Product generation from acidogenic fermentation of nitrogen-deficient wastes

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Abstract

Hydrogen, volatile fatty acid (VFA), and ethanol production were assessed from anaerobic fermentation of nitrogen deficient waste streams in order to determine whether nitrogen fixation would take place without nitrogen supplementation. Continuous stirred tank reactors (CSTR) were run under nitrogen deficient conditions with a synthetic glucose-based wastewater being used as the feed. In this study, stable acidogenic fermentation was achieved with the sole nitrogen source coming from nitrogen fixation.

A mixed culture of bacteria, sourced from Feilding wastewater treatment plant (located in the lower North island, NZ), was input into two IL reactors. The two reactors were initially run under nitrogen-sufficient conditions at pH 5.5, which is considered to be the optimum pH for hydrogen production. The available nitrogen level was systematically reduced in order to investigate the effect of a gradual decrease in available nitrogen (corresponding to an increase in COD to nitrogen ratio (COD:N ratio)) on reactor performance. It was found that total VFA production of acetate, propionate, butyrate and valerate remained similar at all nitrogen levels, though the proportions of each VFA changed slightly; while biomass yield decreased as the COD:N ratio increased. The highest hydrogen output was found to be at the highest nitrogen level, while the hydrogen production decreased with decreasing available nitrogen from a COD:N ratio of 53 to 100 due to methane production and then increased again at a COD:N ratio of 500 and 1000 but not to the same level as was produced initially. In contrast, the carbon dioxide production remained similar at all COD:N ratios. Ethanol production greatly increased as the nitrogen level decreased.

As a second stage to this study the reactors were run at different pHs under nitrogen deficient conditions in order to determine the effect of pH on the behavior of nitrogen-fixing wastewater treatment systems. One reactor was run at pH 4.0 while the other was maintained at pH 5.5. VFA, ethanol and gas production were compared. It was found that hydrogen and VFA yields were higher at pH 4.0 than at pH 5.5, while ethanol and biomass yields were very similar at the two pHs. The predominant VFA
present differed. Though the biomass yields were similar at the two pHs, it was noted that the rate of biological activity was severely reduced at pH 4.0.

Overall it seems that nitrogen fixation is able to take place under nitrogen deficient anaerobic conditions with mixed culture bacteria present. pH seemed to have a large effect on overall reactor productivity. Further study could be performed in order to compare different reactor conditions other than pH, such as HRT under nitrogen deficient conditions. Also, comprehensive microbiological investigation could help to determine which bacterial species are present under which environmental conditions, and whether there is a shift in the biomass population with changes in environmental conditions.
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1. Literature Review

1.1 Overview of anaerobic fermentation.

Anaerobic digestion has been demonstrated to be advantageous for treating wastewater as it requires lower energy than aerobic processing and hence is economically less expensive to run. Anaerobic digestion also leads to the production of useful gases and soluble by-products such as volatile fatty acids (VFAs). For these reasons, anaerobic digestion is widely used for treating industrial wastewater and municipal wastes (Demirel and Yenigün, 2002).

The first step in anaerobic fermentation is hydrolysis. This involves conversion of organic matter to sugars, fatty acids and amino acids. In many laboratory studies on simulating anaerobic digestion of wastewater, glucose is used as a substrate as hydrolysis of α-1,4-glucosidic bonds does not appear to be rate-limiting, while that of β-1,4-glycosidic bonds, as found in cellulose, does seem to be rate limiting (Zoetmeyer et al., 1982). Figure 1 shows the two major phases involved in anaerobic digestion of hydrolysed material. Phase one is acidogenesis and phase two is methanogenesis. Acidogenesis can be further broken down into many smaller processes. Overall acidogenesis involves fermentation of the products of hydrolysis to volatile fatty acids (VFAs), carbon dioxide and hydrogen gases, ethanol and some lactic acid (van Andel and Breure, 1984). In a sub-process, acetogenesis, the reduced products are oxidized to hydrogen, carbon dioxide and acetic acid. Phase two involves the production of methane by methanogens. There are two principal methods of methane formation. The first is conversion of acetic acid to methane. This involves acetic acid conversion to carbon dioxide, with half of the acetic acid further reduced to methane (Klass, 1998). This accounts for approximately 68 percent of methane produced via methanation (Boone, 1982). The second is the formation of methane and water via the reduction of carbon dioxide.
Fats, proteins, carbohydrates

1. Acidogenesis  Acidogenic bacteria

   biomass  VFAs  CO₂ + H₂  ethanol and lactic acid

2. Methanogenesis  Methanogenic bacteria

   biomass  CO₂ + CH₄

Figure 1. An overview of anaerobic digestion processes.

1.2 The substrate.

1.2.1 Complex carbohydrates
Cellulose and hemicellulose are the most plentiful renewable organic compounds on earth (Khan and Trottier, 1978; Schwarz, 2001). According to Niessen et al. (2005), cellulose makes up approximately half of earth’s biomass. As cellulose is such an abundant carbohydrate in nature, anaerobic digestion possibly leading to hydrogen gas formation should be considered as a possible renewable and alternative fuel source in the future.

Lignocellulose is a complex material made up of a matrix of lignin and hemicellulose with embedded cellulose fibres (Saha and Woodward, 1997). Lignin seals cellulose, protecting it by a barrier (Wise, 1984). In most plant materials, the ratio of cellulose:hemicellulose:lignin is 4:3:3. Cellulose is made up of six carbon sugars joined together by β-glycosidic linkages. Hemicellulose is made up of five carbon sugars. Cellulose consists of building blocks with two glucose molecules each (Brown, 2003). Hemicellulose consists of hexoses (D-glucose, D-mannose, D-galactose), pentoses (D-xylose, L-arabinose, D-arabinose) and deoxyhexoses. Lignin
is made up of three monomers of alcohols with functional groups. Lignin is an aromatic polymer covalently bonded to cellulose making up lignocellulose which is highly resistant to microbial degradation (Pometto III and Crawford, 1986).

According to Saha and Woodward (1997), cellulose is very resistant to depolymerization due to its physical properties: it is highly crystalline; it is water insoluble due to the presence of long carbon chains containing more than six carbon atoms. Cellulose can be enzymatically degraded to glucose by: endo-1,4-β-glucanases, exo-1,4-β-D-glucanases and 1,4-β-D-glucan cellobiohydrolase. All cellulases have been found to degrade β-1,4-glucosidic bonds. Niessen et al. (2005) found that anaerobic bacteria use multi-enzyme complexes called cellulosomes to degrade cellulose while minimizing enzyme loss. Only anaerobic bacteria possess cellulosomes, aerobic bacteria do not (Schwarz, 2001).

In order for fermentation of cellulose or hemicellulose to take place, they must first be converted to their component sugars (Saha and Woodward, 1997). Marasabessy (1998) showed that starch had to be pretreated prior to fermentation due to its molecular complexity. This is likely to be the case for cellulose as it too has a complex molecular structure. Pretreatment is used to dissociate lignin from cellulose. Hydrolysis of cellulose and hemicellulose follows to form monosaccharides, which are carried through fermentation (Rahmat, 1991). Rogers et al. (1992) found that anaerobic degradation of pulp and paper effluent is possible. Eleven types of cellulolytic bacteria have been found in the past in a pig-waste digester (Bushell and Slater, 1981).

1.2.2 Nitrogen deficiency and nitrogen fixation
Nitrogen is known to be a required element for microorganisms as it is needed for the production of DNA and proteins. The common chemical formula for biomass is C_{5}H_{7}NO_{2}P_{0.074} (Droste, 1997). According to Ammary (2004), and Metcalf and Eddy (2003), a commonly used COD:N:P ratio for anaerobic wastewater treatment is 250:5:1. Up until now, very little work has been done to see whether a higher COD:N ratio is possible when anaerobically treating wastewater. Brandberg et al. (2007) found the glucose uptake efficiency to be best with a COD:N ratio of approximately
70, while the efficiency dropped as the COD:N ratio increased to 130. Reactor efficiency is the ratio of energy output as products compared to energy input into the reactor. Hence it has been thought that nitrogen must be added to nitrogen deficient wastewater in order for adequate anaerobic wastewater treatment to take place. When wastewater is deficient of nitrogen (having a very high COD:N ratio) then nitrogen is often added in the form of ammonia which can be quite costly. According to Gauthier et al. (2000), pulp and paper mill wastewater tends to be deficient of nitrogen – it has a high carbon to nitrogen ratio. Therefore, ammonia or urea is often added as a nitrogen source. Ammonia is likely to be a major source of nitrogen in digestive processes for fermentative bacteria (Bushell and Slater, 1981) but it should be noted that, according to Reid (1983), organic forms of nitrogen help to degrade cellulose faster than NH₄Cl.

As an alternative to nitrogen supplementation, Kargi and Özmihiçi (2002) found it possible to treat wastewater to a great extent using a nitrogen fixing bacteria. Adding fewer nutrients to wastewater can help to limit eutrophication of the catchment water basins. They noted that the anaerobes Bacillus, Klebsiella, Rhodopseudomonas and some Clostridial genus are capable of fixing nitrogen in treated wastewater.

Nitrogen fixation with respect to anaerobic wastewater treatment is an area where very little is known to date. Nitrogen fixation could be a solution by limiting the need for nitrogen addition to low nitrogen wastewaters. Nitrogen fixation is a reduction reaction involving the conversion of dinitrogen (N₂) to ammonia (NH₃). The ammonia is consequently assimilated into the cells’ nitrogenous compounds. In order for nitrogen fixation to occur it is thought that there must be a carbohydrate source, low fixed nitrogen and low or no dissolved oxygen present. Nitrogen fixation requires a lot of energy (Equation 1) in order to break the N-N triple bond.

\[
\text{N}_2 + 8\text{H}^+ + 8\text{e}^- + 16\text{MgATP} \rightarrow 2\text{NH}_3 + \text{H}_2 + 1\text{MgADP} + 16\text{Pi} \quad \text{Equation 1}
\]

Anaerobic bacteria with the ability to fix nitrogen in nitrogen deficient environments could have a large selective advantage over other bacteria for treating certain types of wastewater. The *Azotobacter* species have previously been selected to treat nitrogen-deficient wastewater.
Bacteria capable of nitrogen fixation (called diazotrophs) require the nitrogenase enzyme. The nitrogenase system consists of iron and molybdenum, and occasionally vanadium. During nitrogen fixation, hydrogen is produced and either oxidized by hydrogenase enzymes present in the cells or lost to the environment (Arp, 1990). Hydrogen has been found to play three roles in nitrogen-fixation: firstly it is a product, secondly it acts to inhibit the nitrogenase enzyme, and thirdly it can be a potential electron donor (Burris, 1971).

Below is a list of the anaerobic bacteria known to be capable of fixing nitrogen according to Silvester and Musgrave (1991).

A. Facultative anaerobic N-fixing bacteria:
   a. Enterobacteriacea:
      i. Klebsiella pneumoniae, Klebsiella rubiaciarum, aerogenes
      ii. Citrobacter freundii, intermedius
      iii. Enterobacter aerogenes, agglomerans, cloacea
      iv. Erwinia herbicola
      v. Escherichia intermedia
   b. Bacillaceae: Bacillus polymyxa and macerans
   c. Vibrionaceae: Vibrio diazotrophicus, natriengens, cincinnatiensis, pelagius

B. Obligate anaerobes:
   a. Bacillaceae:
      i. Clostridium acetobutylicum, beijerinckii, butylicum, butyricum, felsinium, kluyveri, lactoacetophilum, madisonii, pasterurianum, pectinovorum, saccharobutyricum, tetanomorphum, tyrobutyricum
      ii. Desulfotomaculum ruminis, orientis
   b. Uncertain: Desulfovibrio africanis, baculatus, desulfuricans, vulgaris, gigas, salexigens

It has been noted that some Klebsiella species are capable of nitrogen fixing in anaerobic environments (Gauthier et al., 2000; Bruce and Clark, 1994) and Klebsiella bacteria often dominate nitrogen fixing environments. This may be due to a physiological nitrogen fixing advantage present in Klebsiella, not present in other bacterial species. According to Minamisawa et al. (2004), anaerobic nitrogen-fixing bacteria often belong to the genus Clostridium. This genus is very sensitive to oxygen, restricting their growth in anaerobic environments. The varying Clostridium species produce different fermentation products (Cheong et al., 2006). Clostridium
butyricum often produces butyric acid and acetic acid in a 2:1 ratio while also producing hydrogen. *Clostridium acetobutyricum* produces less hydrogen and uses up butyric acid to make butanol. *Clostridium kluyver* often leads to ethanol-acetic acid type fermentation with products of butyric acid, hydrogen and caproic acid formed from ethanol and acetic acid. *Clostridium sphenoides* tends to produce ethanol, acetic acid, hydrogen, carbon dioxide and some lactic acid, but no butyric acid.

### 1.3 VFA, Hydrogen and Ethanol as Products.

Anaerobic digestion of organic wastes carried out to completion produces methane gas and carbon dioxide. As these are both greenhouse gases, alternative cleaner energy sources are sought after. In the first phase of anaerobic digestion, VFAs, short chain alcohols, carbon dioxide and hydrogen gas are produced. Hydrogen is a good alternative fuel source for the future as it is nonpolluting and it has high conversion efficiency. Hydrogen gas has several advantages: it is renewable, it is clean burning as it does not produce any fine particulate matter or hydrocarbons (Valdez-Vazquez *et al.*, 2005), it does not generate any toxic by-products, and it requires less energy to produce than does methane gas production. In a comparison between several alternative fuel sources, hydrogen was found to be the best in terms of renewability and environmental criteria (Veziroglu and Barbar, 1992). The gaseous products of anaerobic acidification reactions are VFA’s, hydrogen and carbon dioxide (Zoetmeyer *et al.*, 1982). The potential to harness the hydrogen gas from anaerobic wastewater treatment processes thus provides an interesting alternative to methanogenesis.

Ethanol is also commonly a product of anaerobic fermentation. *Clostridium* species of bacteria are known to be ethanol producing (Lin *et al.*, 2006). *Clostridium kluyveri* is known to lead to butyric acid, ethanol, acetic acid and hydrogen production (Cheong and Hansen, 2006). *Clostridium sphenoides* also produces ethanol along with hydrogen, carbon dioxide, some lactic acid, acetic acid, but no butyric acid. According to Cheong and Hansen (2006) ethanol production inhibits hydrogen production. In theory the production of ethanol can only be accompanied by a hydrogen production of two mmols of hydrogen per mmol of ethanol (Hwang *et al.*, 2006).
It is known that more ethanol is produced at low pH. According to Zyabreva et al. (2001) 6.7 times more ethanol was produced at pH 5.0 compared to pH 7.0. They noted that acetic acid and ethanol production were enhanced by low pH conditions.

### 1.4 Biochemistry of acidogenic fermentation
(methanogenesis reactions have been excluded as the focus of this study was acidogenesis).

Carbohydrate fermentation has been shown to consistently produce several main products including acetic acid, propionic acid, butyric acid, and ethanol (Parawira et al., 2004; Yang et al., 2004; Fothergill and Mavinic, 2000). A full list of products observed from carbohydrate fermentation is shown in Table 1. As acetic acid is always produced in conjunction with differing amounts of the other acids, the type of fermentation is named by the dominant acid present other than acetic acid (Ren et al., 1997), for example “ethanol-type fermentation”.

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<td></td>
<td></td>
<td>Lactic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*Propionic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*Valeric acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*Caprionic acid</td>
</tr>
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<td></td>
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</tr>
</tbody>
</table>

*These three acids were not included in Klass’ list, but are products of C6 sugar fermentation.
The general pathways by which the main products of fermentation are formed are shown in Figure 2.

Complex Carbohydrates

\[ \downarrow \]

Glucose

\[ \downarrow \]

Glyceraldehyde-3-phosphate $\rightarrow$ Glycerol

\[ \downarrow \]

Phosphoenol pyruvate

\[ \rightarrow \text{Formate} \]

Pyruvate $\rightarrow$ Lactate $\rightarrow$ H$_2$ + CO$_2$

\[ \downarrow \]

Propionic acid

\[ \leftarrow \text{Acetyl-coA} \]

Ethanol

\[ \downarrow \]

Acetylphosphate $\rightarrow$ Acetic acid

\[ \leftarrow \text{Acetoacetyl-coA} \]

ButylcoA

\[ \downarrow \]

Butyric acid

**Figure 2. An overview of the pathways of anaerobic digestion of complex carbohydrates.**

The three main fermentation types are: a) butyric acid, b) propionic acid and c) ethanol-type (Ren *et al.*, 1997), with all three types being observed to coincide with acetic acid production. Butyric fermentation is noted by production of butyric acid, acetic acid, carbon dioxide gas and hydrogen gas. Propionic fermentation involves the production of propionic acid, acetic acid, and some valeric acid with insignificant gas production. Ethanol-type fermentation produces ethanol, carbon dioxide, hydrogen and acetic acid. The stoichiometry for butyric acid, propionic acid and ethanol production, along with acetic acid production, is listed below:
Acetic acid production (observed to coincide with all fermentation processes):

common reaction: \( \text{C}_6\text{H}_12\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 4\text{H}_2 + 2\text{CO}_2 \)  \( \text{Equation 2a} \)

uncommon reaction from ethanol: \( \text{C}_2\text{H}_5\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2 \)  \( \text{Equation 2b} \)

Butyric acid production

\( \text{C}_6\text{H}_12\text{O}_6 \rightarrow \text{CH}_3(\text{CH}_2)_2\text{COOH} + 2\text{H}_2 + 2\text{CO}_2 \)  \( \text{Equation 3} \)

Propionic acid production

\( \text{C}_6\text{H}_12\text{O}_6 + 2\text{H}_2 \rightarrow 2\text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \)  \( \text{Equation 4} \)

Ethanol production

common reaction: \( \text{C}_6\text{H}_12\text{O}_6 + \text{H}_2\text{O} \rightarrow \text{C}_2\text{H}_5\text{OH} + \text{CH}_3\text{COOH} + 2\text{H}_2 + 2\text{CO}_2 \)  \( \text{Equation 5a} \)

\( \text{(Najafpour and Younesi, 2006)} \)

b. uncommon reactions (Datar et al., 2004):

uncommon reactions: \( 6\text{CO} + 3\text{H}_2\text{O} \rightarrow \text{C}_2\text{H}_5\text{OH} + 4\text{CO}_2 \)  \( \text{Equation 5b} \)

\( 6\text{H}_2 + 2\text{CO}_2 \rightarrow \text{C}_2\text{H}_5\text{OH} + 3\text{H}_2\text{O} \)

\( \text{(Datar et al., 2004)} \)

The stoichiometry shows that one mole of glucose gives four moles of hydrogen when converted to acetic acid, and two moles when converted to butyric acid or ethanol. This supports the results of Van Ginkel and Logan (2005), which show that hydrogen yield is higher when acetic acid is formed rather than butyric acid. Hence, the stoichiometry suggests that hydrogen production would be maximized with acetic acid as the predominant VFA. Also, the stoichiometry shows that hydrogen is consumed as a substrate in the production of propionic acid, which supports Ren et al.’s (1997) conclusion that hydrogen production is never associated with propionic acid production.

The short chain volatile fatty acids that are produced during carbohydrate fermentation are the main intermediary products of anaerobic digestion of organic matter to methane and carbon dioxide (Aguilar et al., 1995). Acetic acid can be considered the most important of the intermediates: around 68 % or more of the
methane produced anaerobically is thought to be via acetic acid. Butyric acid is known to convert to hydrogen and acetic acid. Overall anaerobic degradation can be summarized as: initial CO$_2$ production, followed by VFA production via acidogenesis with butyric and acetic acids being the main products. Acidogenesis is normally followed by methane production where VFAs are depleted and pH increases.

1.5 Microbiology of anaerobic fermentation.

Many studies have been performed on anaerobic digestion of wastewater using pure cultures and mixed cultures of bacteria. It seems that the dominant bacteria present may be related to substrate, environmental conditions and the optimal growth conditions of the bacteria themselves (Hearn, 1994).

There are four groups of bacteria present during anaerobic fermentation (Barnett, 1984; and Hearn, 1994):

a) hydrolytic-fermentative bacteria achieving hydrolysis of polymeric substances,

b) acetic acid and hydrogen producing syntrophic acetogenic bacteria,

c) acetogenic bacteria that utilize hydrogen and carbon dioxide to form acetic acid, and

d) methanogenic bacteria.

* In addition to those mentioned above by Barnett (1984) and Hearn (1994) some other main anaerobic bacteria present produce propionic acid, butyric acid, and ethanol

Many studies involving pure bacterial cultures have been performed on anaerobic digestion. Many pure cultures are involved in individual product formation. *Clostridium* species appear to be the most common bacterial species and are capable of producing several fermentation products as seen in Table 2.
Table 2. Product formation via anaerobic digestion in the presence of pure cultures.

<table>
<thead>
<tr>
<th>Product</th>
<th>Pure Culture Bacteria Involved in Production</th>
</tr>
</thead>
</table>
| Acetic acid              | *Acetobacter aceti*  
|                          | *Clostridium thermoaceticum*  
|                          | *Pachysolen tannophilus* |
| Butyric acid             | *Clostridium sp.*  
|                          | *Butyvibrio*  
|                          | *Eubacteria* |
| Propionic acid           | *Propionibacteria*  
|                          | *Clostridium sp.*  
|                          | *Propionibacterium shermanii* |
| Hydrogen                 | *Bacillus*  
|                          | *Enterobacter*  
|                          | *Clostridium sp.* — produces most H2/mol hexose and dominates natural environment mixed cultures  
|                          | - *Clostridium paraputrificum* M-21 produces 1.9 mol H2/mol glucose at optimum conditions of pH 6.5 and 45°C |
| Methane (by converting Acetic acid to methane in presence of hydrogen) | *Methanosarcina barkeri*  
|                          | *Methanobacterium thermoautotrophicum* |

Mixed cultures are the most inexpensive method to use in anaerobic wastewater treatment. Maximum degradation rates vary with the bacterial groups present in the digester’s mixed population (Aguilar et al., 1995). Hence the mixed bacteria present in the digester are important in determining the products of anaerobic fermentation. Mixed cultures involve interactions between microbial species (Table 3), with a limited number of species dominating due to specific environmental conditions present (Blanch and Clark, 1996). Table 3 shows the various interactions possible between bacterial species.

Table 3. Interactions between bacterial species.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Bacterial Species A</th>
<th>Bacterial Species B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutralism</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Commensalism</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Mutualism</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Competition</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amensalism</td>
<td>0 or +</td>
<td>-</td>
</tr>
<tr>
<td>Parasitism</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Predation</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Hydraulic retention time (HRT), temperature (Cha and Noike, 1997) and SRT are noted to determine which bacteria are present. For example, thermophilic bacteria grow well at temperatures above 60°C and have high metabolic rates as compared to mesophilic bacteria which thrive at lower temperatures (Zeikus, 1979). Mesophilic bacteria tend not to respond well to an increase in temperature.

Demirel and Yenigün (2002) found that methanogenic and acidogenic bacteria differ greatly in their growth patterns, nutritional requirements and in environmental sensitivity. Obligate acid-forming anaerobic bacteria grow on carbohydrate and protein under strictly anaerobic conditions, and hence will not grow in the presence of oxygen (Toerien et al., 1967). The obligate anaerobic bacteria have been shown to outnumber aerobic and facultative anaerobes in anaerobic digestion. Hence, conditions suitable for obligate anaerobes should be maintained to enhance acidogenesis as they seem to play a major role in anaerobic digestion processes. For this reason, the two-phase anaerobic treatment reactors are preferred, in order to provide optimum conditions for both types of microorganisms.

1.6 Effect of operating parameters on fermentation.

Bioreactor operating conditions such as pH, HRT, temperature and organic loading rate (OLR) affect the fermentation products in anaerobic systems (Elefsiniotis and Oldham, 1994; von Munch and Greenfield, 1998; Wang et al., 2005; Maharaj and Elefsiniotis, 2001). According to Barnett (1984) there are four environmental factors required for sustained anaerobic digestion: 1/ pH 5-8, 2/ absence of oxygen, 3/ sufficient nutrients, and 4/ absence of toxic materials.

Considering the stoichiometry of anaerobic fermentation, it may be that maximal VFA production and maximal hydrogen production do not occur concurrently. Hence, some compromise may have to be made in order for ‘best’ overall fermentation.
1.6.1 pH

pH has been found to play a main role in determining which products are formed during anaerobic fermentation. The fermentation type, and consequently the specific hydrogen production rate, is pH-dependent (Lin and Chang, 1999). A number of authors have shown that low pH is optimal for hydrogen production (Zoetmeyer et al., 1982; Fang and Liu, 2002; Hawkes et al., 2002; Kisaalita et al., 2004). When operating at low pH, optimal net hydrogen production could be expected for two reasons:

1. The proportion of propionic acid is reduced at low pH. Identifying the exact pH at which propionic acid production is minimized is not straightforward: Kisaalita et al. (2004) concluded that pH below 4.5 is required to prevent propionic acid formation; Inane et al. (1996) and Fang and Liu (2002) have shown that reduced pH (close to 5) prevents propionic acid production; Ren et al. (1997) found that pH between 6.0 and 6.5 limits propionic acid production. At low pH (< 6.5), butyric and acetic acids have been observed to be the dominant VFAs present, followed by ethanol, lactic acid and formic acid (Hawkes et al., 2002), and at very low pH (< 4.5) ethanol-type fermentation dominates (Kisaalita et al., 2004). Further, stoichiometry shows that butyric and ethanol-type fermentation will generate significant hydrogen production, while that of propionic acid-type or involving lactate production will not. This would suggest that in order to harness hydrogen as an energy source from anaerobic wastewater treatment processes, low pH should be maintained.

2. Microbial diversity is increased at higher pHs. Fang and Liu (2002) noted that as pH increases, the percent hydrogen decreases, while those of carbon dioxide and methane increase. Also, according to Vavilin et al. (1995), the optimal pH for acidogenesis is 6.0, while that of methanogenesis is 7.0 (Table 4). This suggests that methanogenesis may be avoided by controlling pH at reduced levels.

According to Annous et al. (1996) net hydrogen production is related to the relative rates of acetic and butyric acid formation. They adjusted the relative rates of acid formation by altering pH. At pH 5.5 the butyric acid to acetic acid ratio increased, while H₂ consumption increased and H₂ production decreased compared to at pH 7.0.
This was thought to be due to more electrons flowing toward butyric acid than being used in hydrogen production.

Though the optimal pH for maximal hydrogen production has been found to differ widely between sources, pH control is known to be a key factor in controlling hydrogen production (Kim et al., 2004). Again, overall it appears that a low pH is best for maximal hydrogen production as it seems the proportion of propionic acid decreases. Optimal pH may vary for different substrates though it appears from the literature that a pH range of 5-7 is optimal for glucose degradation (Ren et al., 1997; Vavilin et al., 1995).

1.6.2 Temperature

Temperature is known to affect fermentation reactions. Overall, VFA production has been noted to be maximal at temperatures between 35-55 °C, with a large decrease at temperatures above 65 or below 25 °C (Penaud et al., 1997). Two optimum temperature ranges are known for fermentations, one in the mesophilic range at 36-38 °C and one in the thermophilic region at 51-53 °C (Zoetmeyer et al., 1982).

Regardless of temperature, acetic acid was always found to be the predominant acid produced. After acetic acid, butyric acid is known to be the next most common acid produced in the mesophilic range. Hawkes et al. (2002) determined that 30 °C may be the desired temperature for hydrogen production, as butyric acid is in high proportions. Though stoichiometry suggests that maximal hydrogen production would be with a high ratio of acetic acid, it has been found that maximal hydrogen production occurs with a high butyric acid to acetic acid ratio. Ethanol is the most common product in the thermophilic range. With decreasing temperature from 60 to 50 °C, propionic acid and acetic acid production increased greatly. As temperature is changed in a reactor, lactate is often produced at high levels during the transition period and disappears thereafter (Zoetmeyer et al., 1982).

With increasing temperature, VFA production is enhanced while methanogenesis is limited (Ahring et al., 2001), likely because the increased VFA production inhibits the activity of the methanogens. However, acidogenesis tends to be performed in the
mesophilic temperature range. Mesophilic temperatures are preferred for anaerobic digestion as the stability is higher and the process is less energy consuming than for thermophilic digestion (Zoetmeyer et al., 1982).

Table 4. Parameters Suggested For Two-Phase Anaerobic Digestion of Municipal Biosolids (Klass, 1998).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acid Phase</th>
<th>Methane Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>PH</td>
<td>5.7-5.9</td>
<td>7.0-7.4</td>
</tr>
<tr>
<td>Retention Time (days)</td>
<td>0.5-1</td>
<td>6.5</td>
</tr>
<tr>
<td>Loading Rate (kg VS/m³/day)</td>
<td>24-43.2</td>
<td>---</td>
</tr>
</tbody>
</table>

From Table 4 above it can be seen that there are certain conditions that are considered optimal for acidogenesis. Overall, it seems that a temperature in the mesophilic range, around 30 °C, may be best for producing a high proportion of butyric acid compared to acetic acid in order to optimize hydrogen production if that is the desired product.

1.6.3 Retention Times

1.6.3a Solids Retention Time (SRT)
SRT represents the average amount of time bacteria (biomass) spend in the system. SRT is a key parameter in environmental biotechnology as different bacterial populations have different growth rates. SRT has a large impact on what takes place within a reactor or wastewater treatment facility as it provides significant metabolic selection pressure to the operation. For example, acidogens and hydrogen producers have fast growth rates (Yu et al., 2002) and require only a short SRT, while methanogens (acid and hydrogen consumers) have slower growth rates (Hawkes et al., 2002) and hence require longer SRT for survival. Hence, SRT affects which products are formed and consumed in a system at any time.

VFA production decreases at shorter SRT, but specific hydrogen production rate has been found to be highest at shortest SRT (Lin and Chang, 1999). This is likely due to the fact that methanogenic bacteria are slow growing and thus require a long retention
time to establish a stable community, and hence a short SRT would lead to the washout of methanogens. According to Nakamura et al. (1993), the number of anaerobic bacteria increase as SRT increases, but the hydrogen gas production decreases. Hence, it seems that at short SRTs there is a tradeoff with having more hydrogen production, but less VFAs produced.

Overall, it seems a shorter SRT leads to higher hydrogen production. Using a short SRT likely helps to avoid hydrogen consumption by methanogens, as methanogens require a long SRT for survival.

**1.6.3b Hydraulic Retention Time (HRT)**

Demirel and Yenigün (2004) found that HRT affected VFA distribution and production; with propionic, valeric, acetic and butyric acids formed predominantly during acidogenesis (Table 5). Acid production was noted to increase as HRT decreased, with acid production proportional to OLR. Acid production and the rate of formation were maximized at 12 hours. According to Demirel and Yenigün (2004), VFA production was greatest at highest OLR and shortest HRT. Penaud et al. (1997) also found that VFA production was optimized as HRT decreased.

Using a short HRT is useful when the substrate is soluble, but for complex material such as cellulose, a longer HRT is required in order for degradation to take place. HRT affects what products are formed in the liquid phase. According to Zhang et al. (2006), HRT can prevent methanogens from utilizing hydrogen as HRT determines what reactions are possible in the liquid phase within a certain time-frame. Hence, if certain microbial populations require longer to perform certain reactions than is available with a given HRT, the process will not be completed under these conditions. Therefore, HRT can be used as a control parameter in determining what reactions take place in a reactor. According to Zhang et al. (2006) a 12 hour HRT is best for maximal hydrogen production with the most efficient glucose conversion rate.
Banerjee et al. (1998) found that increasing HRT from 18 to 30 hours led to an increase in VFA production and COD solubilization. This finding agrees with that of Ueno et al. (1996) who found that VFA production decreased with decreasing HRT. Maharaj and Elefsiniotis (2001) noted that maximum volatile fatty acid production occurred at 30 h HRT, when using a substrate of industrial wastewater consisting of starch-rich diluted sludge with diluted primary sludge.

HRT has been shown to affect hydrogen production rate (Ueno et al., 1996). A short HRT is best as this prevents the acids from being further converted to methane, hence limiting methanogenesis. The HRT affects the type of acid produced, with short HRTs limiting acetogenesis as the longer chained VFAs do not have time to convert to acetic acid.

**1.6.4 Organic Loading Rate (OLR)**

It has generally been found that VFA production increases as OLR increases (Demirel and Yenigün, 2004; Banerjee et al., 1998; Penaud et al., 1997). Overloading can lead to a reduction in the ratio of acetic acid to other longer chained acids (Marchaim and Krause, 1993; Penaud et al., 1997). Zoetmeyer et al. (1982) found that acetic acid and propionic acid were main products formed at low OLR, while butyric acid was formed most at higher OLRS. An increase in the fraction of protein and lipid has particular effect on the ratio of propionic acid to acetic acid (Yu and Fang, 2000). While carbohydrates are degraded regardless of loading rate, protein and lipid degradation are restricted as the loading rate increases. Proteins and lipids are thought to be substrates for propionic acid production; hence by limiting the degradation rate

---

**Table 5. Acidification rate at 1-5 kg COD per 1000 L.d⁻¹ and variable HRT according to Demirel and Yenigün (2004).**

<table>
<thead>
<tr>
<th>HRT (hours)</th>
<th>Acidification Rate (grams.L⁻¹.d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0, no acids produced at 24hr HRT</td>
</tr>
<tr>
<td>22</td>
<td>0.14</td>
</tr>
<tr>
<td>20</td>
<td>0.20</td>
</tr>
<tr>
<td>18</td>
<td>0.23</td>
</tr>
<tr>
<td>16</td>
<td>0.82</td>
</tr>
<tr>
<td>12</td>
<td>3.1</td>
</tr>
</tbody>
</table>
of proteins and lipids, the opportunity for conversion of propionic acid to acetic acid is also limited.

1.7 Hydrogen Production.

1.7.1 Effect of pH on Hydrogen Production

Though there is much variation in the literature, it seems that maximum hydrogen production occurs between pH 5.5 and 5.7, though some sources state between pH 5.7. From table 6 it can be seen that maximal hydrogen production of 711 mmol H\textsubscript{2} \cdot L\textsuperscript{-1} day\textsuperscript{-1} was found at pH 5.7 with an SRT of 0.25 days (Lin and Chang, 1999).

<table>
<thead>
<tr>
<th>pH</th>
<th>OLR (mmol glucose \cdot L\textsuperscript{-1} \cdot d\textsuperscript{-1})</th>
<th>SRT (d)</th>
<th>Amount of H\textsubscript{2} Produced (mmol H\textsubscript{2} \cdot L\textsuperscript{-1} \cdot d\textsuperscript{-1})</th>
<th>Hydrogen Production (mol H\textsubscript{2}/mol glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.7</td>
<td>52</td>
<td>2.0</td>
<td>33.3</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>1.0</td>
<td>104</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>416</td>
<td>0.25</td>
<td>711</td>
<td>1.71</td>
</tr>
<tr>
<td>6.4</td>
<td>52</td>
<td>2.0</td>
<td>46.8</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>1.0</td>
<td>126</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>416</td>
<td>0.25</td>
<td>574</td>
<td>1.38</td>
</tr>
</tbody>
</table>

Fang and Liu (2002) found that the maximum hydrogen yield ranged from 2.1-2.3 mol H\textsubscript{2}/mol glucose (Table 7).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>pH</th>
<th>Hydrogen yield (mol H\textsubscript{2}/mol glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture</td>
<td>5.5</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>5.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>Unspecified</td>
<td>0.7</td>
</tr>
<tr>
<td>\textit{E. aerogenes}</td>
<td>5.5-6.0</td>
<td>1.0</td>
</tr>
<tr>
<td>\textit{E. cloacae}</td>
<td>5.0-6.0</td>
<td>2.2</td>
</tr>
<tr>
<td>\textit{C. butyricum}</td>
<td>6.7</td>
<td>1.4-2.3</td>
</tr>
</tbody>
</table>
Lin and Lay (2004) found maximal hydrogen production under conditions where butyric acid was the most prevalent VFA product, followed by acetic acid, with low propionic acid (Table 8).

Table 8. Optimal conditions for maximizing hydrogen production according to Lin and Lay (2004).

<table>
<thead>
<tr>
<th>Acetic acid (mg COD/L)</th>
<th>Propionic acid (mg COD/L)</th>
<th>Butyric acid (mg COD/L)</th>
<th>pH</th>
<th>ORP (-mV)</th>
<th>Final VSS (grams/L)</th>
<th>H₂ Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2144</td>
<td>536</td>
<td>9172</td>
<td>7.5</td>
<td>340</td>
<td>2.73</td>
<td>53.1</td>
</tr>
</tbody>
</table>

It seems there are contradictions in the literature as to whether hydrogen production and VFA production are maximized with a high butyric acid to acetic acid ratio, or a high acetic acid to butyric acid ratio (though acetic acid and butyric acid are always the dominant products with little propionic acid). This is somewhat surprising as stoichiometry would suggest that a high acetic acid to butyric acid would result in maximal H₂ production (Equations 2 and 3).

1.7.2 Effect of Substrate on Hydrogen Production
It is known that changing the substrate within a system changes the microbial population (Erickson and Fung, 1988), and consequently affects the fermentation products. Logan et al. (2002) noted that lactate, molasses, potato starch and cellulose produced minimal hydrogen gas, while the most hydrogen was produced when glucose or sucrose were used as the substrate.
Table 9. Hydrogen gas production for various substrates according to Logan et al. (2002).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>grams of COD/294 mL bottle</th>
<th>Biogas (mL)</th>
<th>H₂ produced (mL)</th>
<th>H₂ produced (mol/mol substrate)</th>
<th>Theoretical Max production H2 (mol/mol S)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>244</td>
<td>125</td>
<td>0.92</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.0</td>
<td>217</td>
<td>131</td>
<td>1.8</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td>Molasses</td>
<td>1.3</td>
<td>233</td>
<td>134</td>
<td>Could not be calculated</td>
<td>Could not be calculated</td>
<td>Could not be calculated</td>
</tr>
<tr>
<td>Potato starch</td>
<td>1.0</td>
<td>134</td>
<td>90</td>
<td>0.59</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.0</td>
<td>7.2</td>
<td>2.3</td>
<td>0.01</td>
<td>2</td>
<td>0.50</td>
</tr>
<tr>
<td>Cellulose</td>
<td>1.0</td>
<td>0.96</td>
<td>0.6</td>
<td>0.003</td>
<td>4</td>
<td>0.075</td>
</tr>
</tbody>
</table>

From Table 9 it can be seen that the most hydrogen gas was produced using molasses as the carbohydrate substrate, though the best conversion rate to gas was found for glucose and sucrose.

According to Hutnan et al. (2000), sugar beet pulp can be used as a cellulose- and hemicellulose-containing substrate for biogas production. It is thought that most microorganisms require six carbon sugars for fermentation, but that some are capable of breaking down almost any organic compounds (Brown, 2003). Pulp and paper mill effluent is considered to be 40-50% glucose by weight. Lignocellulose found in pulp and paper mill wastewater is considered to be difficult to depolymerise as its basic components are resistant to biological and chemical attack (see Figure 3). The amount of cellulose hydrolysis is thought to depend on its association with lignin (Hearn, 1994). The goal of hydrolysing cellulose is to form glucose to be carried through fermentation.
1.8 Methanogens.

Methanogens are unicellular, gram-variable, strict anaerobes (Klass, 1998). They are obligate anaerobes meaning Eh values should be maintained below -300mV (Mitsumori et al., 2002) and hence could be inhibited in aerobic conditions. This is because anaerobes can only grow under conditions where the Eh value is between -300 and -400 mV (Vorobjeva, 2005). Further, they are known to be much more sensitive than acidogens to conditions such as pH, substrate (Beccari et al., 1996) and mechanical mixing.

According to Girovich (1996), methanogenic bacteria grow slowly, and so are susceptible to washout at short retention times (Hawkes et al., 2002). Methanogens are known to use hydrogen as an electron donor, with CO₂ as an electron acceptor. They are also capable of utilising acetic acid by converting it to methane and carbon dioxide (van Andel and Breure, 1984).
1.9 Nutrient Requirements for Fermentation.

It has been shown that the nutrients present in the wastewater being degraded affects hydrogen production, hence it is important to ensure the necessary nutrients are present for maximizing the desired output.

Hawkes et al. (2002) found that when phosphate was limited alcohols were the major fermentation products. As alcohols are not desired products in the formation of hydrogen it is important to have sufficient phosphate present in order to prevent this.

Iron and molybdenum are required for nitrogen fixation to take place as they are necessary components of the nitrogenase enzyme, hence, with a nitrogen deficient waste stream (see section 1.2.2) sufficient iron and molybdenum present could help to maximize nitrogen fixation capabilities.

Lin and Lay (2004) found that a source of phosphate and carbonate are required for hydrogen production. Increased carbonate leads to a higher proportion of CO$_2$ in the gas; hence this should be limited to enhance hydrogen production. They found that a suitable carbonate and phosphate ratio were required to maximize hydrogen production rate, with NH$_4$HCO$_3$ the most important compound. This must be kept in low concentrations to limit proportions of carbon dioxide gas. A proper phosphate balance may shorten the lag-phase time of the anaerobic microorganisms to increase hydrogen production. An example of a mineral make up for feed can be seen in table 10.
Table 10. Typical concentrations of mineral components required for fermentation according to Stanbury and Whitaker (1984).

<table>
<thead>
<tr>
<th>Mineral</th>
<th>grams L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>1.0-4.0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.25-3.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5-12.0</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>5.0-17.0</td>
</tr>
<tr>
<td>FeSO₄·4H₂O</td>
<td>0.01-0.1</td>
</tr>
<tr>
<td>ZnSO₄·8H₂O</td>
<td>0.1-1.0</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>0.01-0.1</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.003-0.01</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.01-0.1</td>
</tr>
</tbody>
</table>

1.10 Inhibition of anaerobic processes.

1.10.1 Inhibition of hydrogen production

A buildup of hydrogen can lead to a decrease in the metabolic activity of the reactor, hence it is best to release the hydrogen gas produced (Ren et al., 1997). According to Ren et al. (1997), the continuously stirred tank reactor (CSTR) is superior to the plug-flow reactor (PFR) for maximizing hydrogen production via acetogenesis. According to Hawkes et al. (2002), as the liquid partial pressure of hydrogen increases, hydrogen yield is decreased. They found that stirring helped to maintain good hydrogen production by limiting liquid hydrogen partial pressure. Logan et al. (2002) also found that as the hydrogen partial pressure increases hydrogen production becomes inhibited. Acetogenic bacteria require a low hydrogen partial pressure within the reactor in order for acetogenesis to take place (Barnett, 1984).

There seems to be great debate as to whether the acetic acid or butyric acid pathway lead to maximal hydrogen production, though it seems overall from previous findings that higher butyric acid production coincides with maximal hydrogen production at pH 5.5 (Cheong and Hansen, 2006), even though stoichiometry suggests otherwise. Butyric acid is often the dominant product at pH 5.5 (Lin et al., 2006) suggesting that if the butyric acid pathway is associated with maximal hydrogen production that maximal hydrogen production should be possible at pH 5.5.
The bacteria involved in fermentation require nitrogen, phosphorus and metals for survival (Hitte, 1975). Heavy metals, phenols and cyanide were found to be highly toxic to bacteria (Gerardi, 2002). Lin (1993) found that zinc and copper were the most toxic to VFA producing bacteria, while lead was least toxic. Yeningün et al. (1995) noted that cadmium was more toxic than nickel to acetic acid and η–butyric acid production. According to Maillacheruvu and Parkin (1996), propionic acid fermenting microorganisms and acetic acid-utilizing bacteria are very sensitive to sulfide toxicity.

1.10.2 Inhibition of methanogenesis

It appears that in order to have maximum acid and hydrogen production, methanogenesis must be inhibited in order to avoid using the products as an electron sink.

Logan et al. (2002), found that heat-shocking limited the number of methanogens present within an anaerobic inoculum. They also noted that keeping reactors at pH 6 inhibited methanogenesis. Hawkes et al. (2002) found that methanogenesis was best inhibited at low pH. In another study, it was noted that heat treating led to greater hydrogen yields than controlling pH (Oh et al., 2003). However, neither of these methods limited hydrogen loss during acetogenesis.

According to Zoetmeyer et al. (1982), methanogenesis can be inhibited during the first phase of anaerobic digestion using a short residence time, and keeping pH low between 5 and 6.

According to Aguilar et al. (1995), VFA accumulation inhibits many of the microorganisms involved in methanogenesis.

A specific methanogenesis inhibitor has been found to be bromoethanesulfonic acid (BESA). BESA has been shown to completely inhibit methanogenesis at 50 mM in thermophilic anaerobic digestion (Aguilar et al., 1995). McSweeney and McCrabb
(2002) found that bromochloromethane and hemiacetyl inhibit methanogenesis in ruminants.

Methanogenesis was found to be inhibited by: 1/VFA buildup, and 2/high concentrations of phenolic compounds. Nitrophenols are thought to be inhibitory to methanogens at high concentrations. Wang et al. (1991) reported that methanogens that use acetic acid can be inhibited by phenols. Nitrophenols are widely used industrial organic compounds that are considered to be carcinogenic (She et al., 2005). When VFAs were used as substrate, methanogenesis was completely inhibited when 45 mg L\(^{-1}\) 2,4-dinitrophenol (2,4-DNP) was input into the reactor. Nitrophenols were found to have a stronger inhibitory effect on methanogenesis when VFAs were the substrate, than when glucose was the substrate. Below 45 mg L\(^{-1}\) 2,4-DNP and below 100 mg L\(^{-1}\) 3-nitrophenol (3-NP), methanogenesis inhibition was only temporary. Valdez-Vazquez et al. (2005) found acetylene to be a good inhibitor. According to Oh et al. (2003), the inhibition of methanogenesis must be done economically; hence chemical inhibition such as using bromoethanesulfonate should not be used. Cobalt at a total concentration of 280 mg L\(^{-1}\) or as free cobalt of 70 mg L\(^{-1}\) has been noted to completely inhibit methanogenesis (Demirel and Yenigün, 2002). Hydrogen sulfide, sulfate and sulfite have also been found to have high inhibitory effects on methanogenesis when using mixed bacterial cultures (Khan and Trottier, 1978). Sulphide ions are thought to be toxic to most digestion processes (Bushell and Slater, 1981).

Methanogenesis occurs once steady-state has been attained within the reactor. This has been found to sometimes take up to 6 to 8 months (Erickson and Fung, 1988). It is thought that using a short SRT at low temperatures limits methanogen growth opportunities (Oh et al., 2003) as they are known to be slow growing and sensitive to environmental conditions. Methanogenesis occurs well at pH 7; hence to avoid methanogenesis in order to protect the hydrogen produced, perhaps pH 7 should be avoided.
1.11 Research Objectives.

1.11.1 Effect of Nitrogen Deficiency on Anaerobic Fermentation
The primary objective of this work was to determine whether micro-organisms present in a mixed bacterial culture are capable of fixing atmospheric nitrogen in order to support growth in anaerobic environments with insufficient nitrogen. This research will determine if it is possible to produce VFAs, ethanol and hydrogen gas from low nitrogen or nitrogen deficient wastewater.

1.11.2 Products from Nitrogen Deficient Anaerobic Fermentation
Presuming that a community of nitrogen-fixing organisms can be established, this work will consider the biochemical pathways necessary to maximize volatile fatty acid and hydrogen generation.

A specific objective is to maximize the production of VFAs and of hydrogen, as hydrogen can be exploited as an alternative fuel source in the future while VFAs may soon be recycled in the making of biodegradable plastics. It seems that by determining how to control the pathways of VFA production during anaerobic fermentation via hydrolysis, hydrogen production could be maximized. Knowing which VFAs lead to the greatest hydrogen production, and controlling the pathways required to promote these pathways could be very useful. Also, VFAs themselves have value - they could be very useful by-products in the future potentially used in the generation of bioplastics (Scion – Biomaterials Engineering).

1.11.3 Effect of pH on Nitrogen Deficient Anaerobic Fermentation
pH is known to have a large effect on fermentation reactions and on anaerobic digestion processes. A comparison of two pH levels will be considered here under nitrogen deficient conditions in order to determine whether pH 4.0 or pH 5.5 seems to be the best pH for optimum product formation.
Overall this study is a combination of looking at whether nitrogen fixation can take place under low or no nitrogen situations and also what products are formed under these conditions. This study also investigates the effect of pH on anaerobic digestion of nitrogen deficient wastewater.