly used for sulfate reduction.

A significant decrease in propionate levels after day 1 in all the cultures receiving LA was likely due to its consumption by SRBs for sulfate reduction and degradation by acetogens to acetate. Mixotrophic acetate consuming SRB use acetate only when higher VFAs are depleted [Laanbroek et al., 1984]. In sulfidogenic breakdown of VFA, two oxidation patterns are distinguishable. Some SRBs can completely oxidize VFA to CO₂ and sulfide as end products (Eq. 2.44), while others lacking the tricarboxylic acid cycle, carry out an incomplete oxidation of VFA with acetate and sulfide as end products (Table 2.1, Eqs., 2.19 and 2.20) [Lens et al., 1998].

A variety of SRB can be involved in propionate degradation. Propionate degrading Desulfobulbus sp. can couple propionate oxidation with sulfate reduction when sulfate is present in excess. Desulfobulbus propionicus and Desulfobulbus elongates oxidize propionate incompletely to acetate (Table 2.1: Eq. 2.19) [Widdel and Pfennig, 1982]. SRBs compete with the syntrophic acetogenic bacteria for propionate in anaerobic environments when sulfate is present and this competition eventually leads to better growth kinetic properties [Visser et al., 1993; Isa et al., 1986]. The reduction of propionate levels in controls receiving glucose and sulfate coupled with sulfate reduction when compared to the propionate levels in the glucose controls, suggest that SRB out-compete acetogenic bacteria for propionate. However, in this study no distinction could
be made between direct oxidation of propionate by SRB and syntrophic propionate degradation by acetogenic bacteria with hydrogen consuming SRB or MPB.

LA inhibited hydrogenotrophic and aceticlastic methanogens and the level of inhibition increased with the increase in LA concentration. This was clearly illustrated by the reducing methane levels in the cultures with increasing concentrations of LA. The available acetate and hydrogen from glucose were used by the SRBs for sulfate reduction, while methanogens were inhibited due to LA. This was also supported by the trend of acetate levels in these cultures; increase in acetate levels up to day 4 followed by a decrease was observed with the increase in LA levels. Hence, SRBs were able to utilize acetate better in the presence of high levels of LA. Desulfococcus, Desulfosarcina, and Desulfonema are able to oxidize acetate to CO2. Increase in CO2 (data not shown) levels in all the cultures receiving LA suggest that acetate degrading SRB completely oxidized acetate to CO2 and H2S.

The glucose degradation data suggest that LA exerted some inhibition on acidogenic microorganisms, while they were not inhibited to the same degree as hydrogenotrophic and aceticlastic methanogens. Glucose removal from the cultures took place within 10 hrs of glucose injection and propionate levels peaked within a short duration of 24 hrs of glucose injection. The propionate levels were greater in cultures receiving low LA concentrations, which justify the inhibition of acidogens by LA. However, more than 80 % of the propionate was removed from the reactors by sulfidogenic or acetogenic microorganisms. The glucose degradation data suggest that the degree of inhibition increased with the increasing concentration of LA. The initial glucose degradation data ranged between 17 % (in cultures receiving 100 mg l\(^{-1}\)) to 61 % (in cultures receiving
1000 mg l\(^{-1}\)) of the maximum rates in the glucose controls. A similar reduction in the initial glucose degradation was reported in studies conducted by Lalman et al. [2003].

SRBs were likely not inhibited due to the presence of LA. The initial sulfate degradation data suggest that the SRB activity was enhanced with the increasing concentration of LA and this was likely due to an increase in methanogenic inhibition with the increasing LA levels. Selective aceticlastic methanogenic inhibition was reported at as low as 30 mg l\(^{-1}\) LA concentrations and higher concentration impaired the hydrogenotrophic methanogenic activity completely [Lalman and Bagley, 2002; Demeyer and Hendricks, 1967]. Decreasing methane profiles with increasing LA concentrations also validate the selective inhibition of MPB when compared to SRB. The initial sulfate degradation rates ranged between 0.09 ± 0.03 and 0.19 ± 0.07 μg mg VSS\(^{-1}\) min\(^{-1}\) in the cultures receiving LA. The initial sulfate degradation rates in the cultures receiving glucose were 74% less than the maximum rates in the 1000 mg l\(^{-1}\) LA cultures (Table 4.2).

The presence of sulfide could have also inhibited the methanogenic activity, which was clearly demonstrated from the methanogenic activity in the sulfate plus glucose controls. The methane levels in the cultures receiving glucose and sulfate were less than cultures receiving only glucose throughout the experimental duration. The methane levels were 62% more in glucose cultures when compared to the maximum methane levels in cultures receiving glucose and sulfate. The latter could be due to the sulfide inhibition of methanogens. H\(_2\)S produced from microbial reduction of sulfate is inhibitory to MPB and SRB. MPBs are more sensitive than SRBs [Isa et al., 1986] and acidogens [Lens et al., 1998] to H\(_2\)S inhibition. The cause for inhibition is unclear;
however, some researchers argue that inhibition may be the result of an intrinsic toxicity of H$_2$S. The toxic effect may be attributed to the non-ionized state of H$_2$S, which allows it to cross the cell membrane more readily. In the cell, the uncharged species may interfere with the sulfur metabolism or affect the intracellular pH, which denatures native protein or metabolic enzymes [Oude et al., 1994; Reis et al., 1992]. Some researchers have argued that the toxicity might be due to inactivation of iron by converting it into an insoluble ferrous sulfide (FeS). Iron is required for the function of cell constituents such as ferrodoxin and cytochrome [Reis et al., 1992]. In addition, the metal sulfides formed appear to act as barriers, which could prevent the access of reactants to the necessary enzymes [Utgikar et al., 2002]. Researchers have reported that in the pH range of 7.0 - 7.5, the presence of sulfide has no important additional effect on the competition between acetate degrading SRB and MPB [Visser et al., 1996]. These authors reported that the inhibitory effect of un-dissociated H$_2$S on MPBs as well as SRBs was stronger at higher pH values and the growth is more strongly inhibited than the activity. However, in this study at a pH of 7.0 to 7.2, MPBs could have been inhibited more by sulfide. Note the degree of sulfide inhibition on SRBs and MPBs was not assessed during this study.

In control studies with LCFAs plus sulfate, LCFAs did not serve as an electron donor during the experimental duration. This was clear from the percentage of sulfate reduction in the cultures receiving LA. The sulfate reduction in these cultures was approximately same as that of the cultures receiving only sulfate (approximately 10 %). Hence, the electrons used for sulfate reduction was assumed to be provided by glucose in the glucose, sulfate and LCFA experiments. However, note very low levels of acetate were observed in LA controls experiments. LA is degraded to acetate and H$_2$ by a slow β-
oxidation process [Weng and Jesris, 1976]. Hence, LA itself could serve as an electron source for sulfate reduction.

4.5 Conclusion

Sulfate reduction using a batch mixed anaerobic culture acclimated to glucose and sulfate at 37°C and pH 7.0 - 7.2 was assessed in the presence of varying LA concentrations ranging from 100 to 1000 mg l⁻¹. Several studies on sulfate reduction using physical and chemical techniques, to reduce the competition between SRB and MPB have been reported. However, these techniques are reversible, expensive or toxic in terms of practical application, for example use of elevated temperatures, variation of pH and use of chemicals (BES, chloroform, and halogenated carbons). Use of LA to inhibit MPB is a novel approach in enhancing sulfate reduction techniques. LA is slowly degrading and easily available from various industrial sources. The conclusions of this study are as follows:

1. LA caused a metabolic shift in the syntrophic microbial pathway by diverting the electron fluxes to SRB and away from MPB. This was evident from the sulfate reduction and electron balance data. The diversion of electron fluxes was a function of LA concentration and hence, LA selectively inhibited MPB activity.

2. Adding LA resulted in an increase of > 30 % in the electron flow to sulfate reduction compared to the cultures receiving only glucose and sulfate.

3. Sulfate reduction increased with the increasing concentrations of LA in all the cultures. Meanwhile, MPB inhibition was a function of LA concentration, the inhibition increased with the increasing concentration of LA.
4. Greater than 90% sulfate reduction was observed in the cultures receiving 1,870 mg l\(^{-1}\) glucose and 1000 mg l\(^{-1}\) LA.

5. Acetate and propionate were the two major VFA produced from glucose degradation.
4.6 References


CHAPTER 5: INHIBITING METHANOGENESIS AND DIVERTING ELECTRON FLUXES TOWARDS SULFATE REDUCTION IN MIXED ANAEROBIC COMMUNITIES USING OLEIC AND STEARIC ACIDS

5.1 Introduction

Increasingly, regulatory agencies are implementing stringent regulations to control the discharge of effluents containing elevated sulfate levels. In a response to prevent environmental damage from these effluents, researchers are developing chemical and biological treatment technologies. Chemical processes utilize lime and barium salts (barium hydroxide and barium carbonate) to precipitate sulfate salts [Maree et al., 2004; Bosman, 1990]. Biological processes, in comparison, convert sulfate into sulfide utilizing electrons from electron donor compounds. Biological treatment processes are advantageous because expensive chemicals are not utilized and less energy is used [Lettinga, 1995]. Under anaerobic conditions, sulfate is converted into sulfide, a potent toxic compound. During biological treatment, electrons from donor compounds are utilized for sulfate reduction by SRBs. SRBs are obligate anaerobes which utilize organic electron donors.

Biological sulfate reduction occurs in two stages. In the first stage, SRBs oxidize organic substrates and reduce sulfate (SO$_4^{2-}$) into hydrogen sulfide (HS') (Eq. 5.1) under anaerobic conditions. In the second step, hydrogen sulfide (HS') is oxidized to elemental sulfur (S) by chemotrophs (Eq. 5.2). Sulfide produced by SRB in the first step can also be removed by precipitation using heavy metals [El Bayoumy et al., 1999].

\[
\text{Organic matter} + \text{SO}_4^{2-} \rightarrow \text{HS'} + \text{H}_2\text{O} + \text{HCO}_3^- \quad (5.1)
\]

\[
\text{O}_2(\text{g}) + 2\text{HS'} + 2\text{H}^+ \rightarrow 2\text{S(s)} + 2\text{H}_2\text{O} \quad (5.2)
\]
The type of the electron donor carbon source utilized for $\text{SO}_4^{2-}$ reduction varies according to the genus. However, the preferred carbon sources are low-molecular-weight compounds such as organic acids (e.g. acetate, lactate, pyruvate, formate, and malate), alcohols (e.g., ethanol, propanol, methanol, and butanol), occasionally sugars and longer chain fatty acids [Weijma et al., 2002]. The latter compounds are byproducts from the anaerobic conversion of carbohydrates, proteins and lipids. Thus, SRB are terminal degraders, and their role is analogous to that of methanogens.

Sulfidogens and methanogens coexist in mixed anaerobic communities and they compete for electron donors such as hydrogen and acetate [Lens et al., 1998]. Competition between these organisms depends on factors such as organic and sulfate loading rates, COD to sulfur ratio [Bhattacharya et al., 1996; Choi and Rim, 1991;], pH [Isa et al., 1986], reactor design [Isa et al., 1986] temperature [Shin et al., 1996] and hydraulic retention time (HRT) [Omil et al., 1998; Isa et al., 1986]. When compared to methanogens, SRBs have a greater affinity for substrates such as acetate, formate and hydrogen [McCartney and Oleszkiewicz, 1993; Isa et al., 1986]. The affinity for simple substrates leads to more favorable growth conditions for SRBs; however, a fraction of the donor compounds is available for methanogenic growth.

The COD/$\text{SO}_4^{2-}$ ratio and organic loading are major variables controlling the diversion of electron flux to SRBs. Oxidation of lactate with sulfate reduction by SRBs has been shown to proceed without the formation of intermediate products such as propionate and butyrate at low COD/$\text{SO}_4^{2-}$ ratios [Harada, et al., 1994; McCartney and Oleszkiewicz, 1993]. Lowering the COD/$\text{SO}_4^{2-}$ ratio in the range of 0.34 (g g$^{-1}$) is favorable for sulfate reduction over methanogenesis. In continuous treatment systems, increasing the organic
loading from 1.0 kg COD m$^{-3}$ d$^{-1}$ to 3.0 kg COD m$^{-3}$ d$^{-1}$ diverted a larger fraction of electron flow to the SRBs by approximately a factor of 2 to 75% [Harada et al., 1994]. Compared to methanogens, increasing temperatures are more favorable for SRBs growth. In mixed culture treatment systems operating at elevated temperatures, methanogens are strongly suppressed with a large fraction of the electron flow distributed to the SRBs [Shin et al., 1996].

In spite of using engineering as well as environmental factors to control the electron flow between methanogens and SRBs, a competition for acetate and hydrogen always exist. Hence, researchers are developing methods to minimize the electron flow for methane production and instead optimize the fraction for sulfate reduction.

Diverting electron fluxes to optimize the production of a specific product is of a major importance in anaerobic bioprocesses. Diverting a larger fraction of electron fluxes into sulfate reduction increases the process efficiency in the sulfate reducing systems. Using physical and chemical methods to control the electron flow in mixed anaerobic communities has been described in several studies. Work by Oh et al. [2003] and Sung et al. [2002] has demonstrated diverting electron flow by controlling the growth of hydrogen consuming microorganisms such as hydrogenotrophic methanogens in anaerobic communities. Although the latter reports provided compelling evidence of methanogenic growth control in the short-term, these studies; however, did not demonstrate long-term sustained growth control. Over the long-term, after the stress is relieved, microbial growth resumes as conditions become favorable.

Microbial inhibition of selective anaerobic populations and subsequent diversion of electron fluxes has been reported using a variety of chemicals. Inhibition of methane
production has been shown using 2-bromoethanesulphonate (BES) [Scholten et al., 2000; Oremland and Capone, 1988] and ethylene [Oremland and Taylor, 1975]. However, these chemicals are toxic and not cost effective in full-scale applications.

Using methanogenic inhibitors which are cost effective and also degradable is an innovative approach for diverting electron fluxes to SRBs in mixed anaerobic communities. Electron fluxes generated from the degradation of these inhibitor compounds could be useful for sulfate reduction. Recent work by Lalman and Bagley [2002] and Lalman and Bagley [2001] have shown LCFA possessing 18 carbons are suitable methanogenic inhibitors. These acids are easily available, relatively inexpensive and slowly degrading [Lalman and Bagley, 2001; Rinzema, et al., 1994; Angelidaki and Ahring, 1992]. LCFAs usually present in effluents from many food processing industries can serve as useful methanogenic inhibitors and electron donors. The objective of this study was to examine the conversion of sulfate to sulfide by SRBs in the presence of two LCFAs, OA (C18:1) and SA (C18:0) under mesophilic conditions.

5.2 Experimental Methods and Materials

The materials and methods for this phase of experiments are described in Chapter 3: The inoculum source and start-up procedure is described in section 3.1.1. The experimental plan and design are given in section 3.2.1 and the analytical methods used are detailed in section 3.3. Initial glucose and sulfate degradation rates were calculated using Graphpad Prism, a software using a non-linear regression by a one-phase exponential decay model. A sample calculation for initial glucose degradation rate is shown in Appendix E. The initial sulfate degradation rates were calculated in similar manner.
5.3 Results

5.3.1 Control Cultures

Control cultures were prepared with the following substrates: glucose, glucose plus sulfate, and LCFA plus sulfate. In controls receiving glucose plus sulfate, the glucose degradation rate was less when compared to those fed only glucose. In control cultures fed with glucose, the levels were undetectable after 6 h. Noticeable quantities of acetate were detected in all the bottles except in the controls not receiving any glucose. Under all the conditions examined for controls, propionate was observed only in the glucose controls and butyrate was not detected.

The amount of methane produced in the control cultures containing glucose (Figure 5.1) was greater than cultures receiving glucose and sulfate (Figure 5.2). The propionate levels in controls receiving only glucose were greater throughout the experimental duration than in controls fed glucose plus sulfate, except for day 1 (Figure 5.2(A)). Over the duration of the study, the acetate and propionate levels were approximately between 680 mg l\(^{-1}\) to 860 mg l\(^{-1}\) and 250 mg l\(^{-1}\) to 1000 mg l\(^{-1}\), respectively, in cultures receiving glucose (Figure 5.1(A)). As shown in Figure 5.2(A) the acetate levels varied between 640 mg l\(^{-1}\) and 1150 mg l\(^{-1}\) and propionate levels reached approximately 1150 mg l\(^{-1}\) on day 1 and reduced to less than 10 mg l\(^{-1}\) on day 2 while less than 24 % of the sulfate was removed in the cultures containing glucose plus sulfate. In controls containing LCFAs (OA or SA) and LCFAs (OA or SA) plus sulfate, low acetate and methane levels were produced over the duration of the study (Figures 5.3, 5.4, 5.5 and 5.6).
Figure 5.1: Concentration profiles for control cultures fed with 1,870 mg l\(^{-1}\) glucose. (A = VFAs (Acetate and Propionate) and B = Methane. Values shown are mean and standard deviation for triplicate samples).
The quantity of sulfate removed in the controls receiving only sulfate and LCFAs plus sulfate were approximately 10% (Figure 5.7(B) and 11% (1000 mg l⁻¹ OA or SA), respectively (Figure 5.5(A) and 5.6(A)). Higher sulfate reduction (32%) was observed in the glucose controls compared to sulfate and sulfate plus LA controls (Figure 5.2(C)).

Relative to the glucose controls containing sulfate (Figure 5.2(B)), larger quantities of methane was produced in the controls fed with only glucose (approximately 312 μmoles / bottle) (Figure 5.1(B)). Approximately 200 μmoles / bottle of methane were produced in the controls fed with glucose and sulfate (Figure 5.2(B)). Low methane levels (approximately < 80 μmoles / bottle for 1000 mg l⁻¹ OA or SA) were observed in controls fed only LCFAs or sulfate (Figure 5.3(B), 5.4(B) and 5.7(E)). Over the duration of the experiment, the methane levels in the sulfate plus glucose control were approximately 30% less than the glucose control reactors.
Figure 5.2: Concentration profiles for control cultures fed with 1,870 mg l\(^{-1}\) glucose and 1,500 mg l\(^{-1}\) sulfate. (A = VFAs (Acetate and Propionate); B = Methane and C = Sulfate. Values shown are mean and standard deviation for triplicate samples).
Figure 5.3: Concentration profiles for control cultures fed with 100 to 1,000 mg l$^{-1}$ OA. (A = Acetate; B = Methane. Values shown are mean and standard deviation for triplicate samples. C1 = Culture control).
Figure 5.4: Concentration profiles for control cultures fed with 100 to 1,000 mg l\(^{-1}\) SA. (A = Acetate; B = Methane. Values shown are mean and standard deviation for triplicate samples. C1 = Culture control).
Figure 5.5: Concentration profiles for control cultures fed with 1,500 mg l\(^{-1}\) sulfate and 100 to 1,000 mg l\(^{-1}\) OA. (A = Sulfate B = Acetate; C = Methane. Values shown are mean and standard deviation for triplicate samples. C1= Culture control; C2= Sulfate control).
Figure 5.6: Concentration profiles for control cultures fed with 1,500 mg l⁻¹ sulfate and 100 to 1,000 mg l⁻¹ SA. (A = Sulfate; B = Acetate; C = Methane. Values shown are mean and standard deviation for triplicate samples. C1 = Culture control; C2 = Sulfate control).
5.3.2 Cultures receiving glucose, sulfate and LCFA (OA or SA)

Glucose degradation (1,870 mg l\(^{-1}\)) and sulfate (1,500 mg l\(^{-1}\)) removal were examined in presence of varying OA and SA levels. The initial degradation rates for the different conditions examined were used to assess the impact of sulfate and LCFAs on glucose removal (Table 5.1). Statistical differences between the mean values of the initial glucose degradation values were determined using the Tukey’s paired comparison procedure at the 95% confidence level [Box et al., 1978]. Statistically similar initial glucose degradation rates were observed for the cultures receiving sulfate plus OA and controls cultures receiving only glucose and glucose plus sulfate. The data demonstrated that the addition of sulfate and OA affected the initial glucose degradation rates while the presence of SA did not have any detectable effect. In comparison with the glucose control cultures, the initial glucose degradation values were statistically different in the cultures receiving SA and control cultures receiving glucose plus sulfate. However, statistically similar values were observed in the glucose plus sulfate control cultures and cultures receiving SA. Figures 5.7(A) and 5.8(A) show glucose degradation in the cultures receiving sulfate and, OA and SA, respectively. Cultures fed OA had lower glucose degradation rates compared to those receiving SA. Six hours after adding glucose, residual levels of approximately 100 mg l\(^{-1}\) were observed in cultures receiving 100 to 500 mg l\(^{-1}\) of OA. However, the same residual quantity observed in cultures receiving ≤ 500 mg l\(^{-1}\) OA was detected after 8 h in cultures receiving 700 and 1000 mg l\(^{-1}\). OA inhibited glucose degradation more when compared to cultures receiving SA and sulfate. The inhibitory effect caused by SA was greater than that due to sulfate. The initial
The glucose degradation rate trend for conditions examined was as follows: control cultures (only glucose) > sulfate controls (glucose plus sulfate) > SA > OA.

Acetate concentrations in cultures fed OA varied from 400 to 1,400 mg l\(^{-1}\) (Figure 5.7(C)) while lower levels between 100 to 1000 mg l\(^{-1}\) were detected in cultures receiving SA (Figure 5.8(C)). On day 1, the acetate concentrations in cultures receiving OA varied from approximately 800 to 1000 mg l\(^{-1}\). At the end of the study, on day 7, the acetate concentration in cultures receiving ≤ 500 mg l\(^{-1}\) OA reached approximately 1000 mg l\(^{-1}\) while levels of about 500 mg l\(^{-1}\) were observed in cultures fed ≥ 700 mg l\(^{-1}\) OA. In cultures fed with OA, no particular trends in the acetate profiles were observed; however, the peaked level (500 to 1100 mg l\(^{-1}\)) between days 2 to 4 in cultures fed with SA subsequently decreased over the 7 day duration of the study.

### Table 5.1: Initial glucose degradation rates determined using non-linear regression analysis.

<table>
<thead>
<tr>
<th>LCFA Concentration(mg\text{I}^{-1})</th>
<th>Initial glucose degradation rates (\mu{g mgVSS}^{-1} \text{ min}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OA</td>
</tr>
<tr>
<td>0</td>
<td>6.39 ± 0.28 (a)</td>
</tr>
<tr>
<td>(Control (Glucose))</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.81 ± 0.19 (a)</td>
</tr>
<tr>
<td>(Control (Glucose plus Sulfate))</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>5.36 ± 0.49 (a)</td>
</tr>
<tr>
<td>300</td>
<td>4.84 ± 0.21 (a)</td>
</tr>
<tr>
<td>500</td>
<td>4.30 ± 0.12 (a)</td>
</tr>
<tr>
<td>700</td>
<td>3.67 ± 0.28 (a)</td>
</tr>
<tr>
<td>1000</td>
<td>3.22 ± 0.14 (a)</td>
</tr>
</tbody>
</table>

Note: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations \(a\) and \(b\) are used to indicate the means that are statistically different within the same column. The notations cannot be used for comparing data within the same row or between rows.
2. Comparisons are based on adjacent data sets.

Cultures fed with OA, glucose and sulfate produced propionate similar to those receiving glucose. On day 1, the propionate concentration reached a maximum (700 to
1100 mg l\(^{-1}\)) and subsequently decreased to less than 10 mg l\(^{-1}\) in cultures fed OA (Figure 5.7(D)). The propionate profiles in the cultures receiving SA were similar to those fed OA; however, lower concentrations were detected. The levels peaked between days 1 to 3 and subsequently deceased to less than 50 mg l\(^{-1}\).

Sulfate removal was initiated after a 24 hour lag phase in cultures fed OA. The data suggest OA inhibited methanogenesis selectively and sulfate reduction was affected to a lesser extent. Sulfate removal was greater in cultures injected with high concentrations of OA. Significant removals of up to 820 and 1100 mg l\(^{-1}\) of sulfate were detected in cultures receiving 700 and 1000 mg l\(^{-1}\) OA, respectively. Sulfate removal was observed in all cultures receiving sulfate. In the controls receiving only sulfate (Figure 5.7(B)), glucose plus sulfate (Figure 5.2(C)) and LCFA plus sulfate (Figure 5.5(A) and 5.6(A)) the removal was approximately 8 %, 32 % and 11 %, respectively. The percent sulfate removed increased with increasing OA concentrations. The quantity removed were approximately 40 % in cultures fed 100, 300 and 500 mg l\(^{-1}\) OA while in cultures receiving 700 and 1000 mg l\(^{-1}\) OA, the removals increased to 55 and 73 %, respectively (Figure 5.7(B)). Figure 5.11 shows the percentage sulfate removed in the control cultures fed with only sulfate, glucose plus sulfate and glucose, sulfate plus LCFA (OA or SA). Addition of SA to the cultures did not aid in sulfate reduction. The percentage sulfate removal in the glucose plus sulfate control cultures and glucose plus sulfate and SA were similar.

In all the gas samples, CH\(_4\) and CO\(_2\) were detected; however, the quantity of CH\(_4\) varied depending on the type and quantity of substrate added to the culture. Methane production in the glucose controls (Figure 5.1(B)) was greater in comparison to cultures
fed with OA (Figure 5.7(E)) and SA (Figure 5.8(E)). The mass of methane produced in the glucose controls were approximately 65 % and 90 % greater when compared with the amount produced in the sulfate controls on day 1 and day 7, respectively. Increasing inhibition of methane production was observed with increasing OA levels. The extent of the inhibition was similar in cultures receiving 100 to 500 mg l$^{-1}$ OA and the inhibitory effect was greatest in the presence of 700 and 1000 mg l$^{-1}$ of OA. In cultures receiving SA, the inhibition of methane production was not a function of the LCFA concentration. Although methanogenic inhibition was observed in the presence of SA, the extent of the inhibition was less compared to OA.

Figure 5.9 and 5.10 shows the electron mass balance for the cultures receiving glucose, sulfate plus OA and SA, respectively. Sample electron balance calculations are shown in Appendix F.
Figure 5.7: Concentration profiles for cultures fed with 1,870 mg l\(^{-1}\) glucose, 1,500 mg l\(^{-1}\) sulfate and 100 to 1,000 mg l\(^{-1}\) OA. (A = Glucose, B = sulfate, C = Acetate, D = Propionate, E = Methane; Values shown are mean and standard deviation for triplicate samples). (C1 = Culture control; C2 = Sulfate control; C3 = Glucose control; C4 = Glucose plus sulfate control).
The electron mass balance data shows an increased percentage electron flow to sulfate reduction in the cultures fed OA compared to the cultures fed glucose (Figure 5.9(D)). The increase in electron flux to sulfidogenesis increased with increasing OA concentration. The percentage electron flow to sulfate reduction in the cultures fed OA plus glucose was 22% greater than the glucose plus sulfate controls. At the same time, the percentage electron flows decreased to methanogenesis with increase in OA concentrations (Figure 5.9(C)). However, SA did not significantly affect electron flow to sulfidogenesis (Figure 5.10(D)). Significant amounts of electrons were consumed for the formation of VFAs in the cultures receiving OA (Figure 5.9 (B)) or SA (Figure 5.10 (B)).
Electron percentage recovery greater than 100% in some cultures was likely due the biomass decay.

(A) % electron in products and cell biomass

(B) % electron in VFA products

- C3 - C4 - 100 mg l⁻¹ OA
- ▲ 300 mg l⁻¹ OA - ○ 500 mg l⁻¹ OA - ● 700 mg l⁻¹ OA
- ▼ 1000 mg l⁻¹ OA

Time (days)
Figure 5.9: Electron mass balance for cultures fed with 1,870 mg l\(^{-1}\) glucose, 1,500 mg l\(^{-1}\) sulfate and 100 to 1,000 mg l\(^{-1}\) OA. (A = Total electron mass balance; B = Percent electrons diverted to VFAs; C = Percent electrons diverted to methane; D = Percent electrons diverted to sulfate reduction; Assume 15% of the electrons from glucose degradation is converted into biomass; Averages for triplicate samples and error bars (represents standard deviation) for the samples are shown. C3 = Glucose control; C4 = Glucose plus sulfate control).
% electron in VFA products

% electron in products and cell biomass
Figure 5.10: Electron mass balance for cultures fed with 1,870 mg l\textsuperscript{-1} glucose, 1,500 mg l\textsuperscript{-1} sulfate and 100 to 1,000 mg l\textsuperscript{-1} SA. (A = Total electron mass balance; B = Percent electrons diverted to VFAs; C = Percent electrons diverted to methane; D = Percent electrons diverted to sulfate reduction; Assume 15\% of the electrons from glucose degradation is converted into biomass; Averages for triplicate samples and error bars (represents standard deviation) for the samples are shown). (C3 = Glucose control; C4 = Glucose plus sulfate control).
Figure 5.11: Percentage sulfate converted to sulfide in cultures receiving 1,870 mg l\(^{-1}\) glucose, 1,500 mg l\(^{-1}\) sulfate and 100 to 1,000 mg l\(^{-1}\) OA and SA. (Values shown are mean and standard deviation for triplicate samples. C2= Sulfate control; C3= Glucose control).

5.4 Discussion

In this work, diverting electron fluxes to sulfate reduction by inhibiting methanogens was achieved using OA. Relative to the controls receiving sulfate and glucose, the presence of OA caused a diversion of the electron flux from methane production and towards sulfate reduction. OA selectively inhibited methane production without significantly affecting sulfate reduction. The selective inhibition of methane production by LCFAs is supported by work reported by Nieman [1954] who illustrated that unsaturated LCFAs inhibited gram positive bacteria (MPB) but no effect was observed on gram negative microorganisms (SRB).
Evidence demonstrating the diversion of electron fluxes in anaerobic communities has been reported by several researchers. Oh et al. [2003] and Fang and Liu [2002] has shown that electron fluxes in mixed anaerobic communities can be diverted to hydrogen production by inhibiting hydrogenotrophic methanogens. However, in these studies the methanogenic activity reversed with the release of the inhibiting factors. On comparison, the inhibitory effect of LCFAs such as OA on methanogens is reported to be permanent [Rinzema et al., 1994; Angelidaki and Ahring, 1992; Rinzema et al., 1989; Koster and Cramer, 1987]. In this work, inhibition of methanogenesis with OA was likely irreversible. Evidence of this is clear from the electron mass balance data and the methane profiles in the cultures receiving OA, glucose and sulfate. However, note the experiments were performed for a 7-day period.

Compared to OA, SA did not cause a shift in electron flow to sulfate reduction. The initial sulfate degradation rates for cultures receiving SA and OA, suggested that SA did not aid in sulfate reduction, while OA enhanced sulfate reduction (Table 5.2). Statistically similar initial sulfate degradation rates were observed in all the cultures receiving LCFAs and controls (glucose and glucose plus sulfate). SA had no significant effect on sulfate removal because saturated LCFAs such as SA are significantly less inhibitory to methanogens when compared to the LCFAs bearing unsaturated bonds and the extent of inhibition increases with the increase in double bonds [Nieman, 1954]. Aceticlastic methanogens are severely inhibited by OA while SA had no effect [Lalman and Bagley, 2001]. The sulfate reduction data clearly demonstrates a diversion of electron flux towards sulfate reduction. An increase in sulfate reduction and a decrease in methane production strongly indicate that methanogenic inhibition increased with
increasing OA concentration. Hence, the percentage electron flux diverted to sulfate reduction increased with an increase in OA concentrations as methanogenic inhibition increased. LCFA toxicity to methanogens is concentration dependent and increases with the increase in LCFA concentration [Lalman and Bagley, 2001; Hwu et al., 1998; Koster and Cramer, 1987].

Table 5.2: Initial sulfate degradation rates determined using non-linear regression analysis.

<table>
<thead>
<tr>
<th>LCFA Concentration (mg l⁻¹)</th>
<th>Initial sulfate removal rates (µg mgVSS⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OA</td>
</tr>
<tr>
<td>0 (Control (Glucose))</td>
<td>0.02 ± 0.01aid</td>
</tr>
<tr>
<td>0 (Control (Glucose plus Sulfate))</td>
<td>0.05 ± 0.01aid</td>
</tr>
<tr>
<td>100</td>
<td>0.07 ± 0.02aid</td>
</tr>
<tr>
<td>300</td>
<td>0.08 ± 0.00aid</td>
</tr>
<tr>
<td>500</td>
<td>0.09 ± 0.02aid</td>
</tr>
<tr>
<td>700</td>
<td>0.09 ± 0.01aid</td>
</tr>
<tr>
<td>1000</td>
<td>0.10 ± 0.02aid</td>
</tr>
</tbody>
</table>

Note: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations a are used to indicate the means that are statistically similar within the same column. The notations cannot be used for comparing data within the same row or between rows.

2. Comparisons are based on adjacent data sets.

In spite of methanogenic inhibition, no hydrogen was detected in the cultures receiving OA, sulfate and glucose. This was likely due to the fast sulfidogenic reactions using electrons derived from hydrogen for sulfate reduction reactions. The sulfate reduction reaction rates are faster compared to the methanogenic reactions [Lens et al., 1998]. Energetically, sulfidogenic reactions are favored due the low free energies associated with the reactions (Reaction 5.3 to 5.6).

\[
4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \quad (\Delta G^{\circ} = -38.1 \text{ kJ.mol}^{-1}) \quad (5.3)
\]

\[
CH_3COO^- + SO_4^{2-} \rightarrow HS^- + HCO_3^- \quad (-\Delta G^{\circ} = -47.6 \text{ kJ.mol}^{-1}) \quad (5.4)
\]
\[4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \quad (-\Delta G^\circ = -33.9 \text{ kJ.mol}^{-1}) \quad (5.5)\]

\[CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad (-\Delta G^\circ = -31.0 \text{ kJ.mol}^{-1}) \quad (5.6)\]

The major VFAs detected from glucose degradation were acetate and propionate. The elevated acetate levels suggest that acetate produced in the cultures was also produced from sulfidogenic breakdown of higher VFAs. Two oxidation patterns occur in sulfate reduction: some SRB are capable of completely oxidizing VFA to \( CO_2 \) and \( HS^- \) as end products, while others lack the tricarboxylic acid cycle and carry out an incomplete oxidation of VFA with acetate and sulfide as end products [Lens et al., 1998].

Concurrently, faster propionate removal after peaking in the cultures receiving sulfate (in the presence or absence of LCFAs) compared to glucose controls (no sulfate), suggest that propionate was utilized by SRBs along with acetogenic bacteria to produce acetate. Mixotrophic acetate consuming SRBs use acetate only when higher VFAs are depleted [Laanbroek et al., 1984]. Although, a distinguish between the propionate consumed by SRBs and acetogenic bacteria was not possible due to the coexistence of the two in the mixed cultures used in this work.

OA and SA also exerted an inhibitory effect on acidogenesis. OA exhibited higher inhibition on glucose degradation compared to SA. The initial glucose degradation rates were higher in the cultures containing SA when compared to cultures receiving OA. Notice the acidogenic inhibition increased with the increase in LCFAs concentrations. In cultures receiving OA, the initial degradation rates were reduced by approximately 50%. Similar glucose degradation rates trends were also reported in studies by Lalman et al. [2003].

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In addition to OA, methane production data and electron mass balance data suggest that sulfide also exerted methanogenic inhibition. Methane levels in cultures receiving sulfate were approximately 32 % lower than cultures receiving only glucose. H$_2$S produced from microbial reduction of SO$_4^{2-}$ is inhibitory to MPB and SRB. MPBs are more sensitive than SRBs [Isa et al., 1986] and acidogens [Lens et al., 1998] to H$_2$S inhibition.

Because of their slow degradation rates [Lalman, 2000], LCFA did not likely contribute significantly to the electrons for sulfate reduction. Approximately 10 % sulfate removal was noticed in the control experiments conducted with LCFA, which was not significant when compared to the 9 % sulfate reduction in the control cultures containing only cultures. Also, in the LCFA control experiments without sulfate, LCFAs degraded minimally during the seven days period.

5.5 Conclusions

Sulfate reduction was examined using glucose in a mixed mesophilic anaerobic culture at 37°C and neutral pH in the presence of two LCFAs, OA and SA possessing 18 carbons. The findings of this study are as follows:

1. OA selectively inhibited MPB activity and thus enhanced SRB activity by reducing its competition with MPBs for available electrons.
2. SA did not exert significant inhibitory effect on methanogenic activity.
3. Methanogenic inhibition increased with increase in OA concentrations.
4. Significant sulfate conversions of up to 55 % (820 mg l$^{-1}$) and 73 % (1100 mg l$^{-1}$) were observed in cultures fed with 700 and 1000 mg l$^{-1}$ OA, respectively.
5. The percent electron flux diverted to the sulfidogenic reactions were significantly higher than the methanogenic reactions in cultures receiving OA. Increases of up to 22% percent of the electron flow to sulfate reduction were observed due to OA.

6. OA and SA also inhibited acidogenic activity. OA exerted higher inhibition to glucose degradation compared to SA.

7. Acetate and propionate were the major VFAs detected from glucose degradation.

8. OA significantly did not contribute to electrons for sulfate reduction in the examined duration.
5.6 References


CHAPTER 6: EFFECTS OF C18 LONG CHAIN FATTY ACIDS (LCFAS) ON HYDROGEN DEGRADATION IN AN ACCLIMATED MESOPHILIC MIXED ANAEROBIC CULTURE

6.1 Introduction

Under favorable thermodynamic conditions, organic substrates are converted into methane during anaerobic degradation. A vast variety of different microbial consortiums mediating the conversion reaction includes hydrolytic microorganisms, acidogens, acetogens, and methanogens. Hydrogen is an intermediate byproduct produced by acidogens (Eqs. 2.4 and 2.7) and acetogens (Eqs 2.8 and 2.9) and it is mainly a precursor for methane production by hydrogenotrophic methanogens. Nitrate and sulfate may also function as electron acceptor compounds. In addition, homoacetogens uses hydrogen to produce acetate (Eq. 2.45).

Hydrogen producing microorganisms are mainly divided in to two groups, strict anaerobes without cytochrome system such as Clostridia and Micrococi and facultative anaerobes such as, Escherichia coli, Enterobacter, and Citrobacter [Nandi and Sengupta, 1998; Gray and Gest, 1965]. Clostridia and Enterobacter are the most extensively researched groups amongst the hydrogen producing bacteria. Clostridia belongs to the genus Clostridium, a group of gram-positive spore forming rod shaped bacteria [Madigan et al., 2000; Nandi and Sengupta, 1998], whereas Enterobacter belong to a group of gram-negative rod shaped bacteria [Li and Fang, 2007]. Examples of gram-positive hydrogen producing bacteria include Clostridium pasteurianum [Brosseau and Zajic, 1982]; Clostridium beijerincki [Taguchi et al., 1992]; Clostridium butyricum [Kataoka et al., 1997; Suzuki et al. 1980]. Gram-negative hydrogen producing bacteria include
Enterobacter Aerogenes [Tanisho et al., 1983; Tanisho et al., 1998], Aciduric Enterobacter aerogenes [Yokoi et al., 1995], Enterobacter cloacae [Kumar and Das, 2000], Escherichia coli [San et al., 2002], and Rhodopseudomonas palustris [Oh et al., 2002].

Microorganisms producing methane belong to the Archaea domain. Two type of methanogenic reactions occur in anaerobic biodegradation, carbon dioxide is reduced by hydrogen to form methane by hydrogenotrophic methanogens and, in another methanogenic pathway; acetate is converted to methane and carbon dioxide by aceticlastic methanogens. Several hydrogenotrophic methanogens characterized recently include Methanothermobacter thermautotrophicus [Smith et al., 1997], Methanopyrus kandleri [Slesarev et al., 2002], Methanocaldococcus jannaschii [Bult et al., 1996], Methanococcus maripaludis [Hendrickson et al., 2004] and Methanobrevibacter acididurans [Savant and Ranade, 2004]. Inhibiting these microorganisms will prevent hydrogen utilization and cause hydrogen to accumulate in anaerobic communities.

Threshold LCFA levels [Lalman and Bagley, 2001; Lalman and Bagley, 2000; Hanaki et al., 1981; Demeyer and Henderickx, 1967] and low pH conditions [Kim et al., 2004; Fang and Liu, 2002; Van Ginkel and Sung, 2001] are inhibitory to hydrogenotrophic methanogens. LCFA act on methanogens by adsorbing onto their microbial cell wall and eventually promote lysis and leakage of cellular components [Hwu and Lettinga, 1997; Demeyer and Henderickx, 1967].

LCFAs are derived from lipids and fats, which in turn are produced from edible oil refineries, slaughter house, and dairy products industries [Hwu et al., 1998, Hanaki et al., 1981]. Lipids are readily hydrolyzed to LCFA and glycerol (Eq. 2.41). LCFA are
degraded by hydrogen producing acetogens to acetate and hydrogen via β-oxidation (Eq. 2.42) [Weng and Jeris, 1976]. The degradation products from each step are acetate, hydrogen plus a LCFA reduced by two carbons. These reactions progresses until all the LCFA is converted into acetate and hydrogen.

In comparison to methanogenic inhibitors such as 2-bromoethanesulphonate (BES) [Oremland and Capone, 1988] and chloroform [Bauchop, 1967], LCFA are relatively inexpensive, biodegradable and non-toxic if discharged into the environment. Inhibition of hydrogenotrophic methanogens in the absence of hydrogen-consumers causes hydrogen to accumulate.

The significance of this study in understanding LCFA inhibition on hydrogen consumption is important because LCFA can be used to redirect electron fluxes in mixed anaerobic communities. The main objective of this study was to assess the inhibitory effects of individual and mixtures of two LCFA, OA (C18:1) and LA (C18:2) on hydrogenotrophic methanogens at pH 5 and 6.

6.2 Materials and Methods

The materials and methods for this phase of experiments are described in Chapter 3: Inoculum source and start up is described in section 3.1 and the experimental plan and design are given in section 3.2.2. All analytical methods used are detailed in section 3.3. All control cultures and those fed with LCFA received 225 μmoles of hydrogen at 0.5 h and 48 h intervals.

6.3 Results

6.3.1 Hydrogen Consumption in cultures receiving individual LCFA
In the cultures receiving individual LCFAs, hydrogen degradation was examined at pH values of 5 and 6. Control cultures without LCFAs were assessed for hydrogen removal at both pH conditions. Hydrogen removal profiles at pH 5 and 6 for 0.5 h (1st hydrogen injection) and 48 h (2nd hydrogen injection) incubation period are shown in Figure 6.1. Slower hydrogen removal rates were observed in cultures at pH 5 for both first hydrogen injection (Figure 6.1(A)) and second hydrogen injection (Figure 6.1(B)). After 7 h, hydrogen was completely consumed at pH 6 for both incubation periods. In contrast, in cultures at pH 5, 36% and 62% hydrogen was left unconsumed after 7 h after adding hydrogen at 0.5 h and 48 h, respectively. However, after approximately 24 h of incubation, hydrogen was completely removed.

The effect of LA on hydrogen removal at pH values of 5 and 6, are shown in Figures 6.2 and 6.3, respectively. Exponential hydrogen removal profiles were observed for cultures receiving 100 mg l⁻¹ LA at both pH conditions. Note the degree of curvature decreased in hydrogen removal profiles for cultures receiving 500, 1000, and 2000 mg l⁻¹ indicating slower hydrogen removal and possible inhibition at elevated LA levels. When compared to the first hydrogen injection at 0.5 h, greater inhibition of hydrogen degradation was observed after a second hydrogen injection in the cultures receiving LA at both pH values.

In the cultures receiving LA at pH 5 similar hydrogen removal trend was observed at 500 mg l⁻¹ and 1000 mg l⁻¹, while slightly higher inhibition was observed at 2000 mg l⁻¹ following hydrogen injection at 0.5 h incubation period (figure 6.2(A)).
Figure 6.1: Hydrogen degradation profiles for control cultures: (A) H₂ injection at 0.5 h; (B) H₂ injection at 48 h. Values shown are mean and standard deviation for triplicate samples.
Approximately 55% and 59% hydrogen remained in the headspace after injecting at the 48 h incubation period. Significantly high hydrogen levels remained in the headspace following the second hydrogen injection in the cultures receiving LA and at pH 5. The quantity of hydrogen remaining after 48 h were 84%, 80% and 77% in cultures fed with 2000, 1000 and 500 mg l\(^{-1}\) LA, respectively. In cultures receiving 100 mg l\(^{-1}\) LA and at pH 5, hydrogen levels were undetectable within approximately 24 h.

Faster hydrogen removal was observed in the cultures receiving LA at pH 6 compared to pH 5 for both the first and second hydrogen injection. Similar to cultures receiving LA at pH 5, the residual hydrogen remaining in the cultures fed with LA at pH 6 was higher after the second hydrogen injection (Figure 6.3(B)). Hydrogen removal profiles were relatively similar before 9 h after the second hydrogen injection in the cultures fed with high LA (≥ 500 mg l\(^{-1}\)) levels at pH 6. Note the quantity of hydrogen remaining after 48 h in these cultures differed from the values at pH 5. Approximately, 57%, 38% and 17% hydrogen remained in the headspace at 2000, 1000 and 500 mg l\(^{-1}\), respectively at pH 6. The hydrogen versus time profiles for cultures fed with LA at pH 6 after first hydrogen injection were similar; however, the hydrogen removal rate varied under all the LA conditions examined (Figure 6.3(A)).

The quantity of hydrogen utilized by cultures receiving OA at both pH conditions were greater compared to the cultures receiving LA. Hydrogen removal profiles for cultures fed OA at pH 5 and 6, respectively, are shown in Figures 6.4 and 6.5. The quantity of hydrogen remaining after the second injection in the cultures fed 1000 and 2000 mg l\(^{-1}\) OA at pH 5 (Figure 6.4(B)) were relatively similar to those fed similar concentrations of LA at pH 5 (Figure 6.2(B)). The percentage hydrogen not consumed in
cultures fed OA (81\% and 73\% at 2000 and 1000 mg l\(^{-1}\) OA) was between 3 to 7\% less than that for cultures fed with 2000 and 1000 mg l\(^{-1}\) LA, respectively. However, the hydrogen removal rate, after the second hydrogen injection, was significantly different at 500 mg l\(^{-1}\). In cultures receiving 500 mg l\(^{-1}\) OA, the quantity of hydrogen remaining was 32\% less than cultures receiving 500 mg l\(^{-1}\) LA and at pH 5. In addition, the level of hydrogen utilization was greater in OA cultures compared to cultures fed LA at pH 5 after the first hydrogen injection. The amount of residual hydrogen in cultures fed OA at pH 5 was lower and varied in between 18\% to 41\% of the levels for those fed with LA at pH 5 after the first hydrogen injection. The hydrogen removal rate was slower after the second injection in the cultures fed OA at pH 5 compared to first injection. The difference in hydrogen utilization between first and second injection varied in between 38\% to 43\% at varying OA levels (Figure 6.4).

Cultures receiving OA at pH 6 showed higher hydrogen removal rates compared to cultures receiving OA at pH 5 after the first and second hydrogen injection. Hydrogen removal profiles for cultures fed with OA at pH 6 are depicted in Figure 6.5. Approximately 23\% and 49\% of hydrogen was left unconsumed in the cultures receiving OA at pH 6 after the first (at 0.5 h OA incubation) and second (at 48 h OA incubation) hydrogen injection, respectively. Based on the hydrogen consumption profiles, the hydrogen removal rates for the conditions examined are as follows: OA at pH 6 > LA at pH 6 > OA at pH 5 > LA at pH 5.

6.3.2 Hydrogen Consumption in cultures receiving LCFA mixtures

Experiments were also conducted to examine the effect of binary mixtures containing OA and LA at pH values of 5 and 6 (Figure 6.6 and 6.7). Hydrogen removal profiles in
Figure 6.2: Hydrogen degradation profiles for cultures receiving LA at pH 5: (A) 0.5 h injection; (B) 48 h injection. Values shown are mean and standard deviation for triplicate samples.)
Figure 6.3: Hydrogen degradation profiles for cultures receiving LA at pH 6: (A) 0.5 h injection; (B) 48 h injection. Values shown are mean and standard deviation for triplicate samples.)
Figure 6.4: Hydrogen degradation profiles for cultures receiving OA at pH 5: (A) 0.5 h injection; (B) 48 h injection. Values shown are mean and standard deviation for triplicate samples.
Figure 6.5: Hydrogen degradation profiles for cultures receiving OA at pH 6: (A) 0.5 h injection; (B) 48 h injection. Values shown are mean and standard deviation for triplicate samples.)
the control cultures and cultures receiving 100 mg l⁻¹ of OA plus LA at pH 5 were similar for the 0.5 h (1st injection) and 48 h injection periods (2nd injection). The hydrogen removal profiles were exponential (Figure 6.6); however, the removal profiles shape differed for elevated LCFA levels (≥ 500 mg l⁻¹ LA plus OA). Following the 1st hydrogen injection (at 0.5 h OA plus LA incubation), the hydrogen removal profiles showed a decrease in the exponential rate compared to the control cultures. The decrease in the curvature is likely due to a decrease in hydrogen removal rates with increasing LCFA levels. In comparison to the hydrogen removal rates for cultures receiving individual LA or OA, a similar trend was observed in cultures receiving the OA plus LA binary mixture. The rates were greater after 1st hydrogen injection compared to 2nd injection in all the cultures receiving OA plus LA at all concentration and pH conditions.

A greater inhibition of hydrogen utilization was observed in all cultures receiving the LCFA binary mixtures at pH 5 compared to pH 6. The quantity of hydrogen remaining unused in the cultures receiving 1000 and 2000 mg l⁻¹ OA plus LA after 2nd hydrogen injection were similar (approximately 88 % and 82 % at 2000 and 1000 mg l⁻¹, respectively) at pH values of 5 (Figure 6.6 (B)) and 6 (Figure 6.7 (B)). Similarly, the quantity of hydrogen remaining unused by the methane-consuming microorganisms after 1st injection at 1000 and 2000 mg l⁻¹ OA plus LA also varied only by 4 % for pH 5 (57 % and 53 % at 2000 and 1000 mg l⁻¹, respectively) ((Figure 6.6 (A)) and pH 6 (54 % and 50 % at 2000 and 1000 mg l⁻¹, respectively) (Figure 6.7 (A)). On the other hand, the amount of hydrogen remaining unconsumed at 500 mg l⁻¹ after the 2nd hydrogen injection were not similar for the two pH conditions and the values were 80 % and 67 % at pH 5 and 6, respectively. In contrast, similar residual hydrogen (50 % remained unconsumed) was
observed after 1st hydrogen injection in the cultures receiving 500 mg l⁻¹ of the OA plus LA mixtures at both pH conditions, 5 and 6. Overall, at high concentration of LA plus OA similar hydrogen removal profiles were observed for pH 5 and pH 6. However, a greater inhibition of hydrogen removal was observed in cultures receiving < 500 mg l⁻¹ OA plus LA.

6.3.3 Hydrogen degradations constants and reaction half life

The hydrogen removal curves for the controls and cultures receiving individual and mixtures of LCFAs were modeled using a first order reaction rate expression \[ \frac{d[H_2]}{dt} = -k[H_2] \]. A least-square regression model provided \( r^2 \) values greater than 0.9 for all the data sets examined. Note use of the kinetic expression does not imply a 1st order hydrogen uptake mechanism. The rate constant values, k, were used to compare hydrogen removal rates at different conditions examined. Half-life values were determined from the obtained k values and are reported as a percentage of control half-life values. A statistical analysis was performed to compare the mean values of the half-life reaction values using Tukey’s paired comparison test procedure [Box et al., 1978] at a 95 % confidence level.

Hydrogen removal profiles, k and reaction half-life values were used to characterize the hydrogen consumption in the cultures under all the conditions examined. Table 6.1 and 6.2 show reaction half-life values at pH 5 and 6, respectively. The reaction half-life values were statistically similar for control cultures and cultures receiving 100 mg l⁻¹ individual and mixtures of LCFA at both the 0.5 h and 48 h injection periods at both pH conditions. However, the percent reaction half-life values increased in cultures receiving
Figure 6.6: Hydrogen degradation profiles for cultures receiving LA plus OA at pH 5: (A) 0.5 h injection; (B) 48 h injection. Values shown are mean and standard deviation for triplicate samples.
Figure 6.7: Hydrogen degradation profiles for cultures receiving LA plus OA at pH 6: (A) 0.5 h injection; (B) 48 h injection. Values shown are mean and standard deviation for triplicate samples.)
≥ 500 mg l⁻¹ LCFAs compared to the control cultures. Statistically, the reaction half-life values were different in the cultures receiving ≥ 500 mg l⁻¹ LCFAs at pH 5 (Table 6.1).

On the other hand, the reaction half-life values were statistically similar at pH 6 in the cultures receiving LCFA for both the injections compared to the control cultures, except for cultures receiving LA and OA plus LA at 2000 mg l⁻¹ (Table 6.2). For the low pH (pH 5) condition, greater inhibition to hydrogen degradation was observed when compared to the pH 6 condition.

Notice the reaction half-life values at low pH are higher than those at pH 6. After the 1st injection (0.5 h incubation period) the reaction half-life values for hydrogen degradation at all the conditions examined are less than or equal to 12 times the control values. However, the reaction half-life values increased enormously up to 88 times in cultures fed with the binary mixtures and at the low pH condition.

The highest reaction half-life values were observed in the cultures receiving OA plus LA after the 2nd hydrogen injection (48 h incubation period) at low pH (pH 5), which was followed by the cultures receiving LA after 2nd injection at pH 5 (Table 6.1). After the 1st hydrogen injection similar reaction half-life values were noticed for cultures receiving ≥ 1000 mg l⁻¹ LA and OA plus LA at low pH. In contrast, after second hydrogen injection the reaction half-life values increased by 37 % (in 2000 mg l⁻¹ LCFA) and 20 % (in 1000 mg l⁻¹ LCFA) in the cultures receiving OA plus LA compared to the LA cultures (Table 6.1).

At pH 6 the highest reaction half-life values were observed in the cultures receiving ≥ 500 mg l⁻¹ OA plus LA (Table 6.2) after 2nd hydrogen injection, which were lower compared to the reaction half-life values after 2nd hydrogen injection at pH 5 in the
cultures receiving binary mixtures. At pH 6 the reaction half-life values after 2nd hydrogen injection in the cultures receiving 2000 mg l$^{-1}$ individual OA and LA showed similar reaction half-life values, while these values were approximately 15% higher in the LA cultures after 1st hydrogen injection at pH 6 (Table 6.2).

Cultures receiving OA at pH 5 showed lower reaction half-life values and less inhibition to hydrogen removal when compared to LA and OA plus LA at pH 5. Notice the reaction half-life values in the cultures fed with OA at pH 5 were significantly higher than the cultures fed LA at pH 6 after 2nd hydrogen injection.

Table 6.1: Percent hydrogen degradation half-life values (% of Control) for cultures incubated with LCFA at pH 5.

<table>
<thead>
<tr>
<th>LCFA Type</th>
<th>LCFA Concentration (mg l$^{-1}$) (LCFA Incubation for 1 h)</th>
<th>LCFA Concentration (mg l$^{-1}$) (LCFA Incubation for 48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>LA</td>
<td>100 ± 0.03$^a$</td>
<td>100.9 ± 0.07$^a$</td>
</tr>
<tr>
<td>OA</td>
<td>100±0.03$^a$</td>
<td>98.9 ± 0.21$^a$</td>
</tr>
<tr>
<td>LA plus OA</td>
<td>100±0.03$^a$</td>
<td>99.5±0.10$^a$</td>
</tr>
</tbody>
</table>

Note: Average and standard deviation for triplicate cultures are shown. The Tukey's comparison is performed with respect to the control. The superscript notations $a$ and $b$ are used to indicate the means that are statistically different within the same row.

Table 6.2: Percent hydrogen degradation half-life values (% of Control) for cultures incubated with LCFA at pH 6.

<table>
<thead>
<tr>
<th>LCFA Type</th>
<th>LCFA Concentration (mg l$^{-1}$) (LCFA Incubation for 1 h)</th>
<th>LCFA Concentration (mg l$^{-1}$) (LCFA Incubation for 48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>LA</td>
<td>100±0.14$^a$</td>
<td>88.5±0.12$^a$</td>
</tr>
<tr>
<td>OA</td>
<td>100±0.14$^a$</td>
<td>111.2±0.05$^a$</td>
</tr>
<tr>
<td>LA plus OA</td>
<td>100±0.14$^a$</td>
<td>95.6±0.08$^a$</td>
</tr>
</tbody>
</table>

Note: Average and standard deviation for triplicate cultures are shown. The Tukey's comparison is performed with respect to the control. The superscript notations $a$ and $b$ are used to indicate the means that are statistically different within the same row.
The trend of half-life values for the conditions examined is as follows: OA plus LA at pH 5 > LA at pH 5 > OA plus LA at pH 6 > OA at pH 5 > LA at pH 6 > OA at pH 6. Hence, LA at all pH conditions (individually or synergistically) exerted the greatest inhibition on hydrogen removal compared to OA.

6.4 Discussion

The effects of individual and mixtures of LCFAs on hydrogenotrophic methanogens have been reported by several authors [Lalman and Bagley, 2001; Hanaki et al., 1981]. However, the synergistic inhibitory effect of combining low pH conditions and LCFA on hydrogen removal is not reported in these studies. Hence, the combined effects of low pH and LCFA on hydrogen metabolism were examined in this study.

Hydrogen is a precursor to many terminal reactions and is utilized by several microorganisms such as hydrogenotrophic methanogens and sulfidogens. The former uses hydrogen to reduce carbon dioxide for forming methane (Eq. 2.45), while the latter uses sulfate as electron acceptor to produce hydrogen sulfide (Eq. 2.21). No sulfate was present in these experiments and carbon dioxide was the sole electron acceptor. Alternatively, carbon dioxide may be reduced by homoacetogens to form acetate. Nevertheless, thermodynamically homoacetogenesis is less favorable than methanogenesis (Eqs. 2.45 and 2.14). Hence, the hydrogen utilizing metabolic pathway in this study is mediated by hydrogenotrophic methanogens due to the associated energetic advantage.

The results in this study indicate a synergistic inhibitory effect due to low pH and LCFA on hydrogenotrophic methanogens. The reaction half-life values and hydrogen removal profiles clearly demonstrate that the degree of inhibition increased with a
decrease in pH and an increase in the LCFA levels. In addition, the hydrogen removal profiles in the control cultures indicate a greater inhibition to hydrogen removal by hydrogenotrophic methanogens at pH 5 than pH 6.

Low pH is inhibitory to methanogenic activity in anaerobic biological processes. Lowering the pH is also necessary to decrease the activity of hydrogen utilizing microorganisms such as hydrogenotrophic methanogens [Hawkes et al., 2002]. Several authors have reported decreasing the pH in the range of 5 to 6.5 is inhibitory to methanogens, the optimum inhibitory pH to methanogens is between 5 to 5.5 [Khanal et al., 2004; Kim et al., 2004; Hwang et al., 2004; Fang and Liu, 2002; Van Ginkel et al., 2001; Van Ginkel and Sung, 2001; Lay et al., 1999; Tanisho et al., 1989].

The degree of inhibition was a function of LCFA concentration and hydrogen degradation was affected by ≥ 500 mg l\(^{-1}\) LCFA concentrations in all the conditions examined. The threshold inhibitory concentration in this study is lower than reported (1,500 mg l\(^{-1}\)) in a study by Templer et al. [2006] where hydrogenotrophic methanogenic activity was examined at neutral pH conditions in the presence of LCFA. The decrease in the threshold inhibitory levels in this study was likely due to the synergistic effect of low pH and LCFA. Additionally, methanogenic inhibition was affected by the incubation periods. The reaction half-life data in Table 6.1 and 6.2 clearly indicate that in general significantly higher inhibition was exerted after the 2\(^{nd}\) hydrogen injection (48 h LCFA incubation) compared to 1\(^{st}\) hydrogen injection (0.5 h incubation) on hydrogenotrophic methanogens.

LCFA inhibit methanogens by adsorbing on to microbial cell wall causing lysis and leakage of cellular components because of mass transfer limitation of important nutrients.
and ions [Hwu and Lettinga, 1997; Demeyer and Henderickx, 1967]. Because LCFAs are immiscible in polar aqueous mediums, LA and OA sodium salts were used for increased dispersion. A longer contact time could have aided an adequate adsorption of LCFA on to the cell surface causing higher inhibition following 48 h inhibition.

The extent of LCFA microbial inhibition increases with the increase in the number of double bonds in the molecular structure [Lalman and Bagley, 2002; Demeyer and Hendrix, 1967; Nieman, 1954]. In this study hydrogen removal profiles and reaction half-life data suggest that LA has larger inhibitory effect on microbial population compared to OA. Individual or mixtures of LCFAs containing LA illustrated relatively higher inhibition compared to the individual effect of OA. Hence, microbial inhibition due to LCFA in the anaerobic mixed cultures were a function of, pH, LCFA concentration, LCFA contact period and the type of LCFA in this study.

6.5 Conclusions

Hydrogen metabolism by hydrogenotrophic methanogens was assessed in the presence of individual and mixtures of, OA and LA at pH 5 and pH 6. The conclusions of this study are as follows:

1. Hydrogenotrophic methanogenic inhibition was relatively greater at pH 5 compared to cultures maintained at pH 6.

2. A combination of adding LCFAs and decreasing the pH exerted a synergistic inhibitory effect on hydrogen metabolism.

3. The degree of inhibition was a function of LCFA concentration and hydrogen degradation was affected by $\geq 500$ mg l$^{-1}$ LCFA concentrations in all the conditions examined.
4. Methanogenic inhibition was also affected by the incubation periods. Hydrogen removal profiles and reaction half-life values clearly established significantly higher inhibition after the 2nd hydrogen injection (48 h LCFA incubation) compared to the 1st hydrogen injection (0.5 h LCFA incubation) on hydrogenotrophic methanogens.

5. Individual or mixtures of LCFA containing LA illustrated relatively higher inhibition on hydrogenotrophic methanogenesis compared to the individual effect of OA.

6. Based on the hydrogen consumption profiles and reaction half-life values, the greatest inhibition on hydrogenotrophic methanogenesis was observed in OA plus LA cultures after 48 h incubation at pH 5.

7. The trend of reaction half-life values for the conditions examined are as follows: OA plus LA at pH 5 > LA at pH 5 > OA at pH 6 > OA at pH 5 > LA at pH 6 > OA at pH 6. Hence, LA at all pH conditions (individually or synergistically) exerted higher inhibition to hydrogen removal compared to OA.
6.6 References


CHAPTER 7: HYDROGEN PRODUCTION IN A MIXED MESOPHILIC ANAEROBIC CULTURE: IMPACT OF VARYING LINOLEIC ACID (LA) INCUBATION PERIODS

7.1 Introduction

During the anaerobic conversion of complex carbon substrates, hydrogen is produced by several intermediate steps (Eqs. 2.22, 2.23 and 2.28). In an efficient operational anaerobic bioreactor, excess electron equivalences in the form of hydrogen are utilized by microorganisms such as hydrogenotrophic methanogens to produce methane (Eq. 2.14 and 2.21) [Smith and Mah, 1966]. Producing hydrogen in an economical manner under anaerobic conditions could become a major breakthrough towards sustaining cheap energy supplies. Increasing environmental problems associated with global warming and the resulting weather changes as well as energy security are driving researchers to find renewable energy supplies. Therefore, producing hydrogen and other biofuels from renewable agriculture sources is a crucial and developing research area.

Hydrogen is increasingly recognized as a potential fuel for the industry and transport sectors. The latter is mainly due to problems related to the supplies and demand of existing fossil fuel supplies. Among the alternative energy carriers, hydrogen is the most promising for several reasons: greenhouse gases are not a process byproduct [Nath and Das, 2004], it has higher energy content by mass [Lide, 2006], it can be used directly to generate electricity by reversal of electrolysis in fuel cells [Nath and Das, 2004].

Anaerobic fermentation (dark fermentation) is a biological hydrogen production process, which has received widespread attention. Other biological processes which are included into this category include light-dependent photosynthesis, direct biophotolysis
and indirect biophotolysis [Hallenbeck and Benemann, 2002; Das and Veziroglu, 2001; Nandi and Sengupta, 1998]. In addition to biological methods, chemical processes such as steam reforming of naptha and natural gas, coal gasification and electrolysis of water are widely being adopted for hydrogen production [Nath and Das, 2004]. Fossil fuels accounts for the 99% of hydrogen produced currently, and renewable energy sources account for the rest [Nath and Das, 2004]. However, chemical processes are very energy intensive, thereby incurring higher production cost and they are not environmentally benign because many of them generate carbon dioxide.

Recently many researchers have focused their attention on producing hydrogen using biological methods. Current biological processes under investigation are divided between light-dependent (direct photolysis, indirect photolysis, and photo-fermentation) and light-independent (dark fermentation). The latter is generally preferred because of its high reaction rates and non-dependence on light [Fang and Liu, 2002; Hallenbeck and Benemann, 2002].

Anaerobic fermentation of glucose yields 4.0 and 2.0 mole $H_2$ mole$^{-1}$ glucose when acetate (Eq. 2.22), or butyrate (Eq. 2.23) or ethanol plus acetate (Eq. 2.28) are the only byproducts, respectively. Theoretically, oxidizing 1 mole of glucose yields 24 moles of electrons. However, a fraction of the electrons is utilized in products and in the production of biomass. In mixed cultures, the electron equivalents produced by the acidogens are consumed by homoacetogens (Eq. 2.45) and terminal electron acceptors such as methanogens (Eq. 2.14) or sulfidogens (Eq. 2.21) to produce terminal products. Hence, minimizing the hydrogen consumption and subsequently increasing the hydrogen yield is the main focus of this research work. Inhibiting methanogens in sulfate free
organic waste will cause hydrogen to accumulate to very high levels. Using physical and chemical methods to divert the electron flow away from methanogens in mixed anaerobic communities has been studied by several researchers [Oh et al., 2003; Sung et al., 2002; Van Ginkel et al., 2001; Van Ginkel and Sung, 2001; Scholten et al, 2000; Oremland and Capone, 1988; Prins et al., 1972; Bauchop, 1967]. Several researchers have examined heat-treating the inoculum culture prior to hydrogen production. The latter procedure has a tendency to kill the non-spore forming methanogens, thus enhancing the spore-forming acidogens [Oh et al., 2003; Van Ginkel et al., 2001; Van Ginkel and Sung, 2001; Sung et al., 2002]. Microbial inhibition of selective anaerobic populations with subsequent prevention of electron consumption by hydrogenotrophic methanogens has been reported using a variety of chemicals. Inhibition of methane production has been shown using 2-bromoethanesulphonate (BES) [Scholten et al., 2000; Oremland and Capone, 1988] and chlorinated methane analogues, such as chloroform, carbon tetrachloride, and methylene chloride [Prins et al., 1972; Bauchop, 1967]. However, on practical scale, these physical and chemical techniques are not cost effective and the chemicals used are toxic.

Recent studies have shown evidence of inhibiting hydrogenotrophic and aceticlastic methanogens using LCFAs [Lalman and Bagley, 2001; Lalman and Bagley, 2000; Koster and Cramer, 1987]. Inhibiting hydrogenotrophic methanogens using LCFAs is a novel approach because the benefits associated with using LCFAs as inhibitors are multifold. LCFAs are readily available from agriculture sources and they are slowly degradable.

LCFAs inhibit methanogens by interfering with several important cellular mechanisms. LCFAs may exert antibacterial effects in these organisms by disrupting several membrane components and inactivating many energy-linked reactions [Greenway
and Dyke, 1979; Galbraith and Miller, 1973]. For example, they disturb the cell homeostasis by interfering with the K\(^+\), NA\(^+\) regulator proteins and other cell proteins [Cherrington \textit{et al.}, 1991]. Also, unsaturated LCFAs adhere onto the walls of the bacterial cells and changes cellular structures and processes such as permeability and transport of important nutrients [Hwu \textit{et al.}, 1998; Rinzema \textit{et al.}, 1994; Sayed \textit{et al.}, 1988; Henderson, 1973; Demeyer and Henderickx, 1967]. Contradictory theories on the nature of the inhibition by LCFAs have been reported by several researchers. Rinzema \textit{et al.} [1994] and Angelidaki and Ahring [1992] reported the toxic effect of LCFA to be permanent and irreversible, while Pereira \textit{et al.} [2005] and Pereira \textit{et al.} [2004] have argued that LCFA inhibition is not permanent and cultures eventually acclimate to LCFAs.

The main objective of this study was to examine hydrogen production using cultures incubated to LA (0 to 25 days at an increment of 5 days) at pH 5.0 and 37\(^\circ\)C. To date, no studies have been reported on hydrogen production using LA incubated cultures for varying durations. Assessing the effect of various LA incubations on the cultures used for producing hydrogen is important because of issues associated with the degradability of LA and the effects caused by LA degradation byproducts. Hydrogen producing acetogens degrade LCFAs such as LA via a \(\beta\)-oxidation mechanism to hydrogen, acetate and a LCFA with a reduction of two carbons in the alkyl group [Weng and Jeris, 1976]. The \(\beta\)-oxidation reactions proceed until all the LCFA is converted to acetate (Eq. 2.43). Hence, these studies will provide an understanding of the inhibition of hydrogen consumption in the presence of LA and its \(\beta\)-oxidation by-products.
7.2 Materials and Methods

The materials and methods for this phase of experiments are described in Chapter 3: Inoculum source and start up is described in section 3.1. Experimental plan and design are detailed in section 3.2.3, and analytical methods used are detailed in section 3.3. Initial glucose degradation rates were calculated using Graphpad Prism software using a non-linear regression by a one-phase exponential decay model. A sample calculation for initial glucose degradation rate is shown in Appendix E.

7.3 Results

7.3.1 Control Cultures

Triplicate control cultures were prepared with only glucose (5,000 mg l\(^{-1}\)) and only LA (2000 mg l\(^{-1}\)) and no carbon substrate. Three sets of glucose controls were analyzed every consecutive 10\(^{th}\) day and one set of LA bottles (in triplicate) was analyzed for hydrogen production during the entire experimental duration of 32 days. No hydrogen was detected in the cultures fed with only glucose. The main byproducts in the latter cultures included methane, VFAs and alcohols. The major VFAs detected were acetate, propionate and butyrate and the major alcohols were ethanol, i-propanol, and i-butanol. Note a small quantity of formate was detected in glucose cultures (< 143mg l\(^{-1}\)) on day 1 and reached undetectable levels before day 2. In contrast, in the cultures fed with only LA, a small quantity (< 37\(\mu\)moles) of hydrogen was detected during the first 6 days after injecting LA. Other byproducts detected included acetate, palmitic acid, myristic acid, lauric acid and methane. However, the hydrogen in these cultures decreased to below 20 \(\mu\)moles before day 1 and was undetected after day 7.
The methane produced in the control cultures receiving glucose (Figure 7.1(B)) were significantly higher (> 96 %) than control cultures fed with only LA (Figure 7.2(B)). Notice the acetate levels were greater in the controls receiving glucose (Figure 7.1(C)) when compared to the cultures receiving only LA (Figure 7.2(C)). The acetate levels ranged between 600 to 1200 mg l\(^{-1}\) and 580 to 620 mg l\(^{-1}\), respectively, in the cultures receiving only glucose and only LA. Increasing trends in acetate, propionate and butyrate levels were observed in the cultures receiving only glucose. The propionate and butyrate levels ranged between 190 to 530 mg l\(^{-1}\) (Figure 7.1(D)) and 70 to 700 mg l\(^{-1}\) (Figure 7.1(E)), respectively in the cultures receiving only glucose.

Ethanol profiles in the control cultures receiving only glucose are shown in Figure 7.1(F). Ethanol levels reaching up to 880 mg l\(^{-1}\) were detected in the glucose controls and undetectable levels were observed after 8 days from the day of glucose injection. In comparison, a decreasing trend in i-propanol levels was observed in the glucose controls (Figure 7.1(G)) with a peak value of 1420 mg l\(^{-1}\) on day 1. The i-propanol levels were undetected after day 6. Low levels of i-butanol (< 150 mg l\(^{-1}\)) were detected in the glucose controls on day 1. However, butanol was undetected after day 1 after each glucose injection.

LA was degraded in the LA control cultures and was undetectable within 10 days. Saturated LCFA by-products palmitic (C16:0), myristic (C14:0) and lauric (C12:0) acid were observed in the control cultures receiving LA. The distribution of these byproduct LCFAs varied and maxima of 1760, 1050 and 1060 mg l\(^{-1}\) of palmitic, myristic and lauric acid were observed, respectively, on different occasions during the experiments (Figure 7.2(D)). Palmitic, myristic and lauric acid peaked on day 15, 25 and 30, respectively.
Palmitic acid was removed from the cultures before day 30 while myristic and lauric acid levels were 720 mg l$^{-1}$ and 650 mg l$^{-1}$, respectively on day 30.

Glucose was degraded in the glucose control cultures to below 100 mg l$^{-1}$ concentrations within 15 h of glucose injection and was undetected before 24 h (Figure 7.1(A)). The initial glucose degradation rates for the control cultures receiving glucose was 6.2 ± 0.3 µg mg VSS$^{-1}$ min$^{-1}$ (average of the three sets of triplicate glucose controls).

### 7.3.2 Hydrogen and Methane Production

The effect of varying LA incubation time ranging from 0 to 25 days was accessed on hydrogen and methane production using glucose (Figure 7.3). The impact of varying LA incubation times on hydrogen production and accumulation is shown in Figure 7.3(A). Hydrogen production was detected in all LA incubated cultures with incubations ranging from 0 - 25 days. A 12 h lag phase in hydrogen production was observed in all the LA incubated cultures. The lag phase increased as the incubation time increased. The hydrogen levels peaked between days 1 to 2 after glucose injection in all the LA incubated cultures with varying incubations.

Hydrogen yield values in all the LA incubated cultures are shown in Table 7.1. Tukey’s paired comparison test procedure was used to statistically compare the means at a 95 % confidence level [Box et al., 1978]. Hydrogen yields in the cultures were statistically similar under all the conditions examined. The hydrogen yield ranged between 1.70 ± 0.07 and 2.80 ± 0.19 mole H$_2$ mole$^{-1}$ glucose in cultures receiving LA at varying incubation periods. No particular trend was observed in the hydrogen yields with
the varying incubation times (Figure 7.4). However, the largest hydrogen yield (2.80 ± 0.19 mole H₂ mole⁻¹ glucose) was observed in the LA incubated cultures for 0 days, while, the smallest yield was produced in LA incubated cultures for 10 days. In the LA incubated cultures for 5, 15, 20 and 25 days yields of 2.24 ± 0.04, 2.13 ± 0.07, 1.98 ±...
0.58, 2.64 ± 0.26 mole H₂ mole⁻¹ glucose, were observed, respectively. Hydrogen levels in the LA incubated cultures for 0 days decreased slightly after reaching peak values. However, hydrogen accumulated in these cultures and a significant quantity of 80% of maximum yield of hydrogen remained in the headspace on day 7. Similarly, an inhibitory effect on hydrogen removal after reaching the peak values was observed in LA incubated cultures for 25 days. The remaining quantity of hydrogen in the headspace on day 7 was approximately 63% of the maximum in the cultures incubated for 25 days. After reaching peak hydrogen levels, a fast hydrogen removal was observed in the LA incubated cultures for 5, 10, 15, and 20 days. Undetectable levels of hydrogen were achieved on day 7 in all the cultures except for those fed with LA and incubated for 15 days, where the quantity of remaining headspace gas was approximately 50% of the maximum.

Methane was detected in all cultures receiving LA (Figure 7.3(B)). Methane levels below 100 μmoles were detected in LA incubated cultures for 0, 5 and 10 days and the levels remained more or less constant during 0 to 7 days of analysis after glucose injection. A slight increase in methane production levels were observed with the increase in LA incubation time. However, the maximum methane levels in LA incubated cultures were approximately 80% lower than the maximum methane levels in the glucose control.

### 7.3.3 VFAs and Alcohols Production

Acetate, propionate and butyrate were the major VFAs detected from glucose degradation. Low levels (< 150 mg l⁻¹) of formate were detected in all the LA incubated cultures between day 1 and day 3 and none was detected after day 3 following the glucose injection. Increasing trends of acetate levels were observed in all the LA
Table 7.1: Hydrogen yields in LA (2,000 mg l\(^{-1}\)) incubated cultures (0 to 25 days) receiving 5,000 mg l\(^{-1}\) glucose.

<table>
<thead>
<tr>
<th>Experiment (LCFA Incubation Time)</th>
<th>Hydrogen Yield (mole H(_2) mole(^{-1}) glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 mg l(^{-1}) LCFA)</td>
<td>LA (2000 mg l(^{-1}))</td>
</tr>
<tr>
<td>set 1 (0-7 days)</td>
<td>ND(^{a})</td>
</tr>
<tr>
<td>set 2 (5-12 days)</td>
<td>2.80 ± 0.18 (^{b})</td>
</tr>
<tr>
<td>set 3 (10-17 days)</td>
<td>2.24 ± 0.04 (^{b})</td>
</tr>
<tr>
<td>set 4 (15-22 days)</td>
<td>1.70 ± 0.07 (^{b})</td>
</tr>
<tr>
<td>set 5 (20-27 days)</td>
<td>2.13 ± 0.06 (^{b})</td>
</tr>
<tr>
<td>set 6 (25-32 days)</td>
<td>1.98 ± 0.58 (^{b})</td>
</tr>
<tr>
<td></td>
<td>2.64 ± 0.25 (^{b})</td>
</tr>
</tbody>
</table>

Note: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations \(^{a}\) and \(^{b}\) are used to indicate the means that are statistically different within the same column. The notations cannot be used for comparing data within the same row or between rows.
2. Comparisons are based on adjacent data sets
3. ND = Not detectable

![Graph A](image.png)

![Graph B](image.png)
Figure 7.2: Concentration profiles for control cultures receiving 2,000 mg l⁻¹ LA. (A = Hydrogen; B = Methane; C = Acetate; D = LCFA. Values shown are mean and standard deviation for triplicate samples).
Figure 7.3: Hydrogen and methane production in cultures receiving 2,000 mg l\(^{-1}\) LA plus 5,000 mg l\(^{-1}\) glucose. (A = hydrogen; B = methane. Values shown are mean and standard deviation for triplicate samples).
The degradation of LA contributed to the acetate levels in the cultures because residual acetate was present in cultures incubated with LA (5 to 25 days) prior to the glucose injection. The acetate levels after glucose injection in the cultures receiving LA with no incubation (0 day incubation) peaked on days 6 and 7 and ranged between 660 to 1330 mg l\textsuperscript{-1}. In comparison, the acetate levels in the LA incubated cultures were higher with the levels ranging between 840 to 1930 mg l\textsuperscript{-1}.

Butyrate profiles for cultures incubated with LA are shown in Figure 7.5(C). Increasing butyrate levels were observed in all the LA incubated cultures except in cultures incubated for 5 and 10 days. Increasing levels in the production of acetate and butyrate were associated with the increasing hydrogen levels in these reactors. The butyrate concentration in the LA incubated cultures ranged between approximately 130 and 1000 mg l\textsuperscript{-1}. On day 7, the butyrate concentration was less than 150 mg l\textsuperscript{-1} in cultures.
incubated for 5 and 10 days, while the butyrate concentrations in the LA incubated
cultures for 0, 15, 20 and 25 days were greater than 350 mg l\(^{-1}\). Propionate concentration
peaked on day 1 and decreased subsequently to lower levels except in LA incubated
cultures for day 5 and 10 (Figure 7.5(B)). The day 1 propionate concentrations in the LA
incubated cultures ranged between 290 to 1360 mg l\(^{-1}\).

The major alcohols detected included ethanol, i-propanol, and i-butanol. Figure 7.6
shows the alcohol profiles in the LA incubated cultures for varying duration. Low
quantities of ethanol and i-propanol were detected in LA incubated cultures when
compared to the glucose control cultures. Also, the alcohol formed during glucose
degradation were not removed from the LA incubated cultures over the 7 day sampling
duration (Figure 7.6(A)) after glucose injection like the latter. Notice larger quantities of
i-butanol were produced when compared to the control cultures. Unlike ethanol and i-
propanol, butanol was removed after peaking in the LA incubated cultures (Figure
7.6(C)). The maximum ethanol and i-butanol concentration values ranged between 390
to 874 mg l\(^{-1}\) and 200 to 520 mg l\(^{-1}\), respectively, in the various LA incubated cultures.
Meanwhile the average i-propanol levels in the latter were approximately 500 mg l\(^{-1}\)
(Figure 7.6(B)). Small quantities of n-propanol (<50 mg l\(^{-1}\)) and n-butanol (<75 mg l\(^{-1}\))
were detected in the LA incubated cultures.

7.3.4 LA Degradation and byproducts

LA degradation was observed under all the conditions examined. The culture media
were analyzed for LCFA before injecting glucose in the respective incubation sets and
after 7 days at the end of each experiment. \(\beta\)-oxidation degradation byproducts consisted
of acetate and saturated LCFAs with carbon numbers ranging from 16 to 12. Figure 7.7
Figure 7.5: VFA production profiles at varying LA incubation time in cultures receiving 2,000 mg l\(^{-1}\) LA plus 5,000 mg l\(^{-1}\) glucose. (A = Acetate; B = Propionate; C = Butyrate. Values shown are mean and standard deviation for triplicate samples).
Figure 7.6: Alcohol production profiles at varying LA incubation time in cultures receiving 2,000 mg l⁻¹ LA plus 5,000 mg l⁻¹ glucose. (A = Ethanol; B = i-Propanol; C = i-Butanol. Values shown are mean and standard deviation for triplicate samples).
shows the degradation byproducts of LA for all the LA incubated cultures. LA was detected only in cultures incubated for 0 and 5 days Figure 7.7(A). However, notice LA reached undetectable values before day 12 (7th day of analysis after glucose injection) in cultures incubated for 5 days, while approximately 30% residual LA was detected on day 7.

The maximum concentration of individual byproducts did not occur at the same time following each glucose injection. Palmitic acid was detected in all cultures fed with LA (Figure 7.7(B)) and the highest concentration was observed in cultures incubated for 10 and 15 days. Myristic acid was detected in cultures incubated with LA for 15, 20 and 25 days. The maximum concentrations of myristic and lauric acid were detected in cultures incubated for 25 days. Notice lauric acid was detected only in cultures incubated for 25 days (Figure 7.7(D)). No trace of oleic acid was detected in any of the LA incubated cultures.

7.3.5 Glucose degradation

In all the LA incubated cultures, glucose reached undetectable levels within 48 h of glucose injection (Figure 7.8). Except cultures incubated for 0 and 15 days, the half-life for the glucose concentration was achieved after approximately 9 h following the glucose injection. In the case of 0 and 15 day incubation periods, the half-life values were less than 9 h (Figure 7.8). Slower glucose degradation rates were observed in LA incubated cultures when compared to glucose control cultures. Notice the initial glucose degradation rates for cultures fed with LA was less compared to the glucose controls (Table 7.2). No particular trend was observed in the initial glucose degradation rates with increasing incubation time (Figure 7.9).
Myristic acid Conc. (mg l$^{-1}$)

- 0-7 days
- 1-5-12 days
- 1-20-27 days
- 1-25-32 days

Palmitic acid conc. (mg l$^{-1}$)

- 0-7 days
- 1-5-12 days
- 1-20-27 days
- 1-25-32 days

Linoleic acid conc. (mg l$^{-1}$)

- 0-7 days
- 1-5-12 days
- 1-20-27 days
- 1-25-32 days
Figure 7.7: LCFA production profiles at varying LA incubation time in cultures receiving 2000 mg l\(^{-1}\) LA plus 5000 mg l\(^{-1}\) glucose. (A = Linoleic Acid; B = Palmitic Acid; C = Myristic Acid; D = Lauric Acid. Values shown are mean and standard deviation for triplicate samples).

Figure 7.8: Effect of varying LA incubation time on glucose degradation for cultures receiving 2000 mg l\(^{-1}\) LA plus 5000 mg l\(^{-1}\) glucose. (Values shown are mean and standard deviation for triplicate samples).
The greatest inhibition to glucose degradation was observed in LA incubated cultures for 25 days where the reduction in initial glucose degradation rates was 55% compared to the glucose control cultures. Statistically the means were examined using Tukey’s paired comparison test procedure at the 95% confidence level. In the control cultures, the initial glucose degradation rates were statistically different from the LA incubated cultures. However, the initial glucose degradation rate values were statistically similar in all the LA incubated cultures except for cultures incubated for 15 days.

Table 7.2 Initial glucose degradation rates determined using non-linear regression analysis.

<table>
<thead>
<tr>
<th>Experiment (LCFA Incubation Time)</th>
<th>Initial glucose degradation rates (µg mgVSS⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 mg l⁻¹ LCFA)</td>
<td></td>
</tr>
<tr>
<td>set 1 (0-7 days)</td>
<td>6.21 ± 0.33 a</td>
</tr>
<tr>
<td>set 2 (5-12 days)</td>
<td>3.91 ± 0.26 b</td>
</tr>
<tr>
<td>set 3 (10-17 days)</td>
<td>3.52 ± 0.05 b</td>
</tr>
<tr>
<td>set 4 (15-22 days)</td>
<td>3.87 ± 0.25 b</td>
</tr>
<tr>
<td>set 5 (20-27 days)</td>
<td>4.93 ± 0.91 c</td>
</tr>
<tr>
<td>set 6 (25-32 days)</td>
<td>3.33 ± 0.05 b</td>
</tr>
<tr>
<td>LA (2000 mg l⁻¹)</td>
<td></td>
</tr>
<tr>
<td>set 1 (0-7 days)</td>
<td>2.81 ± 0.13 b</td>
</tr>
</tbody>
</table>

Note: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations a, b and c are used to indicate the means that are statistically different within the same column.
2. Comparisons are based on adjacent data sets

Figure 7.9: Percent initial glucose degradation rate versus LA incubation time. (Values shown are mean and standard deviation for triplicate samples).
7.3.6 Electron Balance

An electron mass balance was developed to establish the electrons distributed into the various byproducts. Sample electron mass balance calculations are shown in Appendix F. Electrons present in acetate derived from the degradation of LA (Figure 7.2 (C)) were subtracted while considering the percentage of electron in acetate as a result of glucose degradation. Figure 7.10 shows electron balance for LA incubated cultures and glucose control cultures. In some cultures, reasons for the percent electron to be greater than 100% is likely due to the electron contribution from biomass decay. The electron balance data indicates small fractions of electrons were diverted to methane production in LA incubated cultures when compared to the glucose controls (Figure 7.10(E)). Also, the percentage of electron flow to methanogenesis increased with the increasing LA incubation time. However, this increase was very small compared to the percentage of electrons used for methane formation in the glucose control cultures. A smaller percent of electrons was diverted to alcohols compared to the VFAs (Figure 7.10 (B) and (C)). Higher fractions of electrons were diverted to hydrogen than methane in all the LA incubated cultures. In addition, fewer electrons were diverted to VFAs under elevated hydrogen levels.

7.4 Discussion

In this work, diverting electron equivalents away from methanogens to produce hydrogen was clearly demonstrated using LA at pH 5.0 in LA (2000 mg L⁻¹ LA) incubated cultures from 0 to 25 days. The hydrogen yields and the methane profiles in the LA incubated cultures suggest that hydrogenotrophic methanogens were significantly inhibited in all the LA incubated cultures. This could be due to the combined inhibitory
effect of LA [Lalman and Bagley, 2002, Lalman and Bagley, 2000; Demeyer and Hendricks, 1967] and low pH [Kim et al., 2004; Van Ginkel and Sung, 2001; Fang and Liu, 2002] on the methanogens. High hydrogen yields were observed in all the LA incubation cultures, with a maximum hydrogen yield of $2.80 \pm 0.19$ mole H$_2$ mole$^{-1}$ glucose (70 % conversion efficiency based on theoretical stoichiometry of 4 mole H$_2$ mole$^{-1}$ glucose) in cultures incubated with LA for 0 days. On the other hand, the second highest hydrogen yield was observed in the cultures incubated for 25 days ($2.64 \pm 0.25$ mole H$_2$ mole$^{-1}$ glucose), and no particular trend in hydrogen yield was observed with the increasing incubation times.

The electron mass balance data indicates inhibition of aceticlastic methanogens. No increase in methane levels before and after glucose injection was observed thus, confirming that there was no increase in electron fluxes towards methanogenesis after glucose injection in the LA incubated cultures. On the other hand, a significant and increasing fraction of electron flow towards methane formation and no electron flow towards hydrogen formation in the glucose control cultures indicates that no methanogenic inhibition took place in the absence of LA. Also, the adequate performance of methanogenic population was confirmed by the elevated methane production levels (> 80 to 96 % than LA incubated cultures) with no hydrogen detected in the glucose control cultures. A slight increase in the methane production levels over the various incubation periods was likely due to the growth of methanogenic population which survived over the duration of the study [Rinzema et al., 1994]. In addition, higher acetate (35 % greater than the glucose controls) levels in the LA incubated cultures for 0
Figure 7.10: Electron mass balance. (A = Electron mass balance; B = Percent electrons diverted to VFAs; C = Percent electrons diverted to alcohol; D = Percent electrons diverted to hydrogen; E = Percent electrons diverted to methane; Assume 15% of the electrons from glucose degradation is converted into biomass. Values shown are mean and standard deviation for triplicate samples).
day incubation period (where no acetate was contributed from LA degradation) suggest that aceticlastic methanogens were inhibited.

The findings of this study (hydrogen yields and methane profiles); suggest that the methanogenic inhibition due to LA was not reversed in any of the LA incubated cultures over the period of this study. The exact nature of the inhibitory effect of LCFAs on methanogens is not clearly known so far. Contradictory findings have been reported on the toxicity effects of LCFAs on methanogenic populations. Rinzema et al. [1994] and Angelidaki and Ahring [1992] reported the toxic effect of LCFA to be permanent and irreversible, while others Pereira et al. [2005] and Pereira et al. [2004] have argued that LCFA inhibition is not permanent and cultures eventually acclimate to LCFAs. Nevertheless, the findings of this study in the various LA incubated cultures support the findings of Rinzema et al. [1994] and Angelidaki and Ahring [1992]. Although, in this study the cultures were not examined to assess the reversibility or irreversibility of the inhibitory effect of LA on hydrogenotrophic and aceticlastic methanogens.

The higher hydrogen yields in the cultures incubated for 0, 5, 15, and 25 days compared to the 10 and 20 days incubation periods were likely a result of the inhibitory effect of the β-oxidation byproducts present at the various incubation times. Palmitic acid was the most abundant byproduct from LA degradation, followed by myristic and lauric acid. On a molar basis, the sum of byproduct acids ranged between 84 to 98 % of the added LA (2000 mg l⁻¹) in all the cultures receiving LA. Palmitic, myristic and lauric acid have been reported in work reported by Lalman and Bagley [2000]. However, no trace of OA or SA was observed suggesting that either saturation of LA was not required before the oxidation [Canovas-Diaz et al., 1991] or the hydrogenation of LA to OA and
SA [Lalman and Bagley, 2000] may have occurred inside the cells. Unsaturated LCFAs are not needed to be saturated prior to entering the β-oxidation pathway. Degrading LA to palmitic acid (C16:0) (Eq. 7.1) is energetically more favorable than its degradation to palmitoleic acid (C16:1) (Eq. 7.2) and hydrogenation to oleic acid (Eq. 7.3) [Lalman and Bagley, 2001].

\[
\text{C}_{18}\text{H}_{31}\text{O}_2^- + 2\text{H}_2\text{O} \rightarrow \text{C}_{16}\text{H}_{31}\text{O}_2^- + \text{C}_2\text{H}_3\text{O}_2^- + \text{H}^+ \quad \Delta G_0' = -106.4 \text{ kJ mole}^{-1} \quad (7.1)
\]

\[
\text{C}_{18}\text{H}_{31}\text{O}_2^- + 2\text{H}_2\text{O} \rightarrow \text{C}_{16}\text{H}_{29}\text{O}_2^- + \text{C}_2\text{H}_3\text{O}_2^- + \text{H}^+ \quad \Delta G_0' = -28.1 \text{ kJ mole}^{-1} \quad (7.2)
\]

\[
\text{C}_{18}\text{H}_{31}\text{O}_2^- + 2\text{H}_2 \rightarrow \text{C}_{18}\text{H}_{33}\text{O}_2^- \quad \Delta G_0' = -28.1 \text{ kJ mole}^{-1} \quad (7.3)
\]

Figure 7.11 illustrates that the hydrogen yields in the LA incubated (5 - 25 days) and unincubated (0 days) cultures were a function of the LA or LA β-oxidation byproducts prevalent at the time of the analysis. The LA byproducts likely exerted a lesser inhibitory effect on the methanogenic population compared to LA. This effect was clear from the higher hydrogen yields and significantly slow hydrogen removal after peaking (approximately 80% was present on the day 7 after glucose injection) in the cultures where LA was not completely degraded (LA incubated cultures for 0 days). Of the β-oxidation byproducts detected, higher hydrogen yield was observed in cultures containing elevated levels of lauric acid (25 days LA incubation period) followed by myristic acid and palmitic acid. The later suggest that lauric acid could exert a greater inhibitory effect than myristic and palmitic acid. Koster and Cramer [1987] reported lauric acid to be more inhibitory than myristic acid on aceticlastic methanogens.

The formation of hydrogen is normally accompanied with the production of VFAs (acetate, propionate and butyrate) and alcohols (ethanol, i-propanol, i-butanol and small quantities of n-propanol and n-butanol) at low pH [Sung et al., 2002; Jones and Woods,
Elevated levels of acetate were present in the cultures while hydrogen was formed due to the inhibition of aceticlastic methanogens by LA [Lalman and Bagley, 2000; Hwu and Lettinga, 1997]. Butyrate accumulation was likely due to the inhibitory effect of low pH on butyrate degraders [Fang et al., 2004; Zheng and Yu, 2004] and elevated hydrogen levels in the cultures.

Figure 7.11: Hydrogen yields as a function of LA β-oxidation by-products in the LA incubated cultures. (LCFA levels are the maximum concentrations during the analysis. Values shown are mean and standard deviation for triplicate samples).

In addition, propionate and alcohols (ethanol, i-propanol and i-butanol) accumulation occurred due to the high hydrogen partial pressures. [Speece, 1983]. Propionic and butyric acid fermentation is favored when the hydrogen partial pressure is lower that $10^{-3.5}$ to $10^{-4.4}$ atm, respectively. Instead, ethanol is fermented at hydrogen partial pressures 2 to 3 orders magnitude higher [Fennell et al., 1997]. However, the extent of inhibition to propionate degrading microorganisms was less than that of butyrate degrading
microorganism, which could be due the higher synergistic inhibitory effects of LA and low pH on butyrate degrading microorganisms. Formate detected in the LA incubated cultures suggest that formate was one of the important hydrogen donors (Eq. 2.39) along with the reduction of NADH to NAD$^+$ (Eq. 2.40) and pyruvate to reduced ferredoxine and acetyl-CoA (Eqs. 2.34 and 2.37).

Glucose degradation was inhibited in the LA incubated cultures. A decrease in the initial glucose degradation was observed in all the cultures incubated with LA. This varied from 20 % to 55 % (compared to the glucose controls) (Figure 7.9) and was due to the associated inhibitory levels of the byproducts present at their respective incubation times as a result of LA β-oxidation. A maximum inhibition to glucose degradation was observed in cultures where the lauric acid level was predominant (25 days LA incubated cultures), followed by the cultures where linoleic acid was present (0 day LA incubated cultures). The greatest decrease in initial glucose degradation rates up to 55 % and 43 % (as a percentage of the initial glucose degradation rates in glucose control cultures), respectively, was observed in the cultures where lauric acid and LA were predominant. Lalman and Bagley [2002] reported inhibition to glucose degradation up to 50 % by LA.

The maximum hydrogen yield in this study (pH 5.0; 2.8 mole H$_2$ mole$^{-1}$ glucose) was higher than the yields reported at low pH by Fang and Liu [2002] and Fang et al. [2004] (pH 5.5; 2.1 mole H$_2$ mole$^{-1}$ glucose), and Khanal et al. [2004], (pH 4.5-6.5; 1.7 mole H$_2$ mole$^{-1}$ glucose).

Chowdhury [2005] and Gurukar [2005] reported hydrogen yields, 2.37 and 2.70 mole H$_2$ mole$^{-1}$ glucose, from glucose using cultures unincubated to LA (2000 mg l$^{-1}$) at pH 5.0. However, the effect of longer LA incubations was not assessed in their work. As
mentioned earlier: to address the issues associated with degradation of LA and its effects on hydrogen production longer incubations (6 varying incubations from 0 - 25 days at an increment of 5 days) were assessed in this work. The hydrogen yields in the LA incubated cultures did not decrease with the increasing incubations. Average hydrogen yield in the various incubations was 2.3 ± 0.2 mole H₂ mole⁻¹ glucose. The maximum hydrogen yield in this work (2.8 mole H₂ mole⁻¹ glucose) was greater than that reported by Chowdhury [2005] and Gurukar [2005].

7.5 Conclusions

Hydrogen production from glucose fermentation was examined at 37°C using LA incubated (0 to 25 days at an increment of 5 days) mixed anaerobic cultures under reduced pH conditions (pH 5.0). The conclusions of this work are as follows:

1. Hydrogen was produced and accumulated in all the LA incubated cultures.
2. The methanogenic inhibition due to LA was not reversed during the experimental duration in this study.
3. A maximum of 70% hydrogen conversion efficiency from glucose was observed based on a maximum theoretical yield of 4.0 mole H₂ mole⁻¹ glucose.
4. LA degraded to β-oxidation byproducts (palmitic, myristic, lauric acid and acetate) and was degraded in the cultures during the experimental duration. Palmitic acid was the most abundant byproduct from LA degradation.
5. The highest hydrogen yield was observed in cultures incubated for 0 days (2.80 ± 0.19 mole H₂ mole⁻¹ glucose) when LA acid was not removed from the cultures and the second highest was observed in the cultures incubated for 25 days (2.64 ±...
0.26 mole H$_2$ mole$^{-1}$ glucose) when lauric acid was the predominant β-oxidation by-product.

6. The average hydrogen yield of all the conditions examined with LA was 2.30 ± 0.20 mole H$_2$ mole$^{-1}$ glucose.

7. LA acid β-oxidation by-products exerted less inhibitory effect on methanogenesis when compared to LA itself.

8. Hydrogen was produced using the glucose degradation to acetate, butyrate and ethanol pathways. Lower hydrogen yields were observed when elevated levels of propionate were produced.

9. Glucose degradation was inhibited due to LA and LA β-oxidation by-products. Lauric exerted maximum inhibition to glucose degradation followed by LA.

10. The initial glucose degradation in glucose control cultures was 6.21 ± 0.33 μg mg VSS$^{-1}$ min$^{-1}$. Cultures predominant with lauric acid showed a reduction of 55 % in initial glucose degradation (compared to glucose controls) while, cultures with high level of LA showed a reduction by 43 %.

11. Slower hydrogen removal, after reaching peak values, was observed in the cultures where LA (80 % of maximum hydrogen was present on day 7) and lauric acid (63 % of maximum hydrogen was present on day 7) were present.
7.6 References


CHAPTER 8: HYDROGEN PRODUCTION BY OLEIC ACID (OA) INCUBATED ANAEROBIC MESOPHILIC MIXED CULTURE FOR VARYING PERIODS

8.1 Introduction

Increasing global greenhouse gas levels and depleting fossil energy sources are the major factors driving the development of renewable energy supplies. Hydrogen is an important and promising alternative energy carrier to existing fossil carbon containing energy sources from the viewpoint of carbon dioxide emissions mitigation, and production possibilities. The concept of a hydrogen economy has made a significant progress since the 1970s [Bockris, 2002]. Hydrogen can be produced from a diversity of energy resources such as fossil fuels, nuclear and renewable agriculture residues using thermo-chemical, biological, electrolytic and photolytic routes. Currently, global hydrogen production is based on steam reformation of naptha or natural gas, gasification of coal and electrolysis of water [Nath and Das, 2004]. In the US, 95% of hydrogen is produced from natural gas [Miller, 2003]. Although hydrogen is a clean burning fuel, the process used to produce hydrogen determines its impact on the environment. The global warming potential of hydrogen produced by electrolysis of water is 970 g CO₂ eq kg⁻¹ H₂ and that of steam reforming of methane is 12 times of the former [Amos et al., 2000]. The latter production techniques primarily generate greenhouse gases (CO₂, CH₄ and N₂O). In comparison, biological methods to hydrogen production will use renewable carbon from agriculture resources [Dowaki et al., 2007].

Biological hydrogen production is possible via photosynthesis and fermentation [Nandi and Sengupta, 1998; Benemann, 1996]. These processes are either light-
dependent (photosynthesis) or light-independent (dark fermentation) [Fang et al., 2004; Hallenbeck and Bennemann, 2002]. The latter is advantageous for hydrogen production because it does not rely on the availability of light and the dark fermentation reactions proceed at faster rates and have higher conversion efficiencies compared to photofermentation [Fang and Liu, 2002; Hallenbeck and Benemann, 2002]. In the dark fermentation route, mixed microbial cultures degrades complex organic substrates into hydrogen, organic acids and alcohols (Eqs. 2.22, 2.23 and 2.28).

The coexistence of hydrogen consuming microorganisms is one of the major drawbacks associated with hydrogen production using mixed microbial communities. Hydrogen consuming microorganisms such as hydrogenotrophic methanogens, homoacetogens and sulfate reducing bacteria use hydrogen as electron donors. Hence, harvesting hydrogen in these systems requires minimizing the quantity of hydrogen consumed and subsequently, increasing the yields. Studies conducted on inhibiting the growth and activity of hydrogenotrophic methanogens using pH has shown compelling evidence of methanogenic growth control on a short-term basis. However, over the long-term, after the stress is relieved, microbial growth resumes as conditions become favorable [Oh et al., 2003; Sung et al., 2002; Fang and Liu, 2002].

In this work adding OA at reduced pH conditions was used to inhibit hydrogenotrophic methanogens. LCFAs have been reported to inhibit hydrogenotrophic and aceticlastic methanogens at threshold concentrations [Lalman and Bagely, 2002]. LCFAs are amphiphilic and inhibit methanogens by adsorbing onto the microbial cell wall causing lysis and leakage of cellular components because of a mass transfer
limitation of important nutrients and ions [Hwu and Lettinga, 1997; Demeyer and Henderickx, 1967].

LCFAs are derived from lipids and fats by the hydrolytic microorganisms in mixed microbial communities. Fats and lipids are produced from edible oil refineries, slaughterhouses, and dairy products industries [Hwu et al., 1998, Hanaki et al., 1981]. LCFAs are degraded to shorter chain fatty acids, acetate and hydrogen by hydrogen producing acetogens using β-oxidation [Weng and Jeris, 1976]. Repeated β-oxidation steps convert short chain fatty acid to acetate and hydrogen. Although degradable, LCFAs are inhibitory to microorganisms [Novak and Carlson, 1970; Angelidaki and Ahring, 1992]. LCFAs cause a permanent inhibitory effect on methanogens [Rinzema et al., 1994; Angelidaki and Ahring, 1992; Rinzema, et al., 1989; Koster and Cramer, 1987]. On the contrary, Pereira et al. [2005] reported that LCFAs do not exert a permanent inhibitory effect on methanogens and that their effect is not bactericidal.

Koster and Cramer [1987] observed significant aceticlastic inhibition with OA concentrations ranging from 300 - 1500 mg l⁻¹ at 30°C. On the other hand, Lalman and Bagley [2001] reported above 30 mg l⁻¹ OA concentrations to be inhibitory to aceticlastic methanogens. Evidence of inhibiting hydrogenotrophic methanogens using OA have been reported; however, the extent of inhibition was less than that of aceticlastic methanogens [Lalman and Bagley, 2001; Hanaki et al. 1981].

This research work exploits the combined favorable hydrogen production conditions as result of methanogenic inhibition due to OA and low pH conditions. To date, no study has been reported on hydrogen production using cultures incubated to OA for varying periods. Hence, the main objective of this work was to access hydrogen production from
8.2 Materials and Methods

The materials and methods for this phase of experiments are described in Chapter 3: The inoculum source and start up is described in section 3.1.2, experimental plan and design are given in section 3.2.3, and analytical methods used are detailed in section 3.3. Initial glucose degradation rates were calculated using Graphpad Prism software using a non-linear regression by a one-phase exponential decay model. A sample calculation for initial glucose degradation rate is shown in Appendix E.

8.3 Results

8.3.1 Hydrogen and methane production

Hydrogen production from glucose was assessed using 6 sets of triplicate cultures incubated to 2000 mg l⁻¹ OA for varying periods. The incubation times varied from 0 to 25 days in 5 days consecutive increments. Significantly high levels of hydrogen were detected in all the cultures receiving OA plus glucose for all the conditions examined (Figure 8.3(A)). Hydrogen levels peaked on day 1 in all the OA incubated cultures receiving glucose. The maximum hydrogen yield (2.44 ± 0.12 mole H₂ mole⁻¹ glucose) was observed in cultures incubated with OA for 0 days and those incubated with for 25 days (2.34 ± 0.45 mole H₂ mole⁻¹ glucose). Peak hydrogen yields for OA incubated cultures are shown in Table 8.1. Tukey's paired comparison test procedure at 95 % confidence level was used to compare the hydrogen yield means [Box et al., 1978]. Statistically similar yield values were observed in all the OA incubated cultures [Table
8.1]. In addition, the trend in hydrogen yields with increasing OA incubation time is shown in Figure 8.4. No particular trend was observed in hydrogen yield with increase in incubation time. For all the conditions examined, the average hydrogen yield was 2.12 ± 0.44 H₂ mole⁻¹ glucose.

Table 8.1: Hydrogen yields in cultures incubated (0 to 25 days in increments of 5 days) to OA (2000 mg l⁻¹) receiving 5,000 mg l⁻¹ glucose.

<table>
<thead>
<tr>
<th>Experiment (LCFA Incubation Time)</th>
<th>Hydrogen Yield (mole H₂ mole⁻¹ glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 mg l⁻¹ LCFA)</td>
<td>ND²</td>
</tr>
<tr>
<td>set 1 (0-7 days)</td>
<td>2.44 ± 0.11 b</td>
</tr>
<tr>
<td>set 2 (5-12 days)</td>
<td>1.78 ± 0.40 b</td>
</tr>
<tr>
<td>set 3 (10-17 days)</td>
<td>2.28 ± 0.64 b</td>
</tr>
<tr>
<td>set 4 (15-22 days)</td>
<td>1.66 ± 0.59 b</td>
</tr>
<tr>
<td>set 5 (20-27 days)</td>
<td>2.22 ± 0.42 b</td>
</tr>
<tr>
<td>set 6 (25-32 days)</td>
<td>2.33 ± 0.45 b</td>
</tr>
</tbody>
</table>

Note: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations a and b are used to indicate the means that are statistically different within the same column. The notations cannot be used for comparing data within the same row or between rows.
2. Comparisons are based on adjacent data sets; ND = Not detectable

Controls containing OA were analyzed to confirm that there was no contribution to the hydrogen yield from hydrogen resulting from OA degradation. A negligible quantity (< 1% of the cultures receiving glucose plus OA) of hydrogen was detected in the controls receiving only OA (Figure 8.2(A)) between day 1 and day 4 and after day 4, the levels were undetectable. Control cultures were prepared with glucose to ensure methanogenesis was not inhibited over the duration of the study. Large quantities of methane reaching up to approximately 1268 μmoles were produced while no hydrogen was detected in cultures receiving only glucose (Figure 8.1(B)). In the controls receiving OA only, no significant methane (< 72% of glucose controls) production was detected when compared to the glucose controls (Figure 8.2(B)). After peak hydrogen levels in the OA incubated cultures for 5, 10 and 20 days were attained, the levels decreased and
reached undetectable levels after day 6. In comparison, hydrogen was not removed completely in cultures incubated for 0, 15 and 25 days during the 7 days analysis duration after glucose was injected (Figure 8.3(A)). The quantity of hydrogen detected on day 7 in the OA incubated cultures for 0 day was 66% of the maximum quantity. In comparison, the residual quantity was 30% of the maximum hydrogen production in the OA incubated cultures for 15 and 25 days.

Methane production profiles in OA incubated cultures receiving glucose are shown in Figure 8.3(B). The methane levels increased with increasing incubation times in the OA incubated cultures receiving glucose; however, the quantity of methane produced was significantly less compared to the methane levels in the cultures receiving only glucose. The maximum quantity of methane was observed in the OA incubated cultures for 25 days, which was less than approximately 70% of the glucose controls. No increase in methane production was observed in any of the OA incubated cultures after glucose injection, while hydrogen was produced. In addition, an increase in methane content was not accompanied with a decrease in hydrogen production.

**8.3.2 VFA and Alcohols production**

In the control cultures receiving glucose, acetate, propionate and butyrate were the major VFAs detected (Figure 8.1 (C), (D) and (E)). Elevated acetate levels reaching up to approximately 1,200 mg l\(^{-1}\) were detected in the glucose controls, while maximum propionate and butyrate concentrations in these cultures were approximately 550 and 700 mg l\(^{-1}\), respectively. Less than 143 mg l\(^{-1}\) of formate was detected in the glucose control cultures on day 1 and reached undetectable levels by day 2. On the other hand, in the OA control cultures, the only VFA detected was acetate (Figure 8.2(C)) with levels reaching
(D) Propionate Conc. (mg L⁻¹)

Time (days)

(E) Butyrate Conc. (mg L⁻¹)

Time (days)

(F) Ethanol Conc. (mg L⁻¹)

Time (days)

- ▲ 0 - 10 days  - ▼ 10 - 20 days  - ◊ 20 - 30 days
Figure 8.1: Concentration profiles for control cultures receiving 5,000 mg l\(^{-1}\) glucose. (A = glucose degradation; B = methane; C = acetate; D = propionate; E = butyrate; F = Ethanol, G = i-Propanol, H = i-Butanol. Values shown are mean and standard deviation for triplicate samples).
Figure 8.2: Concentration profiles for control cultures receiving 2,000 mg l⁻¹ OA. (A = Hydrogen; B = Methane; C = Acetate; D = LCFA. Values shown are mean and standard deviation for triplicate samples).
Figure 8.3: Hydrogen and methane production in cultures receiving 2,000 mg l⁻¹ OA plus 5,000 mg l⁻¹ glucose. (A = hydrogen; B = methane. Values shown are mean and standard deviation for triplicate samples).

Figure 8.4: Effect of varying OA incubation time on hydrogen yield for cultures receiving 2,000 mg l⁻¹ OA plus 5,000 mg l⁻¹ glucose. (Values shown are mean and standard deviation for triplicate samples).
a maximum of 1090 mg l\(^{-1}\).

VFA production profiles for the OA incubated cultures receiving glucose are shown in Figure 8.5. Acetate, propionate and butyrate were the major VFAs detected in these cultures. The acetate levels detected in the OA incubated cultures receiving glucose was as a result of glucose degradation and OA β-degradation. The maximum acetate values (the combined maximum acetate values from glucose and OA degradation) in these cultures with varying incubation time ranged between 1,220 to 1,980 mg l\(^{-1}\). The quantity of acetate contributed from glucose were calculated by subtracting the acetate levels (produced as a result of OA β-degradation) in the OA control cultures (Figure 8.2 (C)) from the total acetate levels in the OA incubated cultures receiving glucose (Figure 8.5 (A)). Hence, the maximum acetate quantities from glucose degradation ranged between 960 to 620 mg l\(^{-1}\). The maximum propionate concentrations ranged between, 430 to 780 mg l\(^{-1}\), in the OA incubated cultures receiving glucose. In these cultures, high levels were observed on day 1 followed by a decrease in the levels after day 1 except in the cultures incubated for 5 days with OA. Butyrate was the most abundant VFA from glucose degradation in the OA incubated cultures. The butyrate concentrations peaked in cultures incubated with OA for 0 days. The maximum butyrate levels in the various OA incubated cultures receiving glucose ranged between 800 to 1,320 mg l\(^{-1}\). High butyrate and acetate levels were accompanied with high hydrogen production. Small quantities of formate (< 150 mg l\(^{-1}\)) were detected in all the OA incubated cultures after glucose injection on day 1 and 2.

Ethanol, i-propanol and i-butanol were the major alcohols detected in the control cultures receiving glucose and the OA incubated cultures receiving glucose. The alcohol
profiles for the glucose control cultures are shown in Figure 8.1 (F, G, H). The maximum ethanol and i-propanol production in the glucose controls were approximately 880 (Figure 8.1 (F)) and 1,420 mg l\(^{-1}\) (Figure 8.1(G)), respectively. The average maximum i-butanol concentration in the cultures receiving only glucose was 150 mg l\(^{-1}\). Figure 8.6 shows the alcohol production profiles for cultures fed with OA and glucose. Elevated levels of i-propanol and i-butanol were associated with low hydrogen production in the OA fed cultures for 5 and 15 day incubation periods. Ethanol and i-propanol production profiles from glucose degradation in OA incubated cultures was lower than the glucose controls while higher levels of i-butanol was detected in the former. In the OA incubated cultures receiving glucose, the average maximum concentrations of ethanol and i-propanol were 520 and 540 mg l\(^{-1}\), respectively. The average maximum i-butanol concentration in the OA incubated cultures receiving glucose was 250 mg l\(^{-1}\).

### 8.3.3 OA degradation and byproducts

OA was degraded in all the cultures and was removed completely before day 12 in the OA control cultures (Figure 8.2(D)). The major by-products from the degradation of OA were palmitic (C16:0), myristic (C14:0), lauric (C12:0) acid and acetate. These byproducts peaked on different occasions during the experimental analysis. Palmitic acid was the most abundant byproduct and peaked on day 15 with a concentration of 1770 mg l\(^{-1}\), while myristic and lauric acid peaked on 25 and 30 days, with maximum values of 1,050 and 1,060 mg l\(^{-1}\), respectively. Palmitic and myristic acids were removed before day 30. No SA was detected from the degradation of oleic acid in the OA control cultures. High increasing levels of acetate from day 1 were detected from the degradation of OA in the OA control cultures. On day 1 and 30, the acetate
Figure 8.5: VFA production profiles at varying OA incubation time in cultures receiving 2,000 mg l$^{-1}$ OA plus 5,000 mg l$^{-1}$ glucose. (A = Acetate; B = Propionate; C = Butyrate. Values shown are mean and standard deviation for triplicate samples).
Figure 8.6: Alcohol production profiles at varying OA incubation time in cultures receiving 2,000 mg l⁻¹ OA plus 5,000 mg l⁻¹ glucose. (A = Ethanol; B = i-Propanol; C = i-Butanol. Values shown are mean and standard deviation for triplicate samples).
concentrations in the OA control cultures ranged between 610 and 1,100 mg l\(^{-1}\), respectively.

Similar OA removal and degradation byproducts profiles were observed in the OA incubated cultures receiving glucose (Figure 8.7). These cultures were analyzed for LCFA before glucose injection and at the end of 7 days analysis duration for hydrogen and other parameters. In the OA incubated cultures for the 0 day incubation period and before injecting glucose, OA was not completely removed during the 7-day experimental duration (Figure 8.7(A)). Concurrently, palmitic acid was detected in these cultures which peaked at 1,450 mg l\(^{-1}\) (Figure 8.7(B)). Similarly, OA and palmitic acid were detected in the OA cultures incubated for 5 days before injecting glucose. Approximately 320 mg l\(^{-1}\) OA acid was present in these cultures before glucose injection and were removed completely from the cultures before day 7 from the time of glucose injection, while plamitic acid levels ranged between 1,280 to 1,460 mg l\(^{-1}\). On the other hand, no OA was detected in the cultures incubated for 10, 15, 20 and 25 days before injecting glucose. Plamitic and myristic acid were detected in cultures fed with OA and incubated for 10, 15, 20 and 25 days, while lauric acid was detected in cultures incubated for 25 days (Figure 8.7(D)). Myristic acid concentration up to 790, 1,050, and 1,100 mg l\(^{-1}\) was observed in cultures incubated for 15, 20 and 25 days, respectively, (Figure 8.7(C)) with OA. Lauric acid peaked at 580 mg l\(^{-1}\) in the 25-day OA incubated cultures. However, no SA was detected in any of the cultures for the analyzed durations.

8.3.4 Glucose Degradation

OA and OA degradation products inhibited glucose degradation in cultures fed with OA when compared to the cultures receiving only glucose. The initial glucose
degradation rate in the cultures receiving only glucose was $6.21 \pm 0.33 \mu g \text{ mgVSS}^{-1}\text{ min}^{-1}$ (Table 8.2). The Tukey’s paired comparison test [Box et al., 1978] revealed similar statistical initial glucose degradation values for all the OA incubated cultures (Table 8.2). Lower initial glucose degradation rates were observed in the OA incubated cultures receiving glucose with values between 21 to 42 % less than the glucose control cultures (Figure 8.9/Table 8.2). No particular trend was observed in the initial glucose degradation rates with the increase in incubation time.

Figure 8.8 shows glucose degradation in OA incubated cultures with different incubation times. Glucose was completely removed in these cultures before day 2 except for the 25 day OA incubated cultures where less than 1% glucose was detected. Greater than 98 % of glucose was removed before 15 h in all the cultures receiving only glucose and undetectable levels were observed before day 1 (Figure 8.1(A)).

<table>
<thead>
<tr>
<th>Experiment (LCFA Incubation Time)</th>
<th>Initial glucose degradation rates($\mu g \text{ mgVSS}^{-1}\text{ min}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 mg $l^{-1}$ LCFA)</td>
<td>$6.21 \pm 0.33^a$</td>
</tr>
<tr>
<td>set 1 (0-7 days)</td>
<td>$3.85 \pm 0.18^b$</td>
</tr>
<tr>
<td>set 2 (5-12 days)</td>
<td>$4.08 \pm 0.63^b$</td>
</tr>
<tr>
<td>set 3 (10-17 days)</td>
<td>$3.87 \pm 0.25^b$</td>
</tr>
<tr>
<td>set 4 (15-22 days)</td>
<td>$4.91 \pm 0.40^b$</td>
</tr>
<tr>
<td>set 5 (20-27 days)</td>
<td>$4.35 \pm 0.25^b$</td>
</tr>
<tr>
<td>set 6 (25-32 days)</td>
<td>$3.62 \pm 0.63^b$</td>
</tr>
</tbody>
</table>

Note: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations a and b are used to indicate the means that are statistically different within the same column.
2. Comparisons are based on adjacent data sets

8.3.5 Electron Balance

An electron mass balance comprising the various byproducts is shown in Figure 8.10 and as an example electron mass balance calculations are shown in Appendix F. The mass balance data indicate that when compared to glucose control cultures where no
Figure 8.7: LCFA production profiles at varying OA incubation time in cultures receiving 2,000 mg l\(^{-1}\) OA plus 5,000 mg l\(^{-1}\) glucose. (A = Ethanol; B = i-Propanol; C = i-Butanol. Values shown are mean and standard deviation for triplicate samples).

Figure 8.8: Effect of varying OA incubation time on glucose degradation for cultures receiving 2,000 mg l\(^{-1}\) OA plus 5,000 mg l\(^{-1}\) glucose. (Values shown are mean and standard deviation for triplicate samples).
Figure 8.9: Percent initial glucose degradation rate versus OA incubation time. (Values shown are mean and standard deviation for triplicate samples).

Hydrogen was detected, fewer electrons were diverted to methanogens when hydrogen was produced in the OA incubated cultures (Figure 8.10 (E)). Smaller quantities of electrons were diverted to reduced byproducts such as alcohols, while higher percentage electrons were diverted to VFAs at elevated hydrogen levels in the OA incubated cultures. When calculating the percentage of electrons diverted to acetate only electrons contributed from glucose degradation was considered. In some cases, the electron percentage recovery was a little higher than 100% likely due to the electrons contributed from biomass decay.

8.4 Discussion

In this work, hydrogen production was achieved using cultures incubated to OA for varying periods under reduced pH conditions of 5.0 at 37°C by inhibiting methanogenic
% electron in Alcohols

% electron in VFAs

% electron in products and cell biomass

Time (days)
Figure 8.10: Electron mass balance. (A = Electron mass balance; B = Percent electrons diverted to VFAs; C = Percent electrons diverted to alcohol; D = Percent electrons diverted to hydrogen; E = Percent electrons diverted to methane; Assume 15 % of the electrons from glucose degradation is converted into biomass. Values shown are mean and standard deviation for triplicate samples).
activity and diverting electron equivalents away from methanogenesis. The hydrogen yields attained in the OA incubated cultures (0 - 25 days in 5 days consecutive increments) reached peak and average yield of \(2.44 \pm 0.12\) and \(2.12 \pm 0.44\) \(\text{H}_2\) mole\(^{-1}\) glucose, respectively. The electron mass balance data confirms OA inhibited methanogenic activity and diverted electron fluxes towards hydrogen production under all the incubation periods, which were examined. This suggests that the inhibition on hydrogenotrophic methanogens due to OA was not reversed during the 32-day experimental period. The findings on LCFA inhibition in this work was similar to the findings reported by Rinzema et al. [1994] and Angelidaki and Ahring [1992]. Evidence from work reported by these researchers has shown that the inhibitory effect of LCFAs is permanent and irreversible and the insignificant increase in the methane levels or recovery of methanogens is due to the exponential growth of small number of survivors. Other researchers have argued that LCFA inhibition on methanogens is not permanent and cultures eventually acclimate to LCFAs [Pereira et al., 2005; Pereira et al., 2004]. In this work, the nature of methanogenic inhibition due OA remains unexplained because the cultures were not examined for periods longer than the experimental analysis duration.

Varying hydrogen yields ranging between \(1.66 \pm 0.59\) to \(2.44 \pm 0.12\) mole \(\text{H}_2\) mole\(^{-1}\) glucose observed in the OA incubated cultures with incubation periods from 0 to 25 days were likely due to the effect of different OA degradation byproducts present in the respective cultures. OA likely exerted a higher degree of inhibition on the methanogenic population compared to its degradation byproducts. The latter was clear considering the peak hydrogen yields in the presence of OA (in 0 days OA incubated cultures before...
injecting glucose) (Figure 8.11) and slow hydrogen removal after peak quantities were detected. Examining the effect of longer incubation durations was important to assess the effect of OA degradation and β-oxidation by-products on hydrogen yields. Gurukar [2005] and Chowdhury [2005] reported lower hydrogen yields than the maximum yields achieved in this work with OA unincubated cultures (0 day incubations). In their work, the effect of longer OA incubation on hydrogen production was not assessed. In this work, the average hydrogen yield in the OA incubated cultures (0 - 25 days incubations) was 2.12 ± 0.44 mole H₂ mole⁻¹ glucose, hence, hydrogen yields were not affected by the increasing incubations.

OA degraded via β-oxidation mechanism to saturated LCFAs bearing carbon atoms ranging from 16 to 12, acetate and hydrogen. The appearance of palmitic, myristic and lauric acid were reported in previous studies on OA degradation [Lalman and Bagley, 2001]. Palmitic acid was the most abundant byproduct from OA β-oxidation. OA β-oxidation byproducts also inhibited methanogenic organisms when OA was completely degraded and removed from the cultures. Oleic acid exerts a greater inhibitory effect on methanogens than myristic [Canovas-Diaz et al., 1991] and palmitic acids. Of the β-oxidation byproducts detected, lauric acid exerted highest inhibition, which was evident from the hydrogen yields (2.34 ± 0.45 moles H₂ mole⁻¹ glucose) in the cultures where lauric acid was predominant (Figure 8.11). The hydrogen yields were only 4 % less in the cultures where hydrogen was produced under elevated levels of lauric acid compared to cultures where OA was predominant. In granulated methanogenic microbial communities fed with acetate, lauric acid and OA exerts a greater degree of inhibition when compared to myristic acid [Koster and Cramer, 1987]. In this study, methanogenic
inhibition was due to the combined effect of OA or LCFA degradation byproducts in the respective incubation studies. However, no SA was detected in any of the cultures, which could be due to two main reasons, either saturation of OA was not required before β-oxidation [Canovas-Diaz et al., 1991] or the saturation took place inside the cells.

![Graph showing LCFA concentrations over time](image)

**Figure 8.11:** Hydrogen yields as a function of OA β-oxidation by-products in the OA incubated cultures. (Values shown are mean and standard deviation for triplicate samples).

While the use of OA and OA β-oxidation byproducts inhibited methanogens, consumption of hydrogen could not be prevented from the headspace over the 7 day analysis duration. Complete or partial hydrogen consumption was observed in the OA incubated cultures. Hydrogen consumption in these cultures was likely due to the activity of hydrogen consuming homoacetogens (Eq. 2.45) because the other possibilities of hydrogen removal were remote due to absence of electron acceptors such as nitrate, iron or sulfate in the cultures and inhibition of methanogens. Also, the electron mass balance
data and the quantity of methane produced after injecting glucose confirms that the methane levels in the OA incubated cultures did not increase while hydrogen was consumed in these cultures. Hence, hydrogen was likely not consumed by hydrogenotrophic methanogens but instead it could have been utilized by homoacetogens.

A large fraction of the electron flux was diverted into VFAs formation (acetate, propionate and butyrate) as well as the alcohols (ethanol, i-propanol and i-butanol) production while hydrogen was produced under low pH conditions [Sung et al., 2002; Jones and Woods, 1989]. Butyrate was the most abundant VFA produced from glucose degradation. In the fermentation of glucose, 4.0 and 2.0 moles \( H_2 \) mole\(^{-1}\) glucose are produced when acetate (Eq. 2.22) and butyrate (Eq. 2.23) [Nandi and Sengupta, 1998] or acetate and ethanol (Eq. 2.28) [Gaudy and Gaudy, 1980; Hwang et al., 2004] are the byproducts, respectively. The acetate/butyrate ratio observed in the OA incubated cultures receiving glucose was < 1.0. This ratio ranged between 0.1 to 0.8 in the OA cultures subjected to various incubation periods. The acetate/butyrate ratio and the average hydrogen yield data (2.12 ± 0.44 moles \( H_2 \) mole\(^{-1}\) glucose) indicate that hydrogen production occurred mostly via butyrate type glucose fermentation pathway. The larger fractions of electrons used for VFAs production compared to alcohols suggest that a small fraction of electrons were used in the production of reduced compounds.

The accumulation of VFAs and alcohols imply that aceticlastic methanogens and acetogenic microorganisms were inhibited by OA and low pH conditions. Acetate and butyrate accumulation was mainly due to inhibition of aceticlastic methanogens and butyrate degraders [Lalman and Bagley, 2002; Lalman and Bagley, 2001; Koster and
Butyrate accumulation in the cultures may have also occurred due to low pH conditions in the cultures. Several researchers have reported accumulation of butyrate under low pH conditions [Fang et al., 2004; Zheng and Yu, 2004]. Also, inhibition of the acidogenic products (butyrate, propionate and alcohols) degradation is due to the high hydrogen partial pressures [Speece, 1983].

OA and OA β-oxidation byproducts exerted inhibitory effect on glucose degradation. OA inhibition on glucose degradation was reported in a similar study by Lalman et al. [2003]. The greatest inhibition on glucose degradation was observed when lauric acid was predominant in the OA incubated cultures (25 days OA incubated cultures) followed by OA (OA cultures incubated for 0 days).

The maximum hydrogen yields produced in this work (2.44 ± 0.12 moles H₂ mole⁻¹ glucose) was greater than the yields reported in studies on hydrogen production at low pH by Fang and Liu [2002], Fang et al. [2004] and Khanal et al. [2004]. At pH 5.5, 2.1 moles H₂ mole⁻¹ glucose was reported [Fang and Liu, 2002; Fang et al., 2004]. Khanal et al. [2004] reported a maximum yield of 1.7 moles H₂ mole⁻¹ glucose in a pH range of 4.5 to 6.5.

8.5 Conclusions

Hydrogen production from glucose fermentation was examined using mixed anaerobic cultures incubated to 2000 mg l⁻¹ OA for varying periods (0 to 25 days in 5 days consecutive increments) at 37°C under low pH conditions (pH 5). The conclusions from this work are as follows.

1. Hydrogen was produced and accumulated in all cultures incubated with OA.
2. OA and OA β-oxidation byproducts, palmitic, myristic and lauric acids inhibited methanogenic activity and favored hydrogen production by diverting electron equivalents away from methanogenesis. Methanogenic inhibition due to OA and OA β-oxidation byproducts was not reversed and the cultures did not acclimate to LCFAs over the experimental period.

3. Based on the hydrogen yields, OA and lauric acid inhibited methanogenic activity to the same extent. However, the inhibitory effect due to myristic and palmitic acids was less.

4. A maximum yield of $2.44 \pm 0.12$ moles $H_2$ mole$^{-1}$ glucose was observed in cultures where OA was predominant. During the experiment, lauric acid emerged as a potent methanogenic inhibitor with a yield of $2.34 \pm 0.45$ moles $H_2$ mole$^{-1}$ glucose. The average hydrogen yield detected over the duration of the study was $2.12 \pm 0.44$ moles $H_2$ mole$^{-1}$ glucose.

5. Acetate and butyrate were the preferred VFAs while ethanol was the preferred alcohol produced during glucose degradation.

6. Hydrogen was produced mainly using the glucose degradation to butyrate type pathways.

7. The combined effect of OA and OA β-oxidation by-products, low pH and high hydrogen partial pressures inhibited the removal of the glucose degradation byproducts.
8.6 References


CHAPTER 9: SUMMARY AND GENERAL CONCLUSIONS

9.1 Summary

Wastewater effluents from many major manufacturing sectors such as metallurgical, petroleum, and mining and mineral industries are rich in sulfate while those from diaries, food and vegetable oil processing contain high levels of LCFAs. These effluents are a major pollution concern and their treatment presents several challenges because of toxicity effects they impose on a variety of microbial populations. Although, LCFAs inhibit methanogens and degrade slowly during anaerobic treatment, the diversion of electron fluxes by these acids in anaerobic bioprocesses has only been reported by a few researchers. In this study, diverting electron fluxes from glucose degradation to desired terminal product formation was examined in mixed anaerobic microbial communities, while methanogens were inhibited using LCFAs.

Two cases were examined to understand the pattern of the electron flux in the mixed microbial communities. In the first case, diversion of electron fluxes was examined in the presence of an inorganic terminal electron acceptor, sulfate, and in the second case, the terminal electron acceptor were protons in the absence of sulfate. In both cases, methanogenesis, a terminal metabolic step in the anaerobic degradation of organic matter, was inhibited by LCFAs possessing 18 carbons and the electron fluxes were subsequently diverted toward the desired terminal product formation.

In the first case, the electron equivalents produced from the oxidation of glucose were utilized by SRBs and not consumed by hydrogenotrophic methanogens when unsaturated LCFAs were added to the culture. Without sulfate as the electron acceptor, protons (H+)
were reduced to hydrogen (H₂) when hydrogenotrophic methanogens were inhibited by unsaturated LCFAs.

In this work, the experiments were conducted in batch reactors using inocula enriched in 4 L semi-continuous reactors with glucose plus sulfate or glucose. The VSS concentration in all the batch cultures was 2000 mg l⁻¹ at 37°C.

In diverting electron fluxes to sulfate reduction, a COD/\text{SO}_4^{2-}\) ratio of 1.25 was used [Choi and Rim, 1991]. This ratio was chosen to establish glucose limiting conditions so that excess sulfate was available for SRBs in the presence of varying LCFA levels (100, 300, 500, 700 and 1000 mg l⁻¹) at pH 7.0 - 7.2. In these studies, the LCFAs under consideration consisted of LA, OA and SA. Diverting electron fluxes from glucose to SRBs (for sulfate reduction) was observed in cultures fed LA and OA, while SA had no significant effect on sulfate removal (Figure 9.1). This is due to the inhibitory effect of LA and OA on the methanogenic populations. Several researchers have reported the inhibitory effect of C18 LCFAs on methanogens [Lalman and Bagley, 2002; Lalman and Bagley, 2001; Hwu et al., 1998; Angelidaki and Ahring, 1992; Koster and Cramer, 1987]. In comparison to glucose plus sulfate controls, OA and LA selectively inhibited methanogens at all concentrations and caused a metabolic shift in the syntrophic electron consumption pathway. The highest sulfate reduction level in the cultures receiving glucose plus LA or OA were 92 % and 72 %, respectively (Figure 9.1). In comparison, in cultures receiving glucose plus SA, 31 % of the sulfate was removed while in glucose plus sulfate controls only 24 % was observed. The degree of methanogenic inhibition is reported to increase with the number of double bonds (LA>OA>SA) [Lalman and
Bagley, 2002; Nieman, 1954]. In the case of sulfate reduction in the presence of C18 LCFAs, the same trend was observed.

C18 LCFAs (LA (C18:2), OA (C18:1) and SA (C18:0)) are relatively insoluble (3 mg l⁻¹) [Irani and Callis, 1960] and hence, not readily available to microorganisms. The inhibition imposed by LCFAs is likely due to the surfactant properties associated with these compounds. LA acts as a surfactant (surface-active agents) by reducing the interfacial tension between the bacterial membrane and the bulk aqueous phase of the growth medium [Greenway and Dyke, 1979].

![Figure 9.1: Percentage sulfate reduction (maximum removal) for cultures fed with 1,870 mg l⁻¹ glucose, 1,500 mg l⁻¹ sulfate and 100 to 1,000 mg l⁻¹ LCFAs (C2= Sulfate control; C4= Glucose plus sulfate control. Values shown are mean and standard deviation for triplicate samples).](image)
The ability of a compound such as LA (C18:2) to migrate to cellular surfaces and lower the interfacial tension is related to its surface tension. In comparison to LA, SA (C18:0) was reported not to inhibit growth at 30°C since it is a much poorer surfactant [Greenway and Dyke, 1979]. LCFA adsorption is the main mechanism for these acids to impose their inhibitory effects. After adsorption, LCFAs initiates the inhibition process by interfering with extracellular membrane as well as intercellular processes.

The initial sulfate and glucose degradation rates were affected by LA and OA. The sulfate removal data demonstrate that LA and OA assisted with its reduction while SA had no observable effect (Figure 9.2 (A)). In comparison to their effects on sulfate, increasing LA and OA levels decreased the initial glucose degradation rates (Figure 9.2 (B)). The degree of inhibition on glucose degradation increased with the degree of unsaturation and the LA and OA concentration. LA caused a higher inhibition to glucose degradation compared to SA [Lalman et al., 2003]. Acetate and propionate were the major VFAs detected from glucose degradation in all the cultures analyzed for sulfate reduction.

VFAs accumulation and subsequent pH decrease in the cultures was due to inhibition of acetogens. Hence, prior to performing the electron flux diversion studies in the absence of sulfate (hydrogen production), hydrogen consumption experiments were conducted to assess the effect of low pH and as well as OA or LA (individual and mixtures) on hydrogenotrophic methanogens at pH values of 5.0 and 6.0 and at 37°C. LCFAs under low pH conditions are known to exert a synergistic inhibitory effect on hydrogenotrophic methanogens and the extent of inhibition increases with increasing LCFAs concentrations. Based on the findings of the hydrogen consumption studies, the
hydrogen production studies were conducted at pH 5 and 2000 mg l\(^{-1}\) OA and LA in the batch cultures at 37\(^{\circ}\)C.

In the hydrogen production studies, experiments were conducted using LA or OA incubated cultures with incubations ranging from 0 to 25 days in 5 days consecutive increments. An issue associated with hydrogen production from carbohydrates (glucose) in the presence of LCFAs is the degradation of the LCFAs themselves. The novelty of this work was to explore hydrogen production using varying incubations and assess the effect of degradation of the parent LCFA compound. Typically, the LCFAs are degraded and removed from the anaerobic cultures within 20 - 25 days and hence, a maximum incubation duration of 25 days was selected to determine the minimum dosage (quantity of LCFAs) for maximum hydrogen yields or to determine the effect of LCFAs \(\beta\)-oxidation byproducts. Inhibiting the hydrogenotrophic methanogens resulted in the accumulation of hydrogen under all OA and LA conditions examined. Hydrogen was produced and accumulated in cultures incubated for 0 to 25 days.

The incubation experiments were conducted to confirm or dispute whether LCFA inhibition on hydrogenotrophic methanogens would reverse at some point during the incubation periods under examination. LA inhibited the hydrogenotrophic methanogens to a higher degree when compared to OA. Higher hydrogen yields were achieved in the cultures receiving LA when compared to cultures receiving OA (Figure 9.3). In the hydrogen production studies with LA, the maximum hydrogen yield from glucose reached 2.80 ± 0.20 mole H\(_2\) mole\(^{-1}\) glucose and in experiments with OA, a yield of 2.44 ± 0.12 moles H\(_2\) mole\(^{-1}\) was attained. The average hydrogen yield in cultures fed with LA and OA were 2.30 ± 0.20 and 2.12 ± 0.44 moles H\(_2\) mole\(^{-1}\) glucose, respectively. In the
LCFA incubated cultures, the hydrogen yield was also a function of the type of the predominant LCFA's β-oxidation byproduct present during the time of analysis. Both lauric (a saturated LCFA bearing 12 carbons) and myristic (a saturated LCFA bearing 14 carbons) acids inhibited hydrogen consumption; however, the greatest inhibition was observed in cultures containing only lauric acid. LCFA's also inhibited acidogens (21-50% reduction in initial glucose degradation occurred in various cultures incubated with OA and LA) and possibly acetogens to a lower extent compared to methanogens; however, no evidence of the latter was observed in this work. Evidences of inhibition to VFA removal have been reported in the studies by Mykhaylov et al. [2005] and Lalman and Bagley [2002].

![Graph showing initial sulfate degradation rates vs. LCFA Concentration](image)

(A) Initial sulfate degradation rates (mg mg VSS\(^{-1}\) min\(^{-1}\)) vs. LCFA Concentration (mg l\(^{-1}\))

- SA Batch
- OA Batch
- LA Batch
Figure 9.2: Initial degradation rates (maximum removal) for cultures fed with 1,870 mg l$^{-1}$ glucose, 1,500 mg l$^{-1}$ sulfate and 100 to 1,000 mg l$^{-1}$ LCFA. (A = Initial sulfate degradation rates; B = Initial glucose degradation rates. Values shown are mean and standard deviation for triplicate samples. C2= Sulfate control; C4= Glucose plus sulfate control).

The major VFAs and alcohols byproducts from glucose degradation were acetate, propionate, butyrate, ethanol, i-propanol and i-butanol. However, in the sulfate reduction work at pH 7.0 - 7.2, no alcohols were detected. The formation of alcohols in hydrogen production studies was likely due to the low pH (pH 5.0) of the culture medium. With the decrease in the pH and elevate in hydrogen partial pressures, the glucose degradation pathway shifts to the production of alcohols [Dabrock et al., 1992; Lowe and Zeikus, 1991; Gottwald and Gottschalk, 1985]. The shift toward solvent formation is a response
to unfavorable environmental conditions such as drop in pH because this path way yields less number of ATP and growth yield [Gottschalk and Bhal, 1981].

![Graph showing hydrogen yields](image)

Figure 9.3: Hydrogen yields in cultures receiving 5,000 mg l$^{-1}$ glucose and 2,000 mg l$^{-1}$ LCFAs. (Values shown are mean and standard deviation for triplicate samples).

A two-stage reactor design is recommended for sulfate reduction or hydrogen production to maximize organic removal and treatment efficiency in anaerobic organic biodegradation processes.

### 9.2 General Conclusions

In this study, methanogenesis, a terminal metabolic step in anaerobic biodegradation of organic matter was inhibited using LCFAs possessing 18 carbons and the terminal metabolic pathways were manipulated to produce desired terminal products by diverting the electron fluxes to the desired terminal reactions. The electron equivalents produced
from the oxidation of glucose were diverted to SRBs for sulfate reduction and away from MPBs for hydrogen production, while methanogens were inhibited using LCFAs.

A metabolic shift in the syntrophic microbial pathway by diverting electron equivalents to SRBs was achieved using LCFAs bearing unsaturated C-C double bonds while SA bearing no double bond had no significant effect on sulfate removal. Using OA (LCFA with one unsaturated C-C double bond) and LA (LCFA with two unsaturated C-C double bonds) selectively inhibited methanogens and decreased the competition between SRBs and MPBs for electrons equivalences. The flow of electron fluxes towards sulfidogenesis was a function of LCFA concentration as well as the type of LCFA used. LA increased the overall electron flow to sulfate reduction up to greater than 30 % compared to the cultures receiving only glucose plus sulfate. A maximum of 1380 mg 1⁻¹ (92 %) sulfate reduction was observed in the cultures receiving LA while only 24 % sulfate was reduced in the cultures receiving only glucose plus sulfate. In addition, up to 73 % sulfate reduction and a 22 % increase in overall electron flow was achieved when OA was used to inhibit methanogenesis. The percentage electron flow and sulfate reduction increased with the increasing OA and LA concentrations.

Prior to conducting the studies on hydrogen production from glucose fermentation in the absence of an inorganic electron acceptor such as sulfate, hydrogen consumption studies in the presence of OA and LA provided an understanding of LCFA inhibition on hydrogen consumption. Establishing conditions to decrease hydrogen removal by hydrogenotrophic methanogens was important because LA and OA were used to redirect electron fluxes and subsequently produce hydrogen from glucose degradation. In the hydrogen removal studies, the inhibition was greater in cultures maintained at pH 5 than
at pH 6. Experiments were conducted to assess the combined effect of LCFAs and pH on methanogenic inhibition. Understanding the combined effect of LCFAs and pH on methanogenic inhibition was important because the pH of the degrading medium decreased due to accumulation of VFAs such as acetate, propionate and butyrate, while microbial populations responsible for their degradation are inhibited due to the presence of LCFAs. LCFAs and low pH conditions exerted a synergistic inhibitory effect on hydrogen removal and the extent of inhibition increased with increasing LCFA concentrations. Hence, in the hydrogen production studies, the optimum conditions examined were pH 5 and 2,000 mg l\(^{-1}\) LCFA (OA and LA).

Diverting electron fluxes away from methanogenic population resulted in hydrogen accumulation and an increase in the hydrogen yield under all conditions using LA and OA. Hydrogen accumulation was observed in all the LA and OA cultures incubated for the different incubation periods (6 incubations ranging from 0 to 25 days in 5 days consecutive increments). The findings of this work suggest that hydrogenotrophic methanogenic inhibition due to LCFAs (LA or OA) was likely irreversible during the duration of the study. A maximum of 70 % hydrogen conversion from glucose was achieved based on a maximum theoretical yield of 4.0 mole H\(_2\) mole\(^{-1}\) glucose. In the studies conducted with LA, the maximum hydrogen yield from glucose fermentation was 2.80 ± 0.12 mole H\(_2\) mole\(^{-1}\) glucose and with OA the yield was 2.44 ± 0.12 moles H\(_2\) mole\(^{-1}\). The average hydrogen yields in the LA and OA experiments were 2.3 ± 0.20 mole H\(_2\) mole\(^{-1}\) glucose and 2.12 ± 0.44 moles H\(_2\) mole\(^{-1}\) glucose, respectively. LCFAs and its byproducts inhibited hydrogen removal. In the LA and OA incubated cultures, the hydrogen yield was also a function of the type of the predominant LCFA \(\beta\)-oxidation
byproduct. Of all the detected LCFA byproducts, lauric exerted the greatest inhibition followed by myristic acid.

In general, LCFAs could be successfully used to inhibit methanogens and divert electron flux by manipulating the terminal metabolic pathways. Two treatment systems which could be further developed to establish the effectiveness of LCFAs are the effluents containing sulfate and hydrogen production.
9.3 References


CHAPTER 10: ENGINEERING SIGNIFICANCE AND SUGGESTIONS FOR FUTURE RESEARCH

This research provides a large quantity of data to initiate the development of sustainable anaerobic bioprocesses for addressing two major environmental engineering concerns. The first is the treatment of high sulfate rich effluents and the second is producing hydrogen from renewable-agriculture products.

The results form phase I of this study clearly indicate that use of LCFAs inhibited methanogens selectively without affecting the activity of sulfate reducers. Treatment of sulfate using mixed anaerobic communities is a concern because of a competitive reaction with methanogens and therefore, rendering the sulfate treatment process inefficient. LCFAs are non-toxic, easily available and degradable to acetate and hydrogen. Hence, LCFAs could be employed to maximize the removal of sulfate in sulfate rich effluents from industrial sectors such as mining and metallurgy.

In the sulfate reduction studies, OA (C18:1) and LA (C18:2) levels of up to 1000 mg l$^{-1}$ were used. However, further research needs to be conducted to determine the effect of higher LCFA levels because the industrial effluents from many food processing and dairy industries contain elevated LCFAs levels of up to $> 20,000$ mg l$^{-1}$ [Borja and Banks, 1994]. In addition, additional work should be conducted to assess if LCFAs can serve as electron donors for sulfate reduction. In this work, the results from the control studies with LCFAs plus sulfate indicate that the fractions of electrons which were utilized for sulfate reduction was likely very small.

The data from hydrogen metabolism studies (Phase II) suggest that low pH coupled with LA or OA exerted a synergistic inhibitory effect on hydrogenotrophic methanogens. Based on the data from the hydrogen removal studies, an initial LCFA (LA or OA) level
of 500 mg l⁻¹ was selected as the lower end of the range and 2,000 mg l⁻¹ was the upper range.

The results from studies related to diverting electron equivalents away from methanogens using LCFAs can be used to produce hydrogen. Inhibition of hydrogenotrophic methanogens and other H₂-consumers causes hydrogen to accumulate. Because of issues related to energy security issues, increasing greenhouse gases, and dwindling fossil fuel supplies, hydrogen is emerging as an important renewable fuel.

A major concern associated with hydrogen production by inhibiting methanogens with LCFAs is its degradation and the effect of the β-oxidation by-products on hydrogen production. To assess the impact of LCFAs, 6 incubation periods were established from 0 to 25 with cultures containing LA or OA. Glucose was added at the beginning of each incubation period. The results from this study clearly demonstrate high levels of hydrogen production and accumulation using LA or OA. Data from these findings could be used as a basis for the design of continuous hydrogen production studies.

In this study, hydrogen accumulation was detected in the presence of β-oxidation LCFA byproducts such as myristic, palmitic and lauric acid. Hence, the inhibition was a combined effect of the parent LCFA and the byproducts. Lauric acid exerted the greatest inhibitory effect of all the byproducts detected. Therefore, further research should be conducted to assess the effect of individual and mixtures of LCFAs bearing less than 18 carbons on hydrogen production.

In addition, one of the concerns associated to hydrogen production studies by inhibiting methanogens is the nature of LCFA inhibition. Contradictory findings on the nature of LCFAs have been reported. Some researchers have reported the inhibitory
The effect of LCFAs is permanent and irreversible [Rinzema et al., 1994; Angelidaki and Ahring, 1992] while others have reported that LCFA inhibition on methanogens is not permanent and cultures eventually acclimate to the acids [Pereira et al., 2005 and Pereira et al., 2004]. The findings of this study on methanogenic inhibition support the findings on permanent and irreversible nature of methanogenic inhibition due to LCFAs. However, the cultures were not assessed for longer than the 32-day experimental duration to examine the nature of methanogenic inhibition due to LCFAs over long extended periods. Experiments should be conducted to assess the effect of longer LCFA incubation on hydrogen production and accumulation or the continuous pilot experiments will provide an understanding of the impact of longer LCFA exposure.

Finally, a laboratory study should assess a two-stage reactor system for hydrogen production. The first reactor will be used for hydrogen production and recovery while the second for the treatment or processing of elevated levels of VFAs and alcohols into saleable byproducts or electricity [Logan, 2004].
10.1 References


APPENDICES

Appendix A: Reactor A1 Characterization results

Figure A1: Glucose degradation profile in cultures using inocula from Reactor A1. (Values shown are mean and standard deviation for triplicate samples).

Figure A2: VFA profiles in cultures using inocula from Reactor A1. (Values shown are mean and standard deviation for triplicate samples).
Figure A3: Sulfate removal profile in cultures using inocula from Reactor A1. (Values shown are mean and standard deviation for triplicate samples).

Figure A4: Methane production profile in cultures using inocula from Reactor A1. (Values shown are mean and standard deviation for triplicate samples).
Appendix B: Reactor B1 Characterization results

Figure B1: Glucose degradation profile in cultures using inocula from Reactor B1. (Values shown are mean and standard deviation for triplicate samples).

Figure B2: VFA profiles in cultures using inocula from Reactor B1. (Values shown are mean and standard deviation for triplicate samples).
Figure B3: Methane production profile in cultures using inocula from Reactor B1. (Values shown are mean and standard deviation for triplicate samples).
Appendix C: Calibration Curves

Figure C1: Volatile Fatty Acids Calibration Curves.

Figure C2: Sulfate Calibration Curve.
Figure C3: Alcohols Calibration Curves.

Figure C4: Glucose Calibration Curve.
Figure C5: Long Chain Fatty Acids Calibrations Curves.

Figure C6: Gas Calibration Curves.
Appendix D: Long Chain Fatty Acids Extraction Recovery

Figure D1: Long chain fatty acids extraction recovery plot.
Appendix E: Sample initial glucose degradation rate calculations

Initial glucose and sulfate degradation rates were calculated using Graphpad Prism software using a non-linear regression.

\[
\frac{\Delta C}{\Delta t} = \frac{dC}{dt} = -k \cdot C
\]

Integrating the above equation gives:

\[
C = C_0 e^{-k \cdot t} = C_0 \exp(-k \cdot t) \quad \text{When } t \to 0 \quad C = C_0
\]

\[
\frac{dC}{dt} = -k \cdot C
\]

Where, \( C \) (mg l\(^{-1}\)) and \( k \) (hr\(^{-1}\)) are determined from the software.

Example calculation: Initial glucose degradation rate for the individual glucose control (Figure 4.5 (A)) is shown below. The initial glucose degradation rates reported in Table 4.1 are average of triplicate glucose cultures.

\[
C \cdot k \text{ (mg l}^{-1} \text{ hr}^{-1}) = 795 \text{ mg l}^{-1} \text{ hr}^{-1} (C = 2025 \text{ mg l}^{-1}; k = 0.372 \text{ hr}^{-1})
\]

\[
= (795 \text{ mg l}^{-1} \text{ hr}^{-1}) \times (1000 \mu g \text{ mg}^{-1}) \times (1/2000) \text{ mg (VSS)} \times (\text{hr/60 min}) = 6.62 \mu g \text{ mg VSS}^{-1} \text{ min}^{-1}
\]
Appendix F: Sample electron mass balance calculations

The electron balance only includes the substrates and products, and does not include the LCFA parent compound nor its byproducts. In the sulfate reduction work (Chapter 4 and 5) the electron balance assumes that the degradation rate of LCFA is low enough based on the LCFA control experiments analyzed during the 7-day experiments. On the other hand, in the hydrogen production studies (Chapter 7 and 8), where the experiments were analyzed for 32 days, the electrons contributed from the degradation of LCFAs were subtracted from the total electrons contributed from glucose and LCFAs. This was achieved by subtracting the electrons present in acetate derived from the β-oxidation of LCFAs (Figures, 7.2 (C) and 8.2 (C)) while considering the percentage of electron in acetate as a result of glucose degradation (Figures, 7.5 (A) and 8.5 (A)). The entire electron balance assumes that, 15 % of the electrons from glucose degradation are converted into biomass. The electron balance was calculated using Eq. F.1.

$$\text{Electron mass balance} \ (% ) = \frac{\text{electrons}_{\text{substrate}} + \text{electrons}_{\text{products}}}{\text{electrons}_{\text{initial}}} \times 100$$ \hspace{1cm} (F.1)

**Sample Calculation:** An illustrative example of electron mass balance for control cultures receiving 5000 mg l\(^{-1}\) glucose (Figure 7.1) on day 1 is shown in Figure F.1. The electron mass balance for these cultures are shown in Figure 7.10.

The amount of electrons from each substrate and product were determined from the oxidation or reduction half reactions: Therefore, on a per mole basis, glucose gives 24 electrons (initial electrons in Eq. F.1), acetate gives 8 electrons, propionate gives 14 electrons, butyrate gives 20 electrons, hydrogen gives 2 electrons, ethanol uses 6 electrons, propanol uses 12 electrons and methane uses 8 electrons. The concentrations
of substrates and products were converted to electrons based on the stated relationships and were related as a percentage based on the initial amounts of substrate added. In these cultures, the total electron percentage recovery is 100%. Approximately 38%, 37% and 10% electrons were used in VFAs, alcohols and methane formation, respectively (Figure F.1).

**Figure F.1:** Diagrammatic representation of the electron mass balance in cultures receiving 5,000 mg l⁻¹ glucose.
**VITA AUCTORIS**

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