Product generation from acidogenic fermentation of nitrogen-deficient wastes

Alexa Bowen

2007

A thesis presented in fulfillment of the requirements for the degree of Master of Philosophy in Environmental Engineering at Massey University, Turitea Campus, Palmerston North, New Zealand.

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 1L 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.

Acknowledgments

I would like to thank many people for their contributions and support in helping me with completing this master's project. Firstly, I would like to thank my primary supervisor Dr. Steven Pratt (lecturer of Environmental Technology and Sustainable Energy at Massey University) for being incredibly patient and supportive in helping me get through the many challenges I faced. I would also like to thank my co-supervisor Dr. Daniel Gapes (Eco-Smart Technologies Group Leader) at Scion in Rotorua. He was very helpful in giving advice as to whether I was carrying out experiments in a reasonable manner and spent many hours meeting with Dr. Pratt and I to discuss this project. Without the help of the Centre for Environmental Technology (CETE) laboratory technologist John Sykes and the Microbiology laboratory manager Ann-Marie Jackson at Massey University, this project would not have been possible. They both spent endless hours helping me learn how to use equipment, showing me where things were located and ordering the necessary supplies. I would also like to thank John Edwards, an engineering technician from the Institute of Technology and Engineering for helping me with the necessary gas fittings and setup. I could not have completed this without the wonderful support of my partner Logan Lindsay, and the loving support from my parents Tom Bowen and Avalon Roberts, from overseas. I very kindly had the help of a wonderful lecturer in the Institute of Food and Nutrition at Massey University named Dr. Sue Nicholson, whom allowed me to access her laboratory in order to perform a required test. A big thank you to her also. I would also like to thank Humphrey O'Hagan, the workshop technician in the Institute of Technology and Engineering for helping me with the construction of my reactors and for fixing so many things for me. Lastly, there are many women in the Institute of Technology and Engineering who have helped with the groundwork for this master's project. I would like to say a big thank you to the following: Joan Brookes, Alisha Annan, Glenda Kirk and Trish O'Grady.

Table of Figures

Table of Tables

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 ($_{1}$/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.

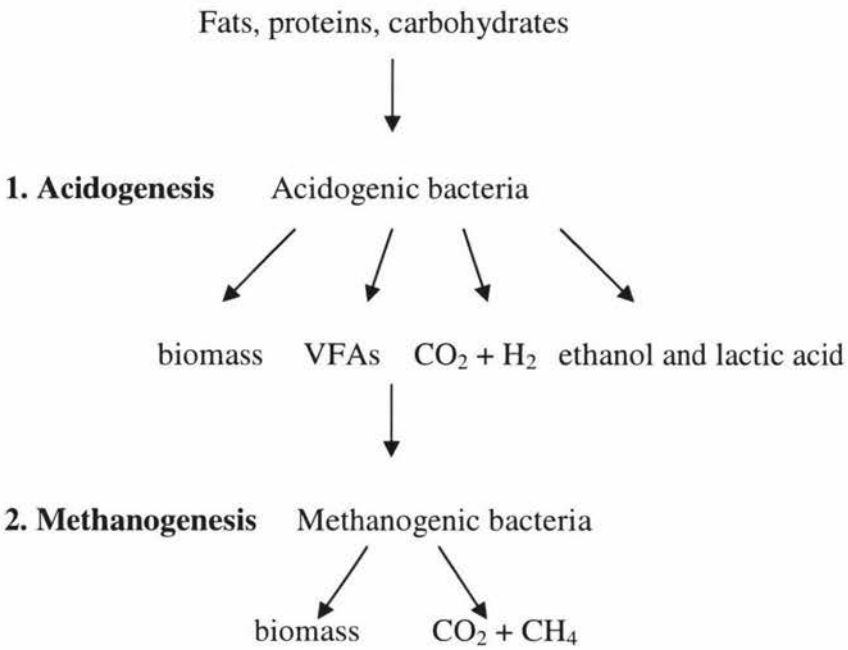


Figure 1. An overview of anaerobic digestion processes.

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-ß-glucanases, exo-1,4-ß-D-glucanases and 1,4-ß-D-glucan cellobiohydrolase. All cellulases have been found to degrade ß-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 $C_5H_7NO_2P_{0.074}$ (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 NH_4Cl.

As an alternative to nitrogen supplementation, Kargi and Özmihçi (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 (N_2) to ammonia (NH_3). 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.

$$N_2 + 8H^+ + 8^{e-} + 16\ MgATP \rightarrow 2NH_3 + H_2 + 1\ MgADP + 16Pi \qquad \text{Equation 1}$$

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. *Desulfatomaculum 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 Barbir, 1992). The gaseous products of anaerobic acidification reactions are VFA's, hydrogen and carbon dioxide ($_2$/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.*,

2004). 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".

Table 1. Products of processing six carbon sugars according to Klass (1998).

Substrate	Processing	Products		
C6 sugars	acidogenic Fermentation	Ethanol	Acetaldehyde	Oxalic acid
		Acetic acid	Acetone	Citric acid
		n-Butyric acid	Glycerol	Amino acids
		Lactic acid	n-Butanol	Antibiotics
		*Propionic acid	Amyl alcohols	Vitamins
		*Valeric acid		
		*Caprionic acid		

* 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.

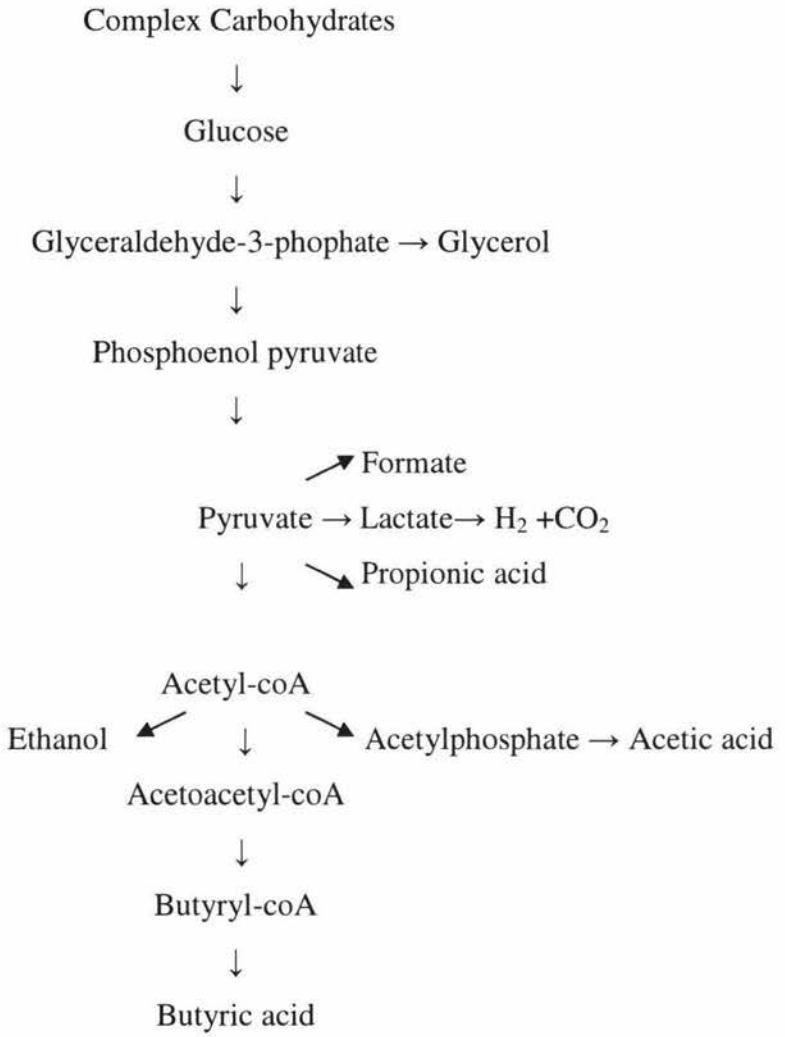


Figure 2. An overview of the pathways of anaerobic digestion of complex carbohydrates.

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: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$ Equation 2a

uncommon reaction from ethanol: $C_2H_5OH + H_2O \rightarrow CH_3COOH + 2H_2$ Equation 2b

Butyric acid production

$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2H_2 + 2CO_2$ Equation 3

Propionic acid production

$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$ Equation 4

Ethanol production

common reaction: $C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2H_2 + 2CO_2$ Equation 5a

(Najafpour and Younesi, 2006)

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

uncommon reactions: $6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$ Equation 5b

$6H_2 + 2\,CO_2 \rightarrow C_2H_5OH + 3H_2O$

(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.

Product	Pure Culture Bacteria Involved in Production
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.

Interaction	Bacterial Species A	Bacterial Species B
Neutralism	0	0
Commensalism	0	+
Mutualism	+	+
Competition	-	-
Amensalism	0 or +	-
Parasitism	+	-
Predation	+	-

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 ($_2$/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; Inanc 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 H_2 consumption increased and H_2 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 (1/ 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 (1/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 (1/ 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 (1/Zoetmeyer *et al.*, 1982).

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

Parameter	Acid Phase	Methane Phase
Temperature (°C)	37	37
PH	5.7-5.9	7.0-7.4
Retention Time (days)	0.5-1	6.5
Loading Rate (kg VS/m^3day)	24-43.2	---

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 (1/ 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.

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

HRT	Acidification Rate (grams.L^{-1}.d^{-1})
24	0, no acids produced at 24hr HRT
22	0.14
20	0.20
18	0.23
16	0.82
12	3.1

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. ($_{1}$/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

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_2 $.L^{-1}$ day^{-1} was found at pH 5.7 with an SRT of 0.25 days (Lin and Chang, 1999).

Table 6. OLR, SRT, and H_2 production at pH 5.7 and 6.4 according to Lin and Chang (1999) using glucose as a substrate.

pH	OLR (mmol glucose. L^{-1} $.d^{-1}$)	SRT (d)	Amount of H_2 Produced (mmol $H_2.L^{-1}.d^{-1}$)	Hydrogen Production (mol H_2/mol glucose)
5.7	52	2.0	33.3	0.64
	104	1.0	104	1.00
	416	0.25	711	1.71
6.4	52	2.0	46.8	0.90
	104	1.0	126	1.21
	416	0.25	574	1.38

Fang and Liu (2002) found that the maximum hydrogen yield ranged from 2.1-2.3 mol H_2/mol glucose (Table 7).

Table 7. Hydrogen yield at various pHs according to Fang and Liu (2002).

Microorganism	pH	Hydrogen yield (mol H_2/mol glucose)
Mixed culture	5.5	2.1± 0.1
Mixed culture	5.7	1.7
Mixed culture	Unspecified	0.7
E. aerogenes	5.5-6.0	1.0
E. cloacae	5.0-6.0	2.2
C. butyricum	6.7	1.4-2.3

Lin and Lay (2/ 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 (2/ 2004).

Acetic acid (mg COD/L)	**Propionic acid** (mg COD/L)	**Butyric acid** (mg COD/L)	**pH**	**ORP** (-mV)	**Final VSS** (grams/L)	**H_2 Content** (%)
2144	536	9172	7.5	340	2.73	53.1

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 H_2 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).

Substrate	grams of COD/294 mL bottle	Biogas (mL)	H_2 produced (mL)	H_2 produced (mol/mol substrate)	Theoretical Max H2 production (mol/mol S)	Conversion (%)
Glucose	1.0	244	125	0.92	4	23
Sucrose	1.0	217	131	1.8	8	23
Molasses	1.3	233	134	Could not be calculated	Could not be calculated	Could not be calculated
Potato starch	1.0	134	90	0.59	4	15
Lactate	1.0	7.2	2.3	0.01	2	0.50
cellulose	1.0	0.96	0.6	0.003	4	0.075

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.

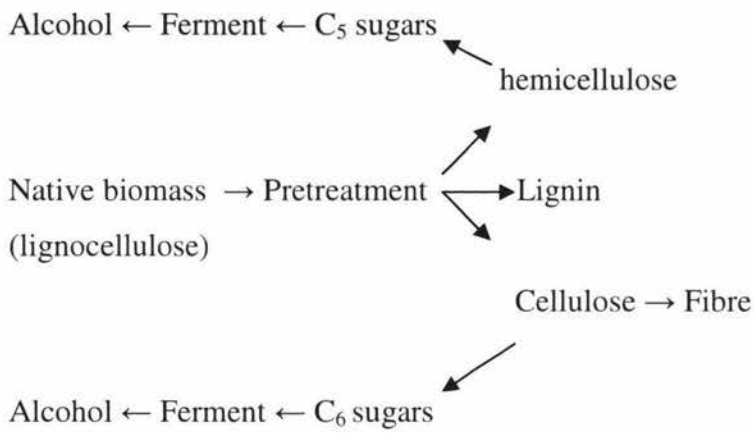


Figure 3. Processes involved in fermentation of lignocellulose from pulp and paper mill waste according to Wise (1984).

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 CO_2 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 (2/ 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_4HCO_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).

Mineral	grams L^{-1}
KH_2PO_4	1.0-4.0
$MgSO_4.7H_20$	0.25-3.0
KCl	0.5-12.0
$CaCO_3$	5.0-17.0
$FeSO_4.4H_20$	0.01-0.1
$ZnSO_4.8H_20$	0.1-1.0
$MnSO_4.H_20$	0.01-0.1
$CuSO_4.5H_20$	0.003-0.01
$Na_2MoO_4.2H_2O$	0.01-0.1

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. ($_{1}$/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.

2. Methods

2.1 Experimental Apparatus.

Two 1L continuously stirred tank reactors were used in this study (Figure 4). Two-stage anaerobic reactors were used as they keep acidogenesis and methanogenesis separate. The feed was stored in a refrigerator at 4 °C. Peristaltic pumps (Masterflex, USA) were used to feed and remove effluent from the two reactors. The effluent pumps were run and stopped alternately for fifteen minute periods. Hence, the reactor volumes varied slightly depending on whether the effluent pumps were on or off. The feed was continually fed to the reactors at 1.3 mL/minute. The effluent pump rates were tested six times to ensure that they were pumping out 1.3 mL/minute as was expected. The effluent pump for reactors A and C was found to pump at an average rate of 1.38 mL/minute while that for reactors B and D was found to pump at an average rate of 1.33 mL/minute. As the volume coming out was equal to what was going in, this suggests that there was negligible loss to evaporation. Nitrogen was sparged through the reactors at 112 mL/minute as determined by mass flow controllers. Bürkert mass flow controllers, type 8711 with an input and output signal range between 0-5 V and an inlet pressure of 3.00 bar were used. Nitrogen gas was supplied to the reactors as a source of available nitrogen to be uptaken via nitrogen fixation. The mass flow controllers determined what volume of gas was allowed to flow by the voltage set on them. The controllers were checked twice throughout experimentation to ensure that the voltage was at the correct setting to allow 112 mL/minute to flow. This helped to ensure the reactors were completely mixed and helped to prevent the buildup of hydrogen in the headspace as this has been shown to inhibit hydrogen production. The reactors were kept on a metal stir plate having stir bars and baffles inside to ensure complete mixing. It turned out that preventing buildup of solids on the reactor walls was quite a challenge and the reactor walls had to be cleaned regularly. Regular cleaning of the walls was to minimize the chance that the SRT was longer than the twelve hour SRT as planned. The reactors'

temperatures were maintained at 35 °C using a hot water bath. The HRT of the reactors was maintained at 12 hours.

The seed sludge for both reactors was obtained from the Feilding sewage treatment plant (lower North Island, New Zealand). The reactors were each initially seeded with 500 mL of sewage sludge and 500 mL of water.

The pH was maintained at 5.5 for the first part of experimentation using Oakton WD-35100-00, -10 1/8 DIN pH/ORP meters that added 1M NaOH whenever necessary via peristaltic pumps. The pH of one reactor was later decreased to 4.0 and maintained there using 1N H_2SO_4. The organic loading rate (OLR) was 5.3 g of COD/L/12 hours throughout experimentation.

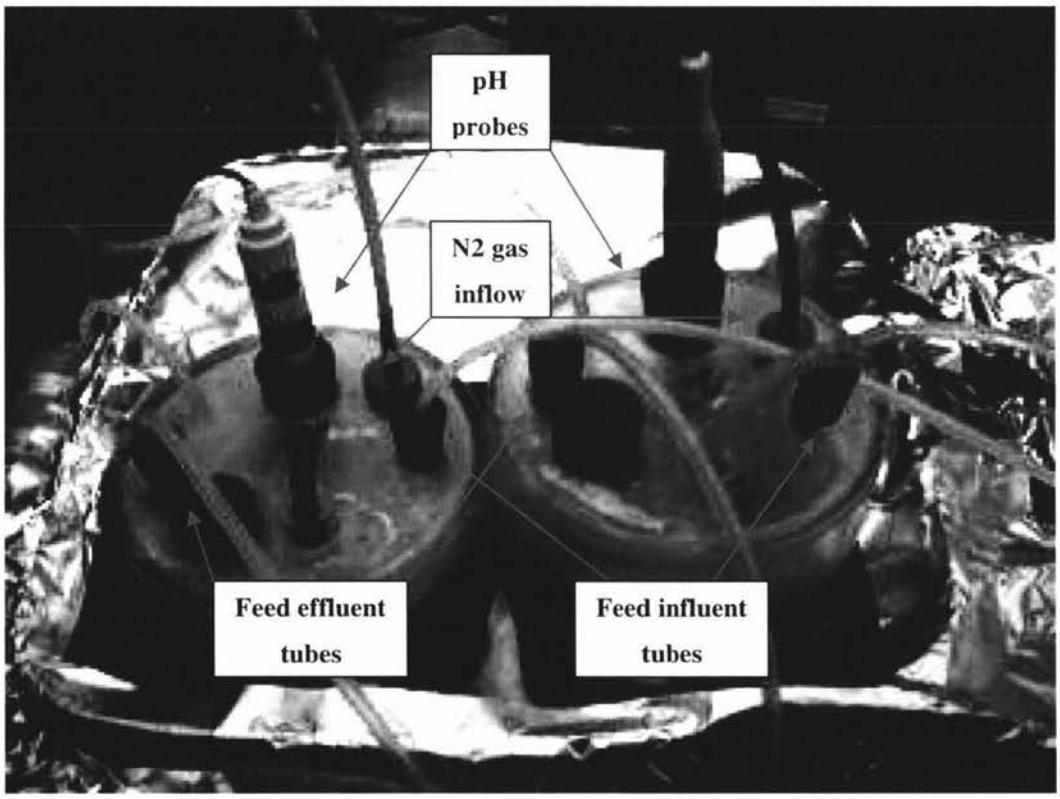


Figure 4. Reactors shown setup in the hot water bath.

The reactors were run under different conditions and over different time periods and will be referred to as in Table 11 below. Reactor A was run as a control at pH 5.5 with a low COD:N ratio of 53. Reactor B was run as a variable N reactor at pH 5.5 with an increasing COD:N ratio over time from 53 to 67 to 100 to 500 to extreme (for

convenience, defined in this work as 1000) when nitrogen was no longer added to the feed. A COD:N ratio of 53 was considered to be similar to the commonly used COD:N ratio of 50:1 for anaerobic wastewater treatment. Reactor C was run under nitrogen deficient conditions at pH 4.0, while reactor D was run under nitrogen deficient conditions at pH 5.5.

Table 11. The naming scheme for the reactors used throughout experimentation.

Reactor	Reactor Name	COD:N ratio	pH	Duration of study (days)
A	Control, Sufficient Nitrogen	53	5.5	100
B	Variable Nitrogen Level	53	5.5	40
		67		20
		100		20
		500		20
*		1000 (0 mg/L N)		30
C	Nitrogen Deficient	Extreme (0 mg/L N)	4.0	30
D*	Nitrogen Deficient	Extreme (0 mg/L N)	5.5	30

*** note: Reactors labeled B (COD:N = 1000) and D were actually the same system**

At 0 mg/L N there is no longer a COD: N ratio, hence the data for 0mg / L N is arbitrarily placed at the 1000:1 mark in order to show that there is a huge amount of COD present relative to nitrogen

2.1.1 Medium Composition

The feed consisted of 5 g/L glucose, or 5.335 g/L COD. The following nutrients were added per litre of feed: 0.357 g NH_4Cl, 0.178 g KH_2PO_4, 0.178 g K_2HPO_4, 0.035 g $CaCl_2$, 0.714 g $NaHCO_3$, and 0.229 g $MgSO_4*7H_2O$. The amount of NH_4Cl was gradually decreased throughout the study period in one of the reactors to determine the effect of increasing the COD:N ratio. The trace element solution consisted of the following per litre: 0.035 g $FeCl_3$, 0.016 g $ZnCl_2$, 0.015 g $CoCl.6H_2O$, 0.007 g $CuCl_2.2H_2O$, 0.021 g $MnCl_2.4H_2O$, 0.05g $Na_2MoO_4.2H_2O$, 0.015 g H_3BO_3, 0.015 g $NiCl_2.6H_2O$. 10mL of the trace element solution was added per litre of feed. The nutrient and mineral solutions were similar to those used by Fang and Liu (2002). Reverse osmosis (RO) water was used to make up all feed, mineral and nutrient solutions.

2.2 Analytical Methods.

Gas Determination

Gas composition was analyzed using a gas chromatograph (Shimadzu - Model GC-8A, Figure 5) with a thermal conductivity detector. The column was a silica gel column. The injector and detector were at 50 °C, and the column was at 30 °C. Nitrogen was used as the carrier gas. Gas standards of 4.2% CH_4, 4.2% CO_2 and 1.11% H_2 were obtained from BOC gases. Gas samples of 200 µL were used to determine the hydrogen, methane and carbon dioxide production as number of mmols gas produced per mmol glucose fed into the reactors. The gas calculations were performed knowing that 112 mL/minute of nitrogen was continually sparged through the reactors, meaning that approximately 112 mL/minute of gas was always coming out of the reactors. It was assumed that the gas flow was constant throughout the study period, and that volumetric gas flowrate out from the reactor was insignificantly different to that of the inflow rate.

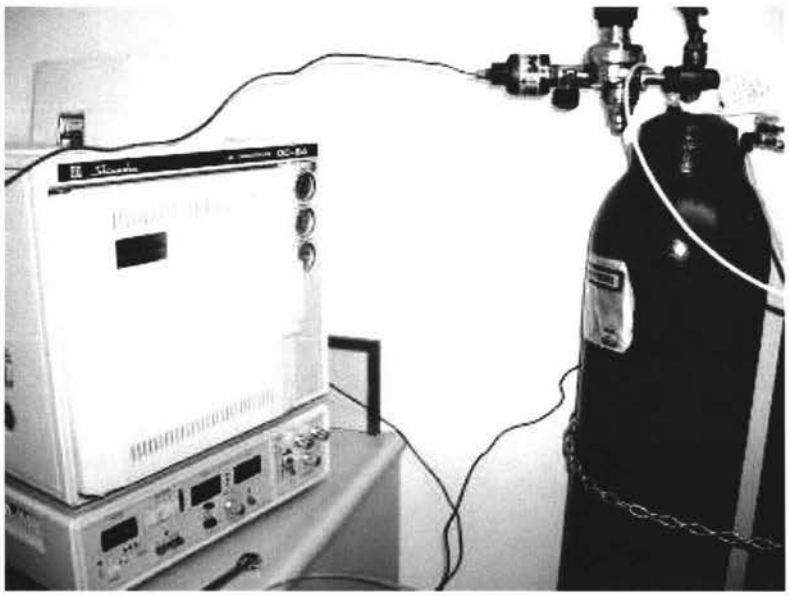


Figure 5. The Shimadzu gas chromatograph used to measure gas production of the two reactors.

Volatile Fatty Acid Analysis

Volatile fatty acids, nitrate, nitrite and phosphate were analyzed using an ion chromatograph (Dionex – Model ICS-2000, Figure 6). The injector and column temperature were 30 °C, and the detector temperature was 35 °C.

Figure 6. The ion chromatograph used to determine the VFA production and anion concentrations of the effluents.

Dextrose and Lactate Measurements

Glucose and lactate concentrations were determined using a YSI-2700 biochemistry analyzer with a calibration standard of 2.50 g/L dextrose and 0.50 g/L L-lactate. The detection limit range for the glucose test was 0-9 g/L for 25 μL samples or 0-25 g/L for 10 μL samples, while that for lactate was 0-2.67 g/L.

Ammonia Concentration

Ammonia concentrations in the effluent were determined using distillation on a Büchi 323 distillation unit (shown in Figure 7), followed by auto-titration (Mettler DL 25 titrator) or manual titration using HCl. 20 mL samples were used.

Figure 7. The Büchi 323 distillation unit used when determining the ammonia concentration in the effluent and for performing TKN tests.

Total Kjeldahl Nitrogen

Total Kjeldahl nitrogen (TKN) was performed as described by the macro Kjeldahl method in Standard Methods (APHA 20th edition, 1998). TKN provides a measurement of total ammonia nitrogen and organic nitrogen. Digestion was performed using Kjeltabs W and left for approximately two hours, or until solutions were clear to ensure complete digestion. Samples were then distilled using a Büchi 323 distillation unit, followed by auto or manual titration to determine the TKN concentration. Eight effluent samples (four from each reactor) from four different COD:N levels in reactor B were sent away to NZ labs in Hamilton, New Zealand, in order to confirm Kjeldahl measurements and to give confidence in the results.

Total and Volatile Suspended Solids

Total and volatile suspended solids were measured following standard methods (APHA 20th edition, 1998). 20 mL samples were used. Filter papers were weighed

on a Mettler AE 200 balance, accurate to 0.1 mg. The filter papers were 90 mm Whatman GF/C.

Ethanol Detection

Ethanol was measured using a Dionex high pressure liquid chromatography machine (HPLC) with an ASI-100 automated sample injector and a TCC-100 (thermostatted column compartment) at 80 °C. The HPLC had a P680 HPLC pump with a flow rate of 0.6 mL per minute and a back pressure of 23 bar.

Nitrogen Fixation Determination

To determine whether or not nitrogen fixation was taking place at each of the five COD:N ratios in the reactors, an acetylene-reduction test was performed at COD:N ratios of 53, 67, 100, 500 and 1000 (used as a very high COD:N ratio for when no nitrogen was added to the feed). This involved flushing glass vials with argon gas, followed by the input of 50 mL of biomass except when the concentration of nitrogen was 0 mg/L, when 100 mL of biomass was used. 1 mL of acetylene was then injected into the vials. The vials were then placed in a hot water bath at 30 °C for half an hour prior to having 1 mL of the headspace gas injected into a Varian-3400 GC fitted with a flame ionization detector. The column was run at 120 °C, and the injector and detector were run at 150 °C. If any acetylene converted to ethylene, it showed that nitrogen fixation was taking place. To confirm that nitrogen fixation is taking place, the ethylene peak should be ten times greater than that of a blank sample..

Sampling Protocol

Samples were taken daily. Gas sampling was performed daily by taking 200 μL samples from each reactor using a 1000 μL syringe and injecting them into a Shimadzu GC. VSS determinations were performed daily. Ammonia measurements were usually performed three times per week. 45 measurements were taken for reactor A. For reactor B, 15 measurements were taken at a COD:N ratio of 53, 8 for a COD:N ratio of 67, 13 for a COD:N ratio of 100, 16 for a COD:N ratio of 500 and 11 for a COD:N ratio of 1000. Dextrose, lactate, and ethanol measurements; TKN tests and VFA tests were all run on pre-frozen samples or on samples filtered and

stored in the fridge. Samples used for measuring dissolved components were all filtered through 0.45 μm membrane filters. Unfiltered samples were taken three times per week and frozen for further analysis.

Microbiological Photos

Samples were taken directly from the reactors using a syringe and placing one small drop onto a slide and then covering the slide with a cover slip. Images were taken of samples from the reactors to undertake microbiological analysis on an Olympus CH30 microscope using 10x and 40x magnification. A Pentax Optio *S4i* camera was used for taking the photographs.

Results and Discussion

3. The Effect of Nitrogen Deficiency on Anaerobic Digestion

Two reactors were run simultaneously, one as a control (reactor A) being fed with nitrogen and a COD:N ratio of 53 and one being fed a reduced nitrogen load (reactor B). By reducing the nitrogen in the feed of the variable N reactor (reactor B), the COD:N ratio was increased from 53 to an extreme COD:N level, at which point no nitrogen was added. This was in order to determine the effects of a decrease in available nitrogen and to determine if nitrogen fixation takes place in anaerobic systems treating nitrogen limited wastewaters. According to Metcalf and Eddy (2003), a COD:N ratio of 50 is commonly used for anaerobic wastewater treatment. Hence, it is expected that the reactor productivity will decrease as the COD:N ratio increases. Both reactors were maintained at pH 5.5. The oxidation-reduction potential of both reactors was between -300 mV and -400 mV throughout experimentation.

All results will be discussed in terms of the COD to nitrogen ratio as this is known to be a key parameter in determining what takes place in biological systems. The COD:N ratios for the five feed levels are shown below in table 12.

Table 12. The COD to nitrogen ratio for the five nitrogen feed levels.

Nitrogen Level in Feed (mg/L N)	COD:N ratio
93.5	53.5
75	66.7
50	100
10	500
0	1000*

* At 0 mg/L N there is no longer a COD: N ratio, hence the data for 0mg / L N is arbitrarily placed at the 1000:1 mark in order to show that there is a huge amount of COD present relative to nitrogen

3.1. Determination of nitrogen fixation

There were two methods attempted in order to determine whether nitrogen fixation was taking place in the reactors.

1. mass balancing.

One method was to calculate whether the nitrogen level found from a TKN test in an unfiltered effluent sample was higher than the nitrogen level in the feed: a positive mass balance would implicate that nitrogen was acquired by fixing nitrogen from the gas phase. For the control reactor, reactor A, approximately 90 % of its nitrogen was recovered, suggesting that nitrogen fixation did not take place in that reactor. For reactor B, at a COD:N ratio of 500 with little nitrogen in the feed, there was approximately 26 mg/L total nitrogen in the unfiltered effluent and under extreme nitrogen deficiency (COD:N labeled 1000) there was approximately 20 mg/L total nitrogen in the unfiltered effluent (Figure 8). This result suggests that for anaerobic fermentation at a nitrogen limitation of (at most) 500:1 on a COD:N basis, the microbial metabolism was relying on nitrogen fixation for obtaining its balanced nutrient requirements.

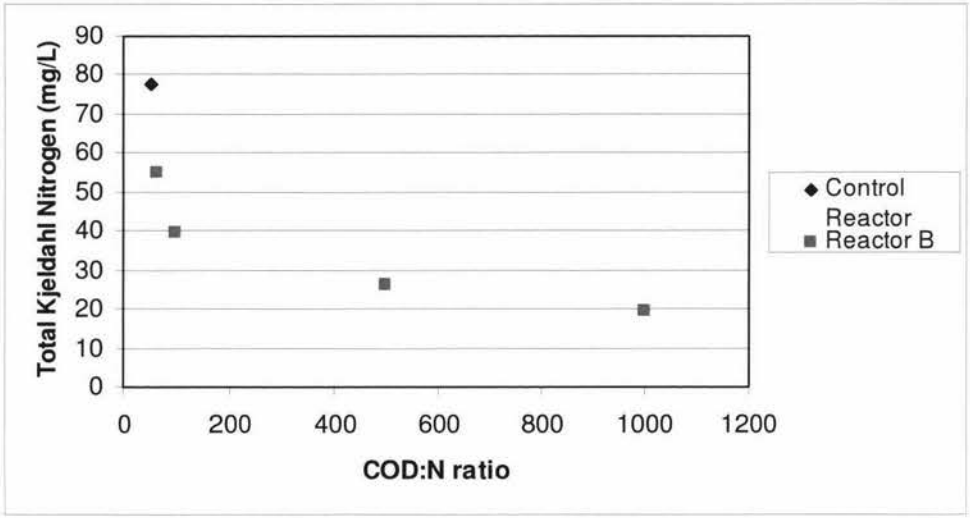


Figure 8. Effluent TKN in the control and experimental reactors.

2. Acetylene reduction

The second method used was the acetylene-reduction test. This biochemical assay confirms the presence of nitrogenase enzyme, with acetylene preferentially reduced to ethylene by this enzyme complex. The acetylene-reduction test confirmed nitrogen fixation at 0 mg/L nitrogen in the feed. Fixation was not confirmed at a COD:N of 500, likely due to initial difficulties in the assay preparation..

Reactor A had an average of 28.14 mg/L ± 6.70 of soluble nitrogen in the effluent. In reactor B, there was an average of 29.19 mg/L ± 6.02 of soluble nitrogen in the effluent when there was a COD:N ratio of 53. With a COD:N ratio of 100, the amount of soluble nitrogen in the effluent declined to an average of 17.71 mg/L ± 5.10. Once the COD:N ratio was increased to 500, there was an average of only 7.33 mg/L ± 5.50 soluble nitrogen remaining in the effluent. As there was still some soluble nitrogen coming out in the effluent even at low levels of nitrogen in the feed, this suggests that the reactor was able to sustain itself and carry on producing biomass with very low nitrogen input.

There was found to be insignificant amounts of nitrate and nitrite present in the effluent of both reactors as expected. There was a small amount of sulphate in both effluents as expected as magnesium sulphate was added to the feed as one of the nutrients. The average sulphate in the effluent for reactor A was 32 mg/L, while that

for reactor B was 19 mg/L. There was 74 mg/L phosphorus being fed into each reactor with an average of 42 mg/L phosphorus in the effluent of reactor A, and an average of 44 mg/L phosphorus in the effluent of reactor B. This suggests that there was sufficient phosphate present for bacterial growth and maintenance, but perhaps the amount of phosphate in the feed should have been reduced as it is unnecessary to have such a high amount of phosphate in the effluent.

3.1.1 Nitrogen Balance

TKN = organic N + NH_3

Total N = organic N + NH_3 + nitrate + nitrite

In this case, nitrate and nitrite are insignificant, hence TKN = Total N.

$$\frac{dN}{dt} = \mathbf{N}_{in} - \mathbf{N}_{out} + accummulation - reaction$$

At steady state, dN/dt = 0

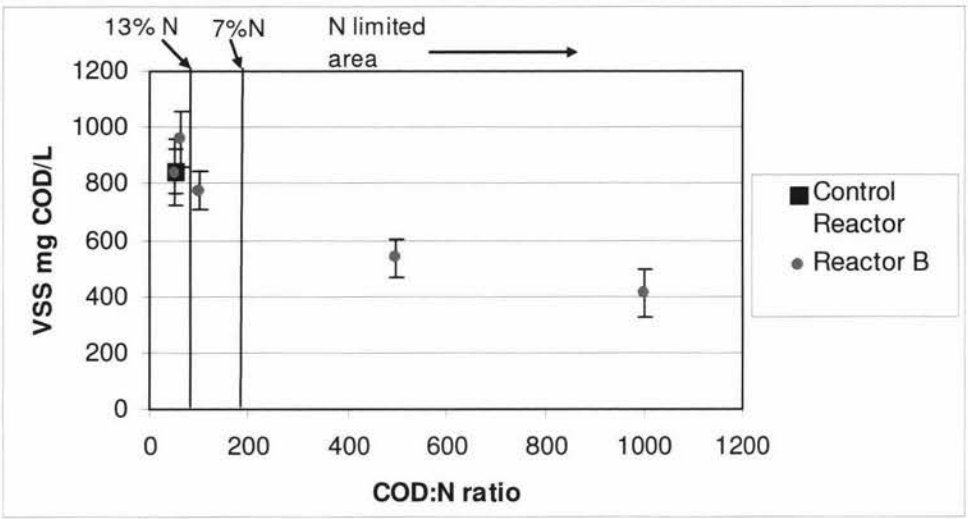
With no nitrogen going into the reactor:

$$0 = 0\,\mathbf{N}_{in} - \mathbf{TKN}_{out} + 0(\textbf{no buildup in reactor}) + \mathbf{N\ fix}$$

$$\mathbf{N\ fix} = TKN_{out}$$

If the biomass content in the reactor is 300 mg VSS/L, with a 12 hour HRT this is 600 mg VSS/L per day washing out of the reactor (refer to section 3.2 below). If it is assumed that the biomass is approximately 10% nitrogen, this means that approximately 60 mg of nitrogen was washed out per day. For one month of running a nitrogen deficient reactor at 0 mg/L N input that is 1800 mg of nitrogen washed out of the reactor. This suggests that nitrogen fixation must be taking place as there is no other way the bacteria could have produced and sustained this amount of biomass. Hence, this study has shown that anaerobic digestion can take place due to nitrogen fixation occurring under nitrogen deficient conditions.

3.2. Biomass production



* The area between the two bars shows the COD:N range beyond which nitrogen limitation is expected for cells ranging between 7-13 % nitrogen.

Figure 9. Effect of COD:N ratio on biomass production as VSS.

From Figure 9, it can be seen that both reactors had very similar solids content at low COD:N levels in their feed. This suggests that the amount of biomass in each reactor was similar at the same COD:N ratio, as to be expected. As the nitrogen level was decreased in the feed and the COD:N ratio was slightly increased, the average amount of VSS remained around 800-900 mg VSS COD/L and then decreased as the COD:N ratio increased greatly as was predicted. According to Ruiz et al. (2006) the COD:N ratio has a large effect on the activity of biomass, with different bacterial species having different optimal COD:N ranges. Zhang et al. (2007) found that biomass production increased as the COD:N ratio was decreased by the addition of nitrogen sources, as was found in this study. With a decrease in available nitrogen, the bacterial cultures have to adapt and hence fewer species are likely to survive. The amount of biomass produced can vary due to a change in biomass yield. As nitrogen fixation is a highly energetic process, the biomass yield often declines when nitrogen fixation is taking place.

Importantly, reactor B was able to effectively remove glucose, and attain a stable steady state biomass level at each COD:N ratio The decrease in biomass is thus due to a decrease in biomass yield.

Assuming that cells typically range from 7-13 % nitrogen, the COD:N range within which cells would be thriving best is shown in Figure 9. Beyond this range, the cells would be nitrogen limited. Nitrogen limitation appears to range from a COD:N ratio of approximately 97 to 178.

Example calculation for 7 % nitrogen:

$$\frac{600\text{mg VSS COD/L} \times 7\ \%\ \text{mg N/mg VSS}}{1.42\ \text{mg VSS COD/mg VSS}} = 30\ \text{mg N/L}$$

$$\frac{5335\text{mg glucose COD/L}}{30\ \text{mg N/L}} = 178\ \text{mg COD/mg N}$$

This suggests that any data recorded throughout this study for the COD:N ratio of 500 is considered to be severely nitrogen limited.

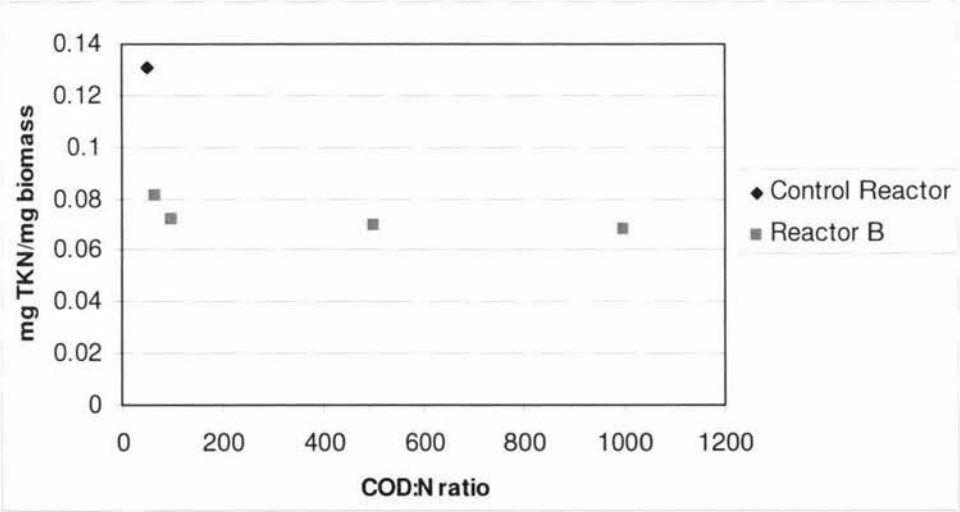


Figure 10. Effect of COD:N ratio on the TKN to biomass ratio.

From Figure 10 it can be seen that the cells in reactor A were approximately 13 % nitrogen, while those in reactor B were approximately 8 % nitrogen at a low COD:N ratio. The cells in reactor B remained around 7 % nitrogen as the COD:N ratio was increased, revealing that they were able to maintain their nitrogen requirements to continue on growing and reproducing even as the amount of available nitrogen was decreased to zero.

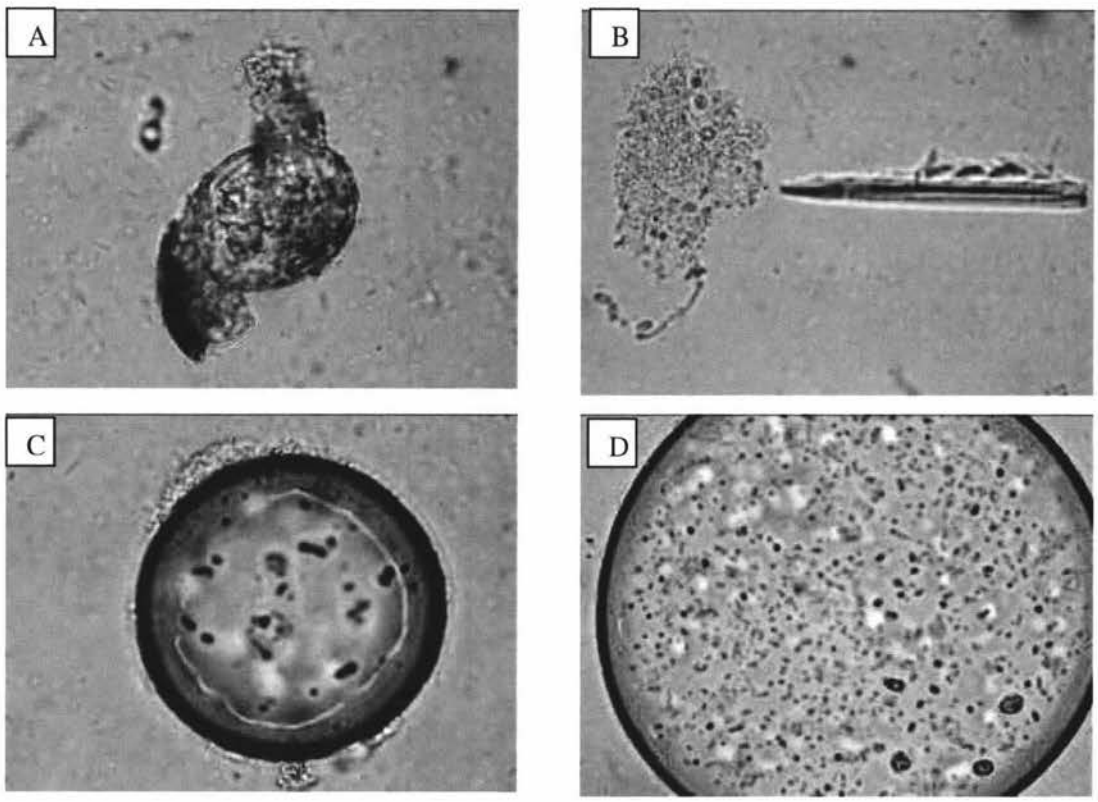


Figure 11. Four images A, B, C, D showing some of the microbiology in the control reactor using 40x magnification. A shows a protozoa specimen. B shows an aggregate of floc, while C and D show very large specimens.

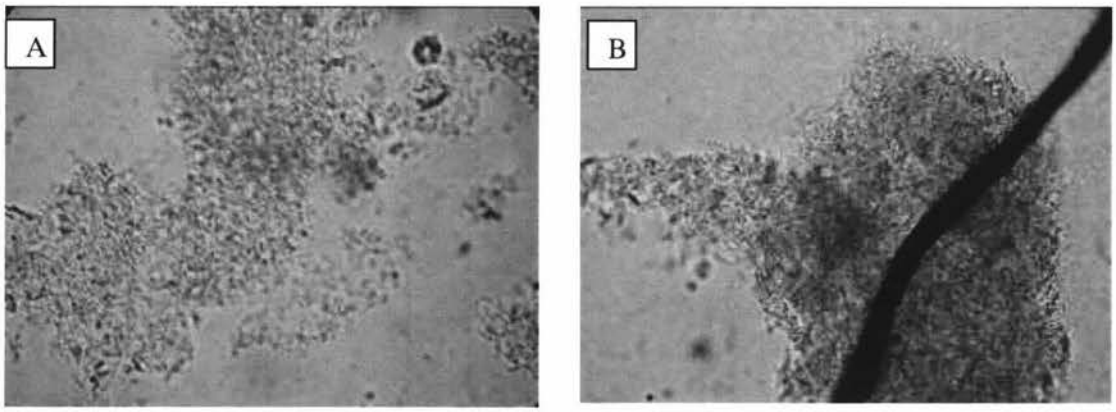


Figure 12. Two images A, and B showing some of the microbiology in reactor B when the COD:N ratio was 100 using 40x magnification. Both photos show an aggregate or floc containing bacteria and organic matter.

Some images showing some microbiology from reactors A and B are shown above in Figures 11 and 12. From qualitative observation, reactors A and B both had large biofilm clusters present consisting of organic matter and bacteria, while only the nitrogen sufficient system (reactor A) was found to support the larger protozoa specimens.

3.3. The effect of decreasing the level of nitrogen on overall VFA, ethanol and gas production.

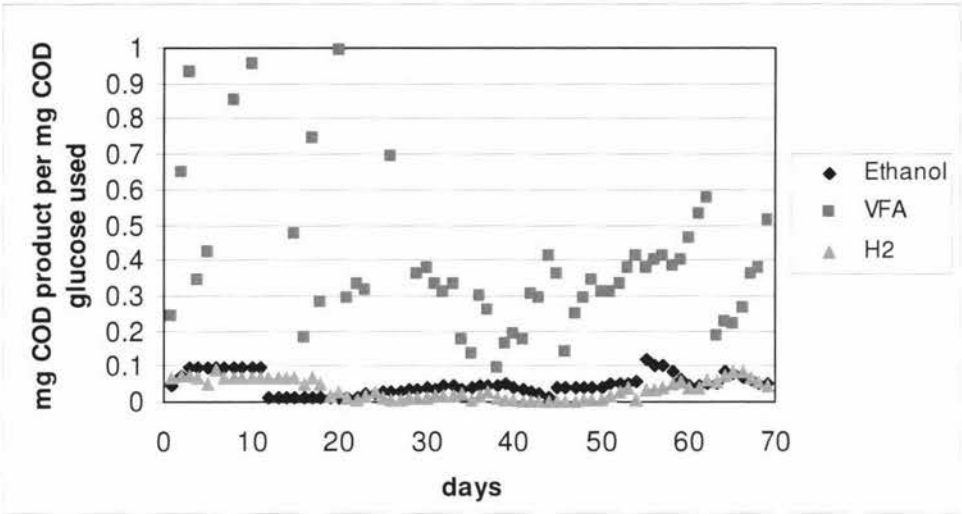


Figure 13. Overall ethanol, VFA and hydrogen product yields for the control reactor.

Figure 13 shows VFA, ethanol and hydrogen production within the control reactor over the experimental period. It can be seen that most of the product yield is attributable to VFAs (acetate, propionate, butyrate and valerate) and little contributed by ethanol and hydrogen.

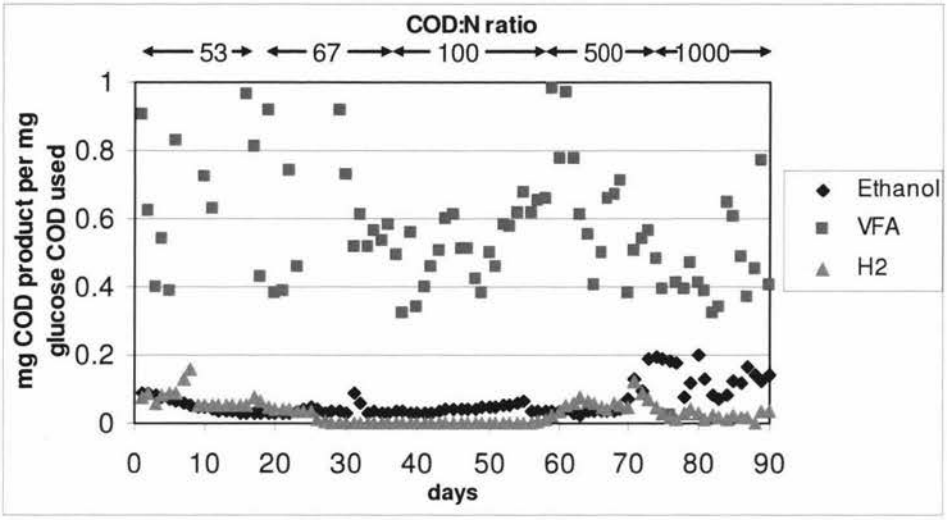


Figure 14. Overall ethanol, VFA and hydrogen product yields for reactor B.

Figure 14 shows VFA, ethanol and hydrogen production within reactor B over the experimental period. As for the control reactor, reactor B product yield was mostly made up by VFAs. As for the control, reactor B had only a small contribution from hydrogen and ethanol to overall product yield, with the amounts produced varying throughout the study period. The yields were impacted by nitrogen deficiency, as discussed below.

3.3.1 Effect of nitrogen deficiency on VFA and ethanol production

Volatile fatty acids are potentially very useful products as they can be used as a feedstock for biotechnological applications such as the bacterial production of polyhydroxyalkanoates (PHAs). PHAs could be used in the future as an environmentally friendly replacement for plastics as they are biodegradable (Ruan *et al.*, 2003). Further, VFA production is useful as these compounds are a good carbon source for use in wastewater treatment applications such as phosphorus removal and denitrification (Elefsiniotis *et al.*, 2004). Hence, knowing how to maximize VFA production during anaerobic wastewater treatment may lead to valuable by-products for future use.

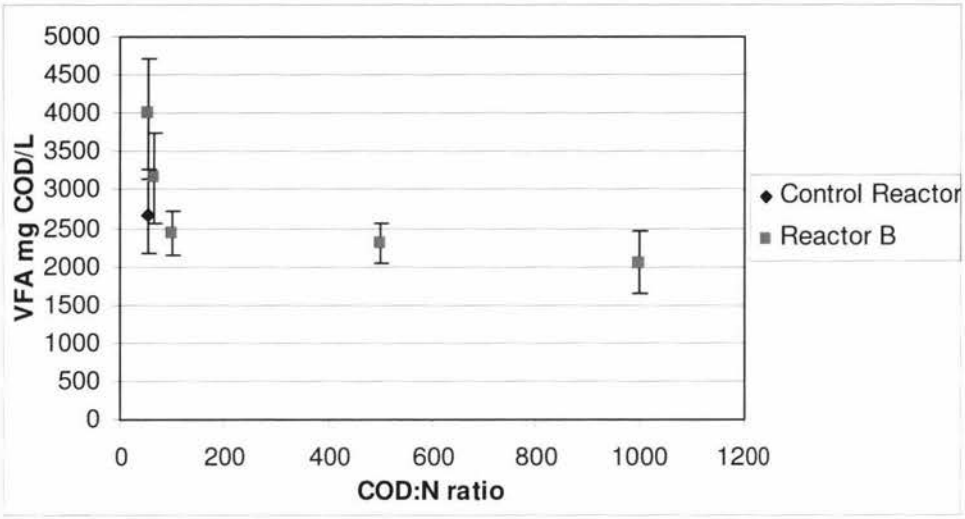


Figure 15. Effect of COD:N level on total VFA production.

When both reactors had a COD:N ratio of 53, reactor A produced on average 2662 ± 485 mg COD/L total VFA, while reactor B produced on average 3992 ± 726 mg COD/L total VFA (Figure 15). For reactor B, the total VFA production decreased proportionately from a COD:N ratio of 53 to 100 and then reached a plateau around 2200 mg VFA COD/L from a COD:N ratio of 100 to 1000. As the total VFA production did not change greatly from a low to high COD:N ratio, this suggests that COD:N ratio does not greatly affect total VFA production. It was expected that the overall reactor productivity would decrease as the amount of available nitrogen decreased and the COD:N ratio increased. The results suggest that the overall VFA production is not highly dependent on the COD:N ratio. Up until now it has not been discussed whether VFAs are produced equally at different COD:N ratios. Here it has been found that approximately 0.42 mg VFA $_{COD}$/ mg glucose can be produced with zero available nitrogen.

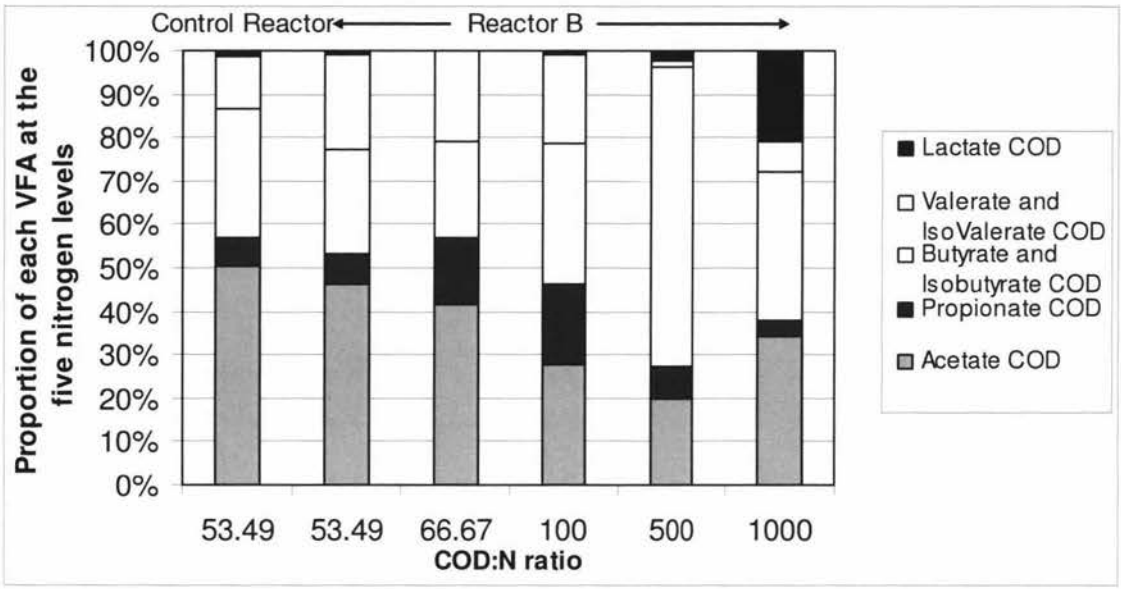


Figure 16. The proportion that each VFA made up at the five COD:N levels at pH 5.5.

From Figure 16 it can be seen that the amount of acetic acid produced decreased with an increase in COD:N ratio from 53 to 500, but increased again when the nitrogen load was reduced to 0 mg/L N. Cheong and Hansen (2006) noted that acetic acid is the major product in unstressed conditions, but as the bacteria are stressed butyric acid becomes the major product. This suggests that reactor B was stressed at a COD:N ratio of 100 and 500 likely due to nitrogen limitation, but perhaps adjusted to the nitrogen deficient conditions when 0 mg/L N was present and nitrogen fixation had become the dominant process for N-assimilation.

According to Cheong et al. (2006), mixed culture anaerobic fermentations are shown to produce large amounts of acetic acid and butyric acid as was found in this study. The most typical VFAs and alcohols produced via *Clostridium species* are butyric acid, propionic acid, acetic acid, butanol and ethanol, though certain species of *Clostridium* also lead to the production of other products. Commonly, acetic acid and butyric acid are produced with some hydrogen and some carbon dioxide. There are many biochemical pathways thought to be possible under anaerobic fermentative conditions, such as the production of ethanol along with acetic acid, hydrogen and carbon dioxide as shown in equation 5a.

From most previous studies, acetic acid and butyric acid tend to be the dominant products from anaerobic digestion at similar conditions. There is much debate as to whether acetic acid or butyric acid is usually the dominant product, but it seems at pH 5.5 that butyric acid tends to be dominant. The butyric acid to acetic acid ratio is thought to be useful for predicting the hydrogen output. Zhang et al. (2006) found that there was a 2.1:1 ratio of butyric acid to acetic acid produced via fermentation accounting for 82-94% of total products, with ethanol making up another 4.6-7.3%. The butyric acid to acetic acid ratio was found to be close to 1 in this study at low and extreme COD:N ratios, but was found to be close to 3.5 at a COD:N ratio of 500.

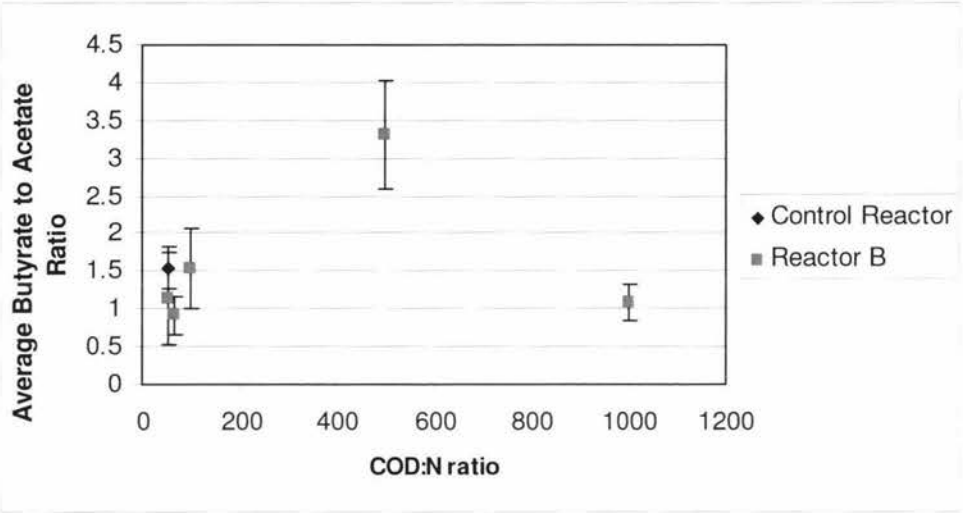


Figure 17. The effect of COD:N ratio on the butyric acid to acetic acid ratio.

From Figure 17 it can be seen that the butyric acid to acetic acid ratio varied greatly with COD:N ratio. At low and extremely high COD:N ratios there was approximately equal amounts of butyric acid and acetic acid produced, though butyric acid slightly dominated. At a COD:N ratio of 500 there was much more butyric acid than acetic acid produced. This is a very interesting finding as this suggests that the most butyric acid was produced while the reactor was nitrogen limited, while less was produced with sufficient nitrogen or with complete nitrogen deficiency. According to Cheong and Hansen (2006) butyric acid is the dominant product at pH 5.7 as was often found here at pH 5.5. It seems overall that the butyric acid to acetic acid ratio varied with nitrogen level and seems to suggest butyric acid production greatly dominates when stressed and nitrogen limited. This suggests that the greatest hydrogen production should be at low and high COD:N ratios according to stoichiometry or under nitrogen

limited conditions according to a high butyric acid to acetic acid ratio. Overall, there was generally more butyric acid than acetic acid present as found by many authors in the past (Khanal *et al.*, 2004; Zhang *et al.*, 2006).

Lactate production remained consistent and low, at around 50 mg COD/L from a COD:N ratio of 53 to 500, and then increased to 400 mg COD/L when completely nitrogen deficient (Figure 16). This is a very interesting finding as Zhang et al. (2007) found that lactic acid production is highest at low C:N ratios. The production of lactate could potentially be very useful as it is used in preserving goods and may be used in the production of biodegradable plastics in the future. However, the lactate pathway involves consumption of hydrogen (Shin and Youn, 2005), hence should be limited if hydrogen production is the goal of the system. The spike in lactate production with zero available nitrogen suggests the hydrogen production will be low at this nitrogen level (see Section 3.3.2a).

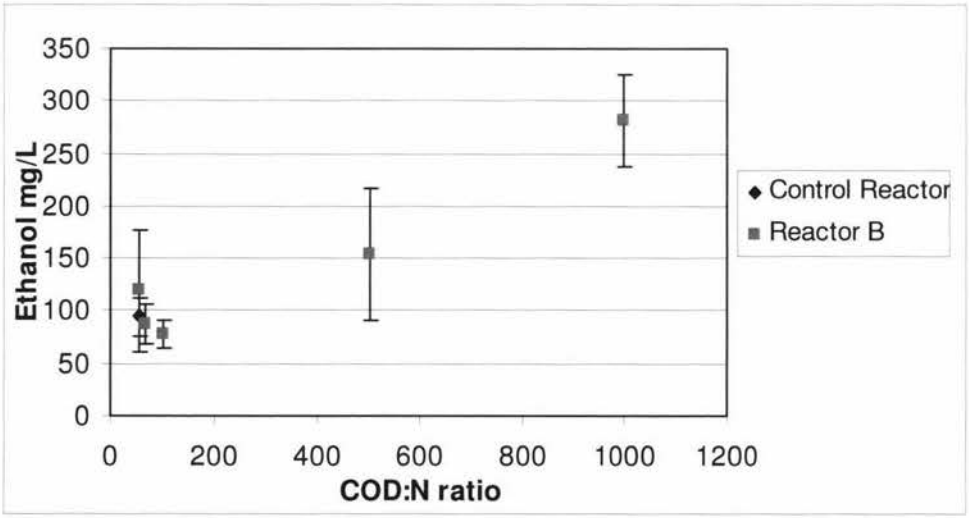


Figure 18. The effect of COD:N ratio on ethanol production.

Figure 18 shows that the amount of ethanol produced at pH 5.5 did not vary much between a COD:N ratio of 53 to 100, but increased slightly at a COD:N ratio of 500 and increased greatly when there was no longer any available nitrogen. Hence, as the reactor became nitrogen limited and then nitrogen deficient, the ethanol production continuously increased. It is unclear as to why this happened though it is possible that the nitrogen deficiency caused the ethanol to be produced in greater amounts as different pathways can be exploited under different reactor stress levels and

conditions. Brandberg et al. (2007) also found that ethanol production is greatest when nitrogen is limiting. On the other hand, Zhang et al. (2007) found that ethanol was produced in greater amounts with a low COD:N ratio. This is likely due to a shift in bacterial population present with nitrogen deficiency. It is likely that the predominant pathways shifted with a deficiency of nitrogen to ethanol producing pathways such as those seen in equation 5 above.

3.3.2 Impact of nitrogen deficiency on gas production

It has been found that the ratio of the various VFAs produced is directly related to the types and amounts of gases produced (Van Ginkel and Logan, 2005). In order to maximize hydrogen production it is thought that a high butyric acid to acetic acid ratio is desirable (Khanal *et al.*, 2004). The varying *Clostridium* species produce different fermentation products (Cheong *et al.*, 2006).

The production of propionic acid, and lactate should be minimized or limited in order to prevent hydrogen from being consumed (Kyazze *et al.*, 2006; Hawkes *et al.*, 2002). It is thought that hydrogen is produced during ethanol production, but many of the hydrogen atoms produced are used in the making of the alcohols hence the overall hydrogen yield is lower. To date, the highest hydrogen yield found under similar anaerobic conditions to those used in this study is 2.6 mol H_2/mol glucose as found by Taguchi et al. (1996).

a) Hydrogen

The amount of hydrogen produced varied greatly over the study period for reactor A as described in Figure 19. The decrease in hydrogen production during days 11-41 is attributed to unwanted methanogenesis reactions, as will be discussed. The reactor walls were cleaned in order to prevent methanogens from growing in the reactors once it was determined that this was the cause of the methane production and the loss of hydrogen.

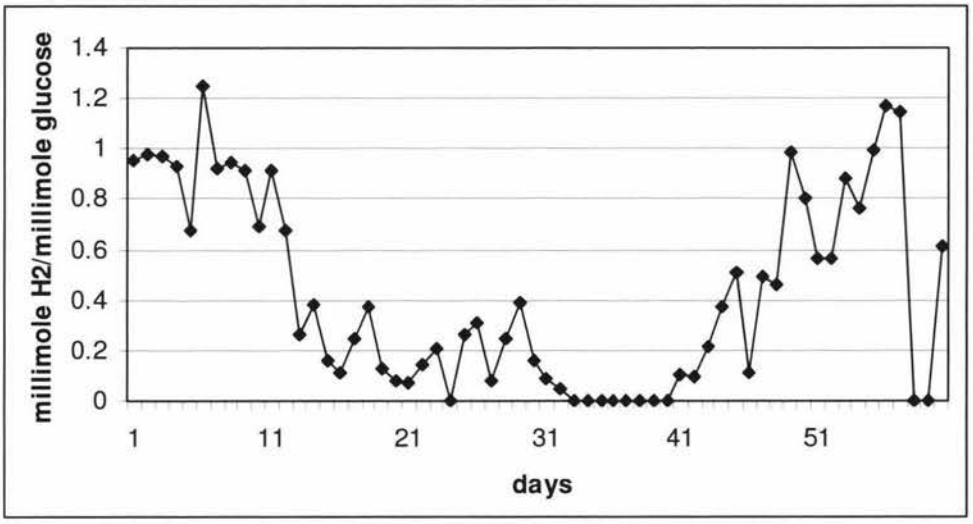


Figure 19. The average number of mmoles of hydrogen produced per mmol of glucose for the control reactor over the study period.

The number of mmoles H_2/mmole glucose varied as the COD:N ratio increased (Figure 20). It has been found that the C:N and hence the COD:N ratio affects the production of hydrogen (Tao *et al.*, 2007). It can be seen that the hydrogen production was greatest at approximately 0.95 mmol H_2/mmol glucose when the COD:N ratio was low at around 53. A COD:N ratio of 180 has been noted to be optimal for hydrogen production when compared to COD:N ratios of 110, 250 and 360 (Tao *et al.*, 2007). Tao et al. (2007) found that hydrogen production was only partially limited at COD:N ratios of 250 and 360, but was severely limited at a COD:N ratio of 110. In this study, the hydrogen production was approximately 0.90 mmol H2/mmol glucose at a COD:N ratio of 500 and 0.40 mmol H2/mmol glucose with no available nitrogen. It seems that hydrogen production decreased by 50-60% as a result of nitrogen deficiency. This drop in hydrogen production at 0 mg/L N is possibly due to the surge in lactate production with nitrogen deficiency. Hence, from this study it seems that hydrogen production is best with a low COD:N ratio of approximately 50, but is still possible with complete nitrogen deficiency.

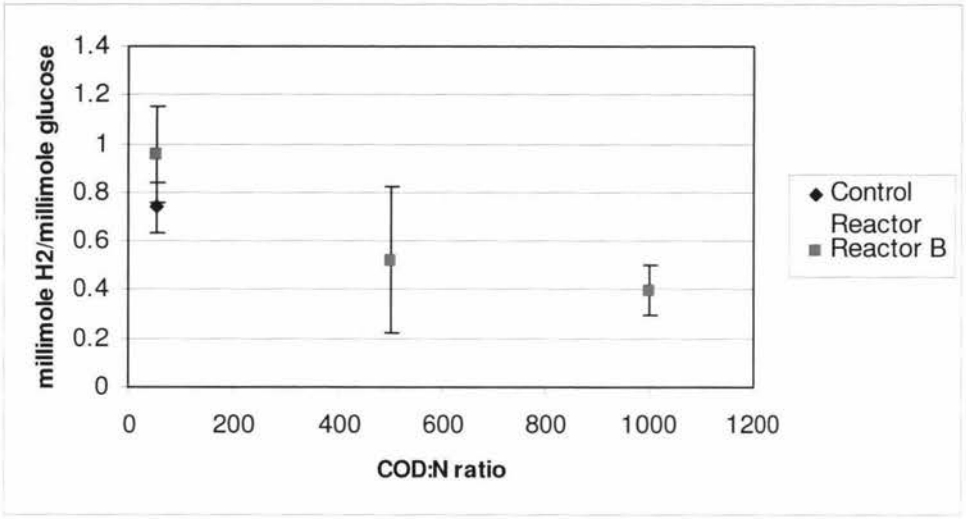


Figure 20. The effect of COD:N ratio on the number of mmoles of hydrogen produced per mmol of glucose.

From the spike in the butyric acid to acetic acid ratio with a COD:N ratio of 500, it would be expected that the hydrogen production would be high at this level of nitrogen according to what other authors have found with respect to a high butyrate to acetate ratio, though stoichiometry suggests a higher acetate ratio would lead to higher hydrogen production. This was not the case, suggesting that the low level of available nitrogen causing nitrogen limitation may have inhibited hydrogen production.

According to Zheng and Yu (2005) large amounts of VFAs can be inhibitory to hydrogen production via fermentation, hence perhaps low levels of hydrogen were associated with periods of high VFA production. Hydrogen production may also decrease due to consumption by acetogenesis as seen in equation 6.

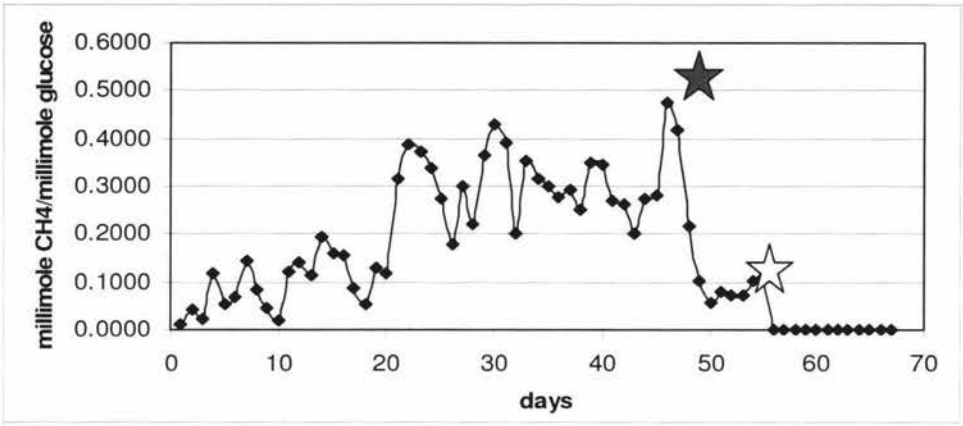
Hydrogen consumption during acetogenesis:

$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$ Equation 6

It is possible that the overall hydrogen production at the low COD:N ratios was lower than expected (1-2 mmol hydrogen per mmol glucose) as Ueno et al. (2001) noted that when NH_4Cl was used instead of peptone as a nitrogen source for their CSTRs the hydrogen production was halved. As NH_4Cl was used as the nitrogen source in this study, it is possible that that the hydrogen production was not as great as previously found for these conditions.

b) Methane

From the findings of previous studies (Yu *et al.*, 2002; Hwang *et al.*, 2004), it was not expected that any methane would be produced at pH 5.5 and with an SRT of 12 hours. However, methane was produced in both reactors A and B. The amount of methane produced varied in reactor A from zero to 0.476 mmols methane produced per mmol of glucose (Figure 21). Once the reactor walls were scrubbed regularly there was no longer methane produced (Figure 21). Regular scrubbing was to ensure that there were no solids remaining in the reactor longer than 12 hours. Kim et al. (2006) found that keeping anaerobic reactors running long-term led to methane gas production and they noted that using a short SRT such as 4 hours washed out methanogens present. It is possible that the hydrogen consuming methanogens were able to survive at this weakly acidic pH as it has been proposed that hydrogen consuming methanogens may be able to withstand acidic pHs better than some other methanogens (Hwang *et al.*, 2004). Preventing methanogenesis was an unexpected challenge and revealed that running small volume reactors over a long period of time is quite difficult. Even with nitrogen bubbling through the reactors and with a stir bar and baffles to ensure mixing, the methanogens were still able to buildup on the reactor walls due to the high surface area to volume ratio. Therefore, it is very important to ensure there is strong mixing in all parts of the reactor to try to prevent solids buildup on the walls. It is also important to ensure there are no solids building up in the reactor as this increases the SRT and hence the dynamics of the system.



★ Cleaned reactor walls

☆ Cleaned reactor walls again

Figure 21. The average number of mmoles of methane produced per mmol of glucose in the control reactor.

For reactor B, there was little methane being produced to begin with at a COD:N ratio of 53 (Figure 22). However, there was 0.035 to 1.26 mmoles CH_4/mmole glucose from a COD:N ratio of 67 to 500. This methane production was likely due to solids building in the reactor allowing for a longer SRT than 12 hours, permitting methanogens to grow. A similar finding was found by Kraemer and Bagley (2005). At the time when the methanogens were present there was a high butyrate to acetate ratio due to an increase in butyrate production. This coincided with a decrease in hydrogen production. As there was no significant decrease in acetate production at this time it is likely that the methane was produced via the carbon dioxide pathway and not the acetic acid consuming pathway.

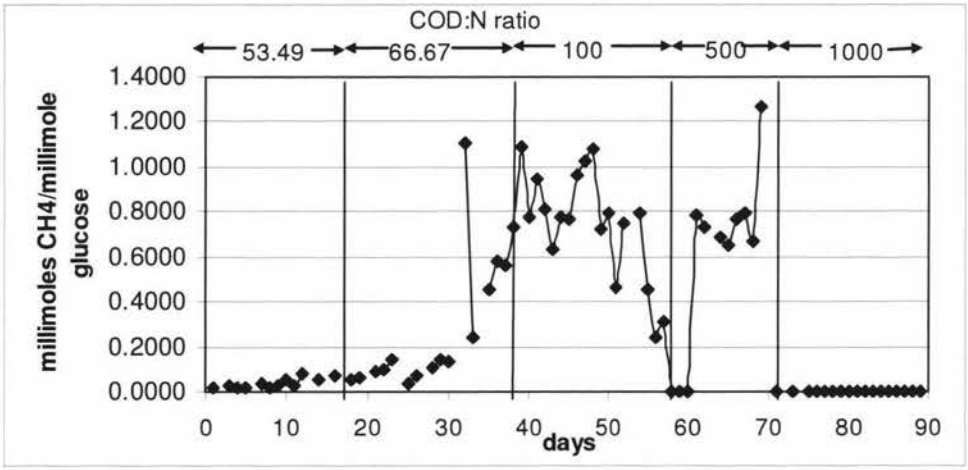


Figure 22. The effect of COD:N ratio on the average number of mmoles of methane per mmol of glucose produced at various nitrogen levels.

Though bacteria are known to dominate nitrogen fixing environments, Archaea (of which some methanogens belong) are capable of fixing nitrogen. Many methanogens are known to have the nitrogenase enzyme present (Kessler *et al.,* 1998), suggesting that many methanogen species are capable of nitrogen fixing. It is likely that no methanogens existed in the environment once the walls were scrubbed regularly and hence no methanogens were present once the nitrogen level was turned down to zero. It is unknown whether methane could have been produced at pH 5.5 with zero nitrogen had any of the conditions such as SRT, OLR or the feed been different. Due to methanogenesis taking place at COD:N ratios of 67, 100 and 500, it is difficult to interpret the data as this reduced the hydrogen production and likely also changed the quantitative values for other products.

c) CO_2

Reactor A averaged 3.51 mmoles CO_2/mmole glucose at a COD:N ratio of 53 nitrogen (Figure 23). The maximum theoretical yield in terms of mmoles CO_2/mmole glucose is four (equation 5b).

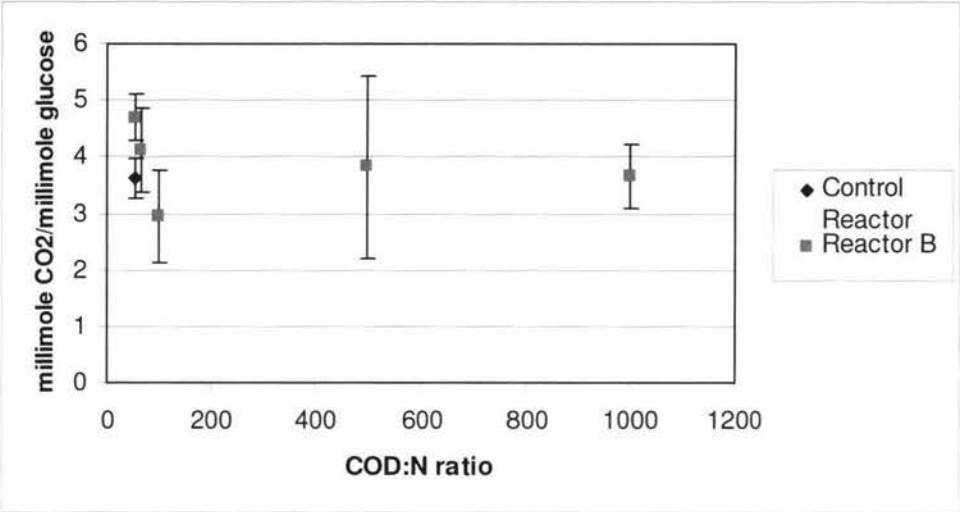


Figure 23. The effect of COD:N ratio on the number of mmoles of CO_2 produced per mmole of glucose.

The number of mmols of CO_2 produced per mmol of glucose was highly variable as the amount of nitrogen was decreased in the feed and the COD:N ratio increased for reactor B (Figure 23). It is possible that the amount of CO_2 produced with a COD:N ratio of 500 was an over-estimate as the GC being used did not calibrate very well for CO_2 and tended to calibrate a bit high. The average number of mmols of CO_2 produced per mmol of glucose was 4.69 ± 0.41 at a COD:N ratio of 53; 4.13 ± 0.74 at a COD:N of 67; 2.93 ± 0.82 at a COD:N of 100, 3.83 ± 1.61 at a COD:N of 500 and 3.66 ± 0.58 at 0 mg/L nitrogen (as seen in Figure 23). Carbon dioxide production does not seem to vary with nitrogen level as CO_2 production was approximately 3.5 mmols per mmol of glucose throughout the study period.

Between pH 4.5 to 7.9, the most gas was found to be produced at pH 5.7 with 41 % being carbon dioxide ($_2$/Zoetmeyer *et al.*, 1982). The ratio of hydrogen gas to carbon dioxide is generally 0.6, hence the finding here was slightly lower than usual likely due to overestimating the CO_2 production or due to the possibility of stripping out inorganic carbon. For reactor A, CO_2 made up approximately 75 % of total gas production while hydrogen made up approximately 20 % when methane was not being made, but hydrogen contributed less to overall gas production when methane was being made.

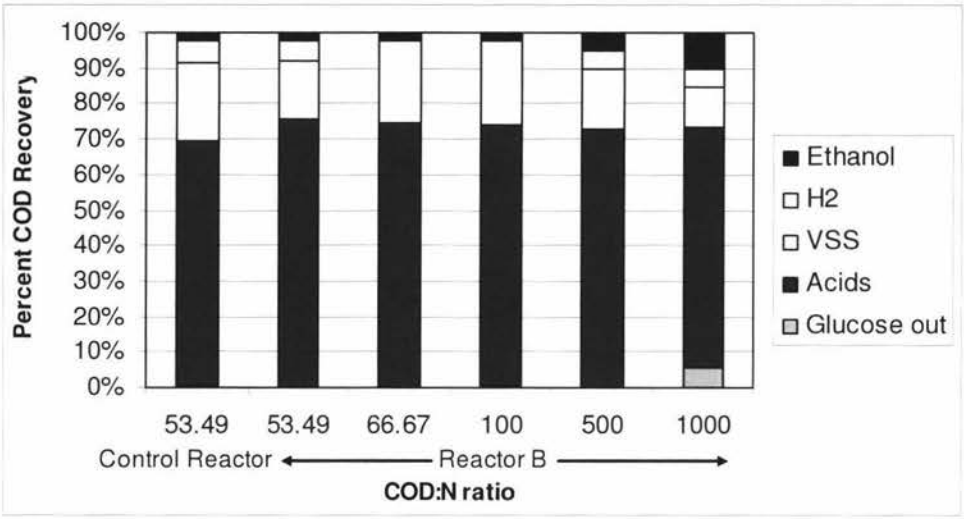
For reactor B, CO_2 made up approximately 80 % of gas production while hydrogen made up the other 20 % at a COD:N ratio of 53. At 0 mg/L N CO_2 made up 92 % of the gas production while hydrogen only made up 8%. This increase in carbon dioxide as the COD:N ratio increased may be due to the increase in ethanol production as one of the ethanol pathways can lead to 4 moles of carbon dioxide being formed as a by-product, while there are usually only 2 moles produced via the other pathways. Hydrogen was made at both very low and very high COD:N levels to some degree though it appears that the percent of total gas that hydrogen makes up decreases as nitrogen decreases and the COD:N ratio increases. This suggests that hydrogen production is greatest with sufficient nitrogen supply, though it is likely that methane production at COD:N ratios of 67, 100 and 500 affected hydrogen production at these levels.

3.4 COD and Carbon balances.

3.4.1 COD balance

Control reactor A

From Figure 24, it is evident that most of the COD was converted into volatile fatty acids, followed by biomass (solids). Hydrogen and ethanol made up approximately 10% of COD, while there was very little glucose COD remaining in the effluent. This suggests that the bacteria present were processing the incoming glucose almost 100 %. COD recovery for reactor A varied from 65 to 98%, and averaged 86%. It is possible that when the COD recovery was far from 100% that other products were being formed that were not measured, such as butanol or other alcohols. Caproate is also a product that was not tested for in this study which has been found to be a minor product in several similar studies (Tao *et al.*, 2007; Yu and Mu, 2006) and may have contributed somewhat to the overall COD.



* VSS were assumed to have a composition of C4H7O2N as Kyazze et al. (2006) assumed in converting mg VSS to mg VSS COD

Figure 24. The distribution of COD into products of ethanol, hydrogen, biomass, volatile fatty acids and glucose in the effluent for reactors A and B.

Reactor B

From Figure 24 for it can be seen that volatile fatty acids made up the largest percent of COD at all nitrogen levels in the experimental reactor B. The biomass (solids) contributed the second largest amount as for reactor A. Hydrogen contributed most to overall COD at a COD:N ratio of 53, though this is likely because it was consumed by methanogenesis at the higher COD:N ratios of 67 and 100. As the COD:N ratio increased to 500 and upwards, the hydrogen production again became significant making up approximately 5 % of the COD distribution. Ethanol contributed very little to the COD distribution from a COD:N ratio of 53 to 500, but contributed approximately 10 % with 0 mg/L N. The COD recovery for reactor B was as follows: COD:N of 67, 103 %; COD:N of 100, 103%; COD:N of 500, 99 %; COD:N of 1000 (completely N-deficient), 81 %. This suggests that there may have been one or more other products being produced at 0 mg/L N that was not being accounted for and that was not being produced at the higher nitrogen levels. At 0 mg/L added N, 3 % of glucose input was found in the effluent suggesting that the dilution rate (=1/HRT) may have been approaching the maximal growth rate of the microbial population, while less than 0.5 % of input glucose was found in the effluent at the other COD:N ratios.

$$\text{Product Yield, } Y = \frac{\text{g COD produced}}{\text{g glucose COD removed}}$$

Table 13. Effect of nitrogen deficiency on product yield.

Reactor	Percent glucose converted to products (mg COD converted to products/mg COD input)	VFA *	VSS *	CH4 *	H2 *	Ethanol *
A	99.6	0.61	0.19	0.05	0.05	0.02
B 53.47	99.8	0.85	0.18	0.01	0.07	0.03
B 67	100	0.66	0.20	0.15	0.003	0.02
B 100	99.7	0.62	0.20	0.19	0	0.02
B 500	99.9	0.55	0.13	0.24	0.04	0.04
B 1000	97	0.56	0.10	0	0.04	0.08

* The units of yield for these products were $mgCOD/mgCOD_{converted}$

From Table 13 it can be seen that the yield of VFAs did not vary much with different COD:N ratios. It appears that the biomass yield was very similar when the COD:N ratio was between 53 and 100. As the nitrogen became limited at a COD:N ratio of 500, the biomass yield decreased from around 0.20 to 0.13. The yield again decreased to 0.10 when the reactor was deficient of nitrogen. This suggests that the reactor was most 'efficient' when it was deficient of nitrogen, as the carbohydrate source was being made into products and not into biomass. In terms of other products aside from biomass, it seems that the reactor was most 'efficient' at a low COD:N ratio as less carbon dioxide was being produced proportionately as this is considered to be a waste product in this case. In contrast to the findings of this study, Larsson et al. (1997) found that biomass yield was greatest with high COD:N ratios, with a yield of 0.10 g biomass per g of glucose. When the energy source was in excess as was the case here with a COD:N ratio of 53, 67 and 100, they found that there was a 50 % decrease in yield. The yield of methane increased upto a COD:N ratio of 500 due to solids' building up on the walls of the reactor, but then decreased to zero with proper cleaning of the reactor walls. The hydrogen yield remained approximately was slightly higher at a COD:N ratio of 53 than with no nitrogen present, but it is possible that the hydrogen yield may be even higher at COD:N ratios around 70 or 100 which cannot be determined from this study as the hydrogen was being converted to methane at these levels. The ethanol yield remained consistent from a COD:N ratio of 53 to 100, but then increased at a COD:N ratio of 500 and again at a COD:N ratio of 1000 suggesting that ethanol yield increases as the COD:N ratio increases.

3.4.2 Carbon Balance

The percent carbon recovery for reactor A was approximately 123 % while that for reactor B was 133 % at a COD:N ratio of 53. The carbon recovery for reactor B was as follows: COD:N of 67, 152 %; COD:N of 100, 154 %; COD:N of 500, 161 %; no nitrogen, 91 %. It is likely that the percent carbon recovery was overestimated as the GC gave higher than true values for carbon dioxide due to calibration problems or due to the fact that inorganic carbon was added in the feed. Hence at 0 mg/L N the true percent carbon recovery was likely lower than 90 % suggesting that other products were likely being made that were not considered.

3.5 The effect of shocking reactors and their recovery.

3.5.1 The effect of leaving reactors unfed for twelve days and their recovery period upon being fed again

It seems that following reactor shutdown for twelve consecutive days with no feed that both reactors were able to recover gradually. For nine days following the introduction of a glucose feed again, both reactors had high glucose in their effluents suggesting that there was low biomass activity present. After nine days, the glucose level in the effluent dropped to almost zero. During the nine day period, the biomass levels increased gradually. The reactor productivity reached a plateau and remained stable for months to follow. Hence, it seems that it is possible for mixed bacterial biomass to survive for short periods of time with no feed supply. It is not yet known how long a bacterial population could sustain itself with no feed input.

3.6 Summary.

This work has demonstrated that acidogenic fermentation can take place under nitrogen deficient conditions. CSTRs were successfully run under nitrogen deficient conditions using a mixed bacterial culture showing that nitrogen fixation takes place with no nitrogen supplementation. Overall as the nitrogen level approached zero mg/L and the COD:N ratio increased, the TKN decreased but never went to zero suggesting that nitrogen fixation was taking place. From the nitrogen balance, it was shown that nitrogen fixation was taking place at high COD:N ratios. The acetylene-reduction test confirmed that nitrogen fixation was taking place under nitrogen

deficient conditions. The amount of biomass production decreased as the nitrogen level decreased and the COD:N ratio increased, but remained at approximately 300 mg VSS COD/L with no nitrogen available, suggesting that the bacteria present were able to adapt to an environment that was deficient of nitrogen. It is possible that there was a shift in the bacterial population from a mixed population at low COD:N ratios, to a more specific culture of nitrogen fixing bacteria such as *Klebsiella* under nitrogen limited and nitrogen deficient conditions.

The total VFA production only slightly decreased as the nitrogen level decreased, suggesting that the VFA production did not vary with COD:N ratio. Butyric acid was the dominant VFA produced at all COD:N ratios aside from at a COD:N ratio of 67 when the butyric acid to acetic acid ratio was just under one. Butyric acid was highly dominant at a COD:N ratio of 500 with nitrogen limitation. Ethanol production and lactate production greatly increased as the COD:N ratio increased and the nitrogen level approached zero mg/L, suggesting that these two products may be mostly produced at low nitrogen levels. Hydrogen production decreased as the COD:N ratio increased to 100 due to methanogen growth in the reactor, but once the reactor walls were cleaned the hydrogen production was re-generated to a slightly lower level than with a low COD:N ratio. Carbon dioxide production remained approximately the same at all nitrogen levels. It was unusual to find that methane was produced at pH 5.5 with an SRT of 12h, though this was likely due to solids building up on the reactor walls altering the SRT and allowing time for methanogens to grow. Regular scrubbing of the reactor walls was able to prevent methane production. From the biomass yield it seems that running the reactor with a very high COD:N ratio under nitrogen deficient conditions was the most efficient, though this was not the case in terms of hydrogen production. Overall, it appears that the best reactor conditions depend on the desired output. A low COD:N ratio is best for producing hydrogen and VFAs, though this is costly in terms of nitrogen input to low nitrogen wastewater. However, a high COD:N ratio is best for ethanol and lactate production, though not as effective at producing hydrogen, whilst the maximal growth rate of the biomass is implicated as being lower.

4. The Effect of Low pH on Nitrogen Deficient Acidogenesis

It is known that pH has an effect on microbial population diversity (2/ Mu *et al.*, 2006). It is also known that the main products formed vary with pH. According to Rodríguez et al. (2006), with a decrease in pH the main product changes from acetic acid to butyric acid with concurrent ethanol production. Hydrogen production is also affected by a change in pH as the hydrogenase enzyme activity is highly pH dependent (Cheong *et al.*, 2006). Maximum hydrogen production tends to be at pH 5.7, while pH 4.0 is thought to be the minimum pH useful in anaerobic biohydrogen production (Hwang *et al.*, 2004). In this section, the effect of a reduction in pH on acidogenic fermentation under nitrogen deficient conditions is investigated.

4.1 Biomass production pH 5.5 and 4.0.

Figure 25 shows the average biomass populations over the period of study. Stable biomass populations were observed despite the severe nitrogen deficiency of the feed to the two systems; neither reactor was receiving nitrogen in their feed but both were continuously producing between 300-400 mg COD/L VSS.

From Figure 25 similar biomass levels were produced at pH 5.5 than at pH 4.0 under nitrogen deficient conditions. The biomass yield was found to be the same at 0.09 mg VSS COD per mg glucose COD for both reactors.

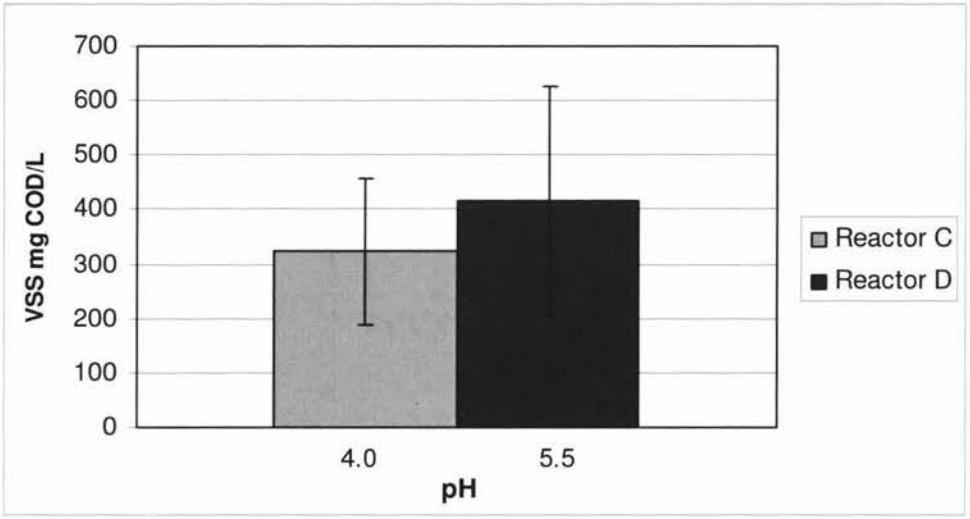


Figure 25. The effect of pH on biomass production under nitrogen deficient conditions.

Samples were taken from both reactors C and D in order for microbiological investigation to be performed using a microscope. From microbiological study performed by Jon Palmer (assistant lecturer in the Institute of Food Nutrition and Human Health, Massey University) it was found that reactor C had *Clostridium* and *Bacillus* species present, while reactor D had mixed microbes. Certain species of *Clostridium* and *Bacillus* are known to be nitrogen fixing, hence reactor C was likely to be containing nitrogen fixing bacteria. Reactor D was confirmed to be nitrogen fixing by the acetylene-reduction test. (The acetylene-reduction test used to confirm nitrogen fixation was not successful for reactor C as the assay was conducted immediately after the reactor had suffered a pH shock). It is interesting that reactors C and D were found to have different bacterial populations (Figures 26 and 27). Reactors C and D were both found to have biofilm clusters present consisting of bacteria and organic matter, though reactor C was noted to have yeast present while reactor D seemed to show large mixed bacterial culture clumps. This suggests that pH has a significant effect on bacterial population. This difference in bacteria present also suggests that the products in reactors C and D should differ.

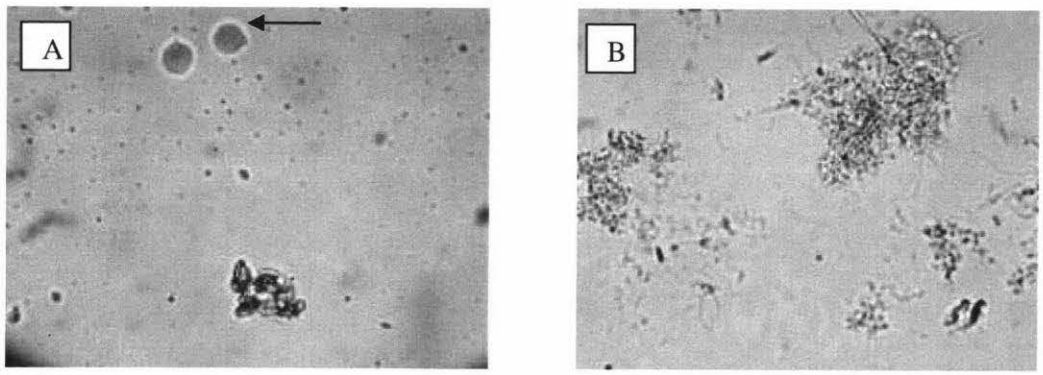


Figure 26. Two photos (A and B) showing some of the microbiology in reactor C (pH 4.0) using 40x magnification. The structure labeled with the black arrow in A is yeast, while B shows a biofilm cluster.

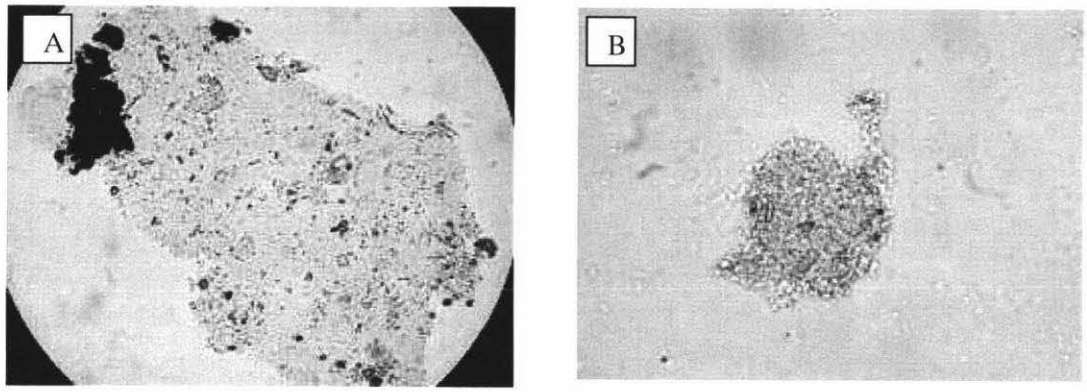


Figure 27. Two photos (A and B) showing some of the microbiology in reactor D (pH 5.5) using 40x magnification for A and 10x for B. A shows a biofilm cluster with organic matter and bacteria, while B shows a mixed bacterial population.

It was found that the biomass TKN concentration was much higher at pH 4.0 than at pH 5.5 (Figure 28). It is possible that much of the material in reactor D (pH:5.5) was EPS, which has a very low nitrogen content. Extracellular polymeric substances (EPS) are produced by biomass during organic matter consumption and vary in form depending on environmental conditions (Sheng and Yu, 2007). EPS can be soluble or bound to cells and can be consumed as energy if other energy sources are lacking. The presence of EPS within wastewater play a role in determining the properties of the flocs present and their settling ability (Hoa *et al.*, 2003). EPS are thought to be produced in greater amounts with nitrogen limitation (Punal *et al.*, 2000). EPS components have also been found to vary with available nitrogen level, with protein

and carbohydrate level decreasing as nitrogen level increases (Hoa *et al.*, 2003). Hoa et al. (2003) thought that the EPS structure may be best at high nitrogen concentrations (COD:N $\geq$ 10:1) suggesting that high nitrogen concentrations would be best for optimizing wastewater sludge properties for settling out solids. Further study could be done to observe EPS properties at varying COD:N ratios.

There was never found to be any ammonia in the effluent under nitrogen deficient conditions at pH 4.0 or pH 5.5.

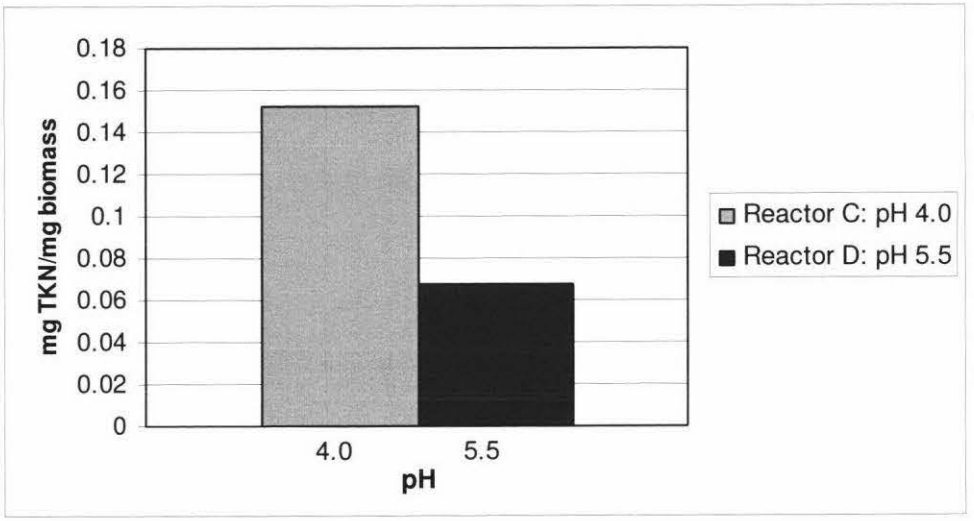


Figure 28. The amount of TKN per mg of biomass at pH 4.0 and pH 5.5.

The results indicate that the N content of cells in Reactor C was 15 % and in reactor D was remarkably lower at approximately 7 % (Figure 28). It is interesting that the two reactors had different nitrogen content in their cells under nitrogen deficient conditions, the reasons for this being unknown.

4.2. VFA, ethanol and gas production.

4.2.1 VFA Production

The VFA production over time is shown in Figure 29. At pH 4.0 an average of 1690 $\pm$ 300 mg/L COD VFAs was produced, while at pH 5.5 an average of 2670 $\pm$ 380 mg/L COD VFAs was produced. In terms of product yields, reactor D produced far more VFAs than reactor C. Reactor C produced 0.47 mg COD VFA/mg COD

glucose, while reactor D only produced 0.56 mg COD VFA/mg COD glucose. The overall VFA production at pH 4.0 and pH 5.5 greatly varied day to day as seen in Figure 29.

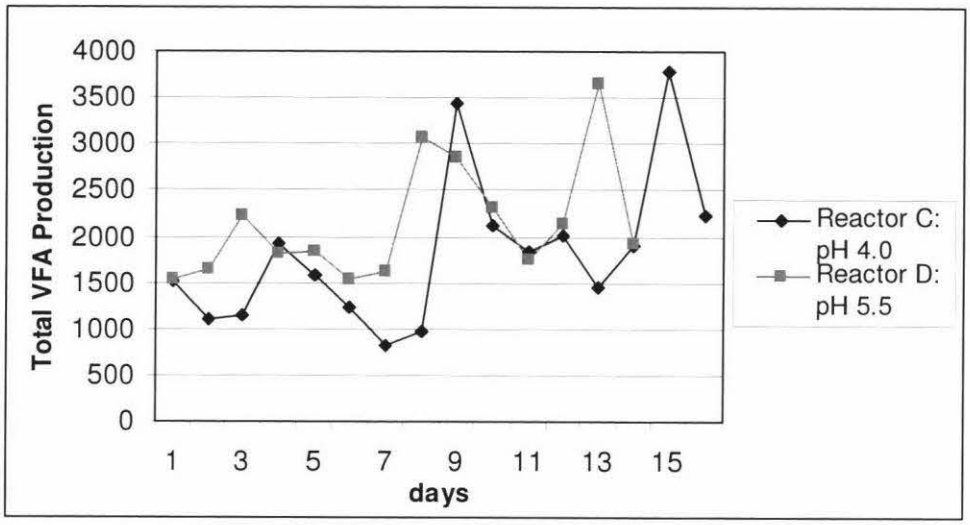


Figure 29. The effect of pH on total VFA production.

Acetic acid and butyric acid continued to be the dominant VFAs (Figure 30). From Table 14 it can be seen that acetic acid yield was dominant at pH 4.0, while butyric acid dominated at pH 5.5. From the literature, it was expected that there would be more butyric acid than acetic acid at pH 5.5, as butyric acid is known to increase and dominate at pH 5.5.

Table 14. Product yields (mgCOD acid/mgCOD glucose) for the different acids at pH 4.0 and pH 5.5.

Reactor	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Lactate
C: pH 4.0	0.23	0.03	0.15	0.03	0.04
D: pH 5.5	0.13	0.03	0.19	0.02	0.09

The production of propionic acid did not vary with pH, while slightly more lactate was produced at pH 5.5 than at pH 4.0. It is interesting to find that there was no difference in propionic acid production at low pH as Ren et al. (1997) found that propionic acid production decreased with decreasing pH.

Overall, pH did exercise some control over VFA production under nitrogen deficient conditions.

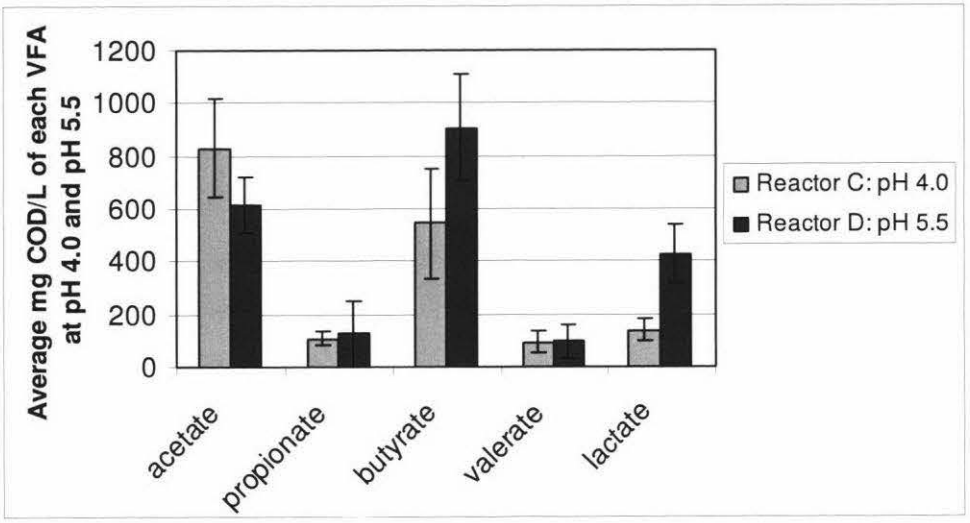


Figure 30. The average amount of acetic acid, propionic acid, butyric acid and valeric acid produced in mg_{COD} /L at pH 4.0 and pH 5.5.

The butyric acid to acetic acid ratio at pH 4.0 was 0.65 ± 0.34, while at pH 5.5 it was 1.46 ± 0.24. It has been shown that having a higher ratio increases hydrogen production, suggesting that more hydrogen should be produced at pH 5.5 than at pH 4.0. Overall, it seems that pH affected the VFA distribution. Mu et al. (2007 2006) found VFA distribution to vary with pH, with acetic acid being lower at pH 4.2 than at pH 6.3, while butyric acid was higher at pH 4.2 and lower at pH 6.3. Interestingly, the findings in this study are the opposite.

From Figure 30 it can be seen that significantly more lactate was produced at pH 5.5 than at pH 4.0. In terms of product yields, 0.09 mg lactate/mg glucose was produced at pH 5.5 while only 0.04 mg lactate/mg glucose was produced at pH 4.0. It is possible that reactor C (pH 4.0) was better adapted to its environmental conditions than reactor D as lactate production has been found to be formed mostly during times of change to the environment and tends to decrease once the changes are over (1/ Zoetmeyer *et al.*, 1982). Zoetmeyer et al. (2/ 1982) found there to be more lactate produced at pH 4.5 than at pH 5.7 which is opposite to the findings in this study. These results suggest that there may be less hydrogen produced at pH 5.5 than at pH 4.0 as lactate production results in hydrogen consumption.

4.2.2 Ethanol Production

From Figure 31 it can be seen that overall ethanol production was no greater at pH 4.0 than at pH 5.5. At pH 4.0 the product yield of ethanol was 0.08 mg COD ethanol/ mg COD glucose, while at pH 5.5 it was 0.06 mg COD ethanol/ mg COD glucose. Ethanol production is known to increase as pH decreases, and is thought to be the main product of fermentation at pH 4.5 (Ren *et al.*, 1997). Wang et al. (2006) found that as pH decreased to 4.6, ethanol production greatly increased. According to Cheong and Hansen (2006) butyric acid is the dominant product followed by ethanol and then acetic acid at pH 4.6. Different to previous findings it appears that ethanol production varied with nitrogen level and not with pH.

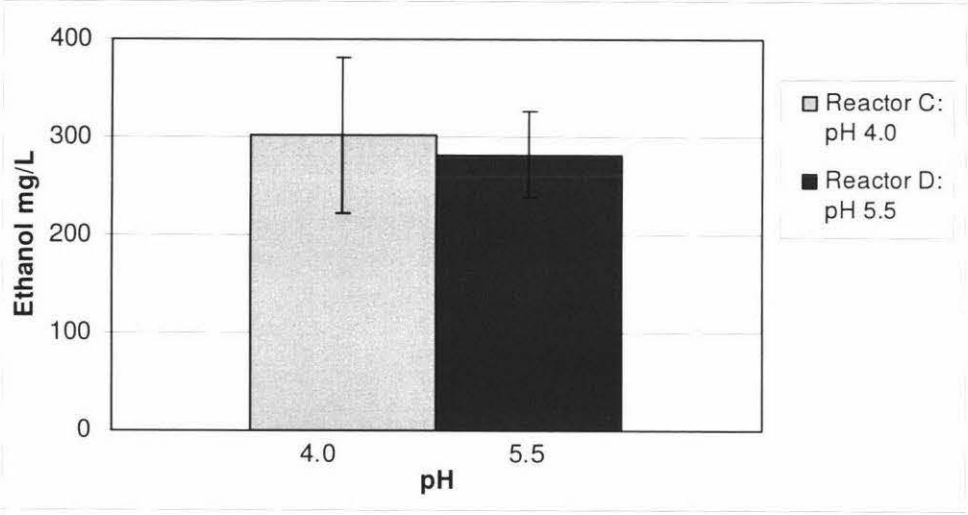


Figure 31. The effect of pH on ethanol production under nitrogen deficient conditions.

Rodríguez et al. (2006) found that below a pH of 5.6 ethanol production dominated. Significant levels of ethanol production were attained at both pH 4.0 and pH 5.5 under nitrogen deficient conditions. Nitrogen deficient conditions have been well examined with relation to ethanol production in the past, with this work thus presenting a new opportunity for exploration.

4.2.3 Gas Production

a) Hydrogen

It is expected that hydrogen production will decrease at low nitrogen levels as the microorganisms are likely to shift towards nitrogen fixers, which may include *Clostridium* species that are thought to lead to maximum hydrogen production (Park *et al.*, 2005) and often with mixed cultures. It has been shown that hydrogen can be liberated during nitrogen fixation as nitrogenase enzymes are often catalysts for hydrogen production (Hansel and Lindblad, 1998). Hydrogen can also be consumed during nitrogen fixation.

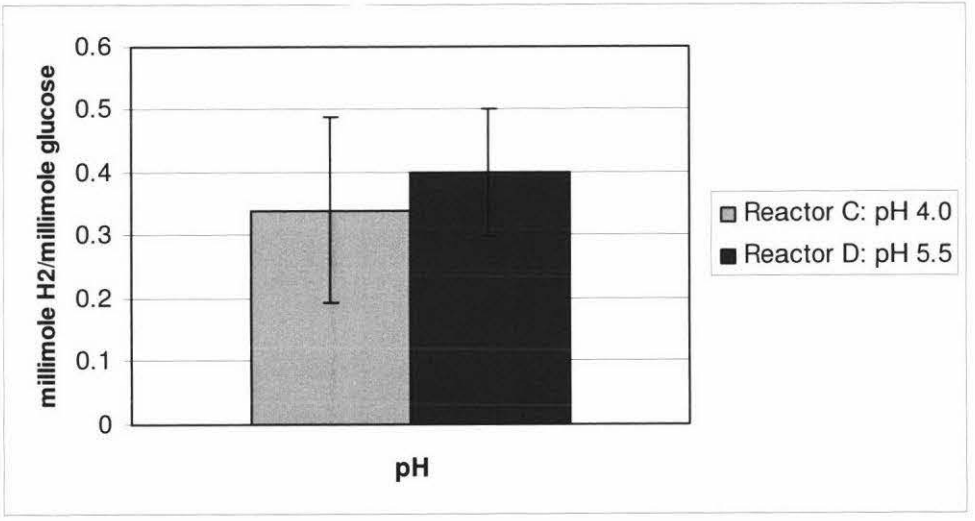


Figure 32. The effect of pH on hydrogen production under nitrogen deficient conditions.

From Figure 32 it can be seen that hydrogen production was similar at pH 5.5 and at pH 4.0. At pH 4.0 the product yield of hydrogen was 0.02 mg COD/mg COD glucose, while at pH 5.5 it was 0.04 mg COD/mg COD glucose. It was expected from the findings of previous literature that more hydrogen would be produced at pH 5.5 than at pH 4.0 (Lin and Chang, 1999; Fang and Liu, 2002). According to Kapdan and Kargi (2006), for hydrogen production to take place under the conditions considered in this study, nitrogen is required. Lin and Lay ($_{1/}$ 2004) found that the carbon to nitrogen ratio is related to hydrogen production. This suggests that with no nitrogen present hydrogen production will be limited as was found here. However, Hafner

(2006) found that the same amount or more hydrogen was produced using nitrogen deficient wastes than with wastes with NH_4Cl as a nitrogen source. It is interesting that pH was not found to affect hydrogen production, contrasting with the work of Khanal et al. (2004) who found that hydrogen production decreases as pH decreases, and Cheong and Hansen (2006) noted that the hydrogenase enzyme required for hydrogen production is affected by pH. Similarly, Kapdan and Kargi (2006) noted that hydrogen production slows as pH decreases due to a decrease in hydrogenase enzyme activity. Khanal et al. (2004) found that pH 5.5 was optimal for hydrogen production. It appears that the COD:N ratio has a much greater effect on hydrogen production than pH.

b) Methane

It has previously been found that methanogens are inhibited below pH 5.5 or 6 (Kyazze *et al.*, 2006). Hence it was expected that no methane production would occur at pH 4.0. No methane production was found at pH 4.0 or pH 5.5 when operating at a 12 hour SRT under nitrogen deficient conditions. It has been shown that methane production is inhibited at pHs below 5 (2/ Kim *et al.*, 2004). Hafner (2006) and O'connor and Young (1993) also found there was no methane produced during anaerobic digestion of nitrogen deficient wastes as was found in this study.

c) CO_2

Figure 33 shows similar CO_2 production at the two pH levels. In terms of product yields, there was 0.45 mg CO_2 carbon/mg glucose carbon while at pH 5.5 there was 0.57 mg CO_2 carbon/mg glucose carbon. This may have been due to having different bacterial populations present in the two reactors leading to different overall gas production. Having different bacterial populations likely led to different biochemical reactions occurring in the two reactors, leading to different product formations.

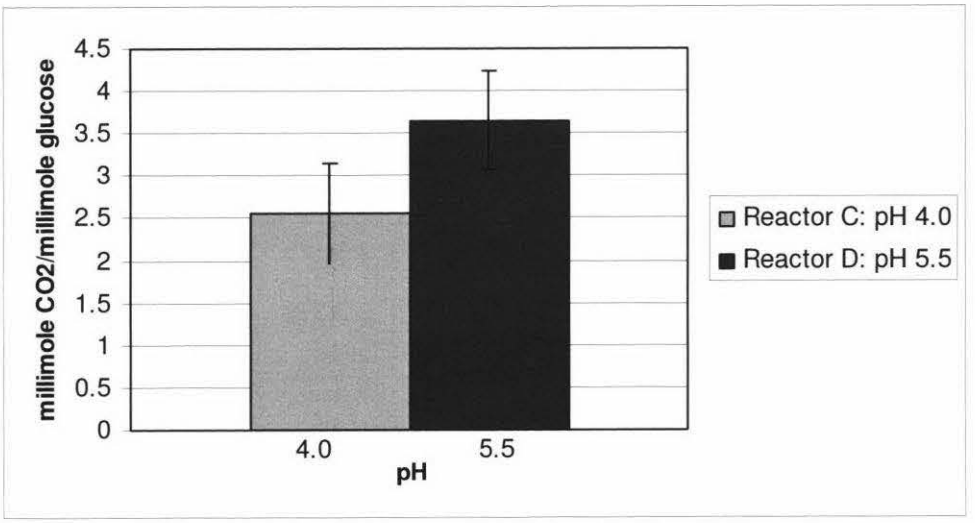


Figure 33. The effect of pH on carbon dioxide production under nitrogen deficient conditions.

4.3 Carbon and COD balance for nitrogen deficient conditions at pH 4.0 and 5.5.

4.3.1 Carbon Balance

The carbon recovery was close to 100 % for both reactors suggesting that most products are accounted for. It is possible that caproate was being produced and would have contributed to the carbon recovery. Caproate production has been found to be pH-dependent (Mu *et al.*, 2006) and may have been a relevant product to measure in this study.

4.3.2 COD Balance

The COD distribution was quite different at pH 4.0 and pH 5.5 as seen in figure 34. At both pHs, VFAs were the dominant proportion of the COD output. At pH 4.0 there was quite a large portion of COD as glucose in the effluent while this was not the case at pH 5.5. This suggests that at pH 4.0 the reactor could not cope as well with the glucose load as at pH 5.5. More biomass was produced at pH 5.5 than at pH 4.0. Both reactors had approximately 10 % COD as ethanol.

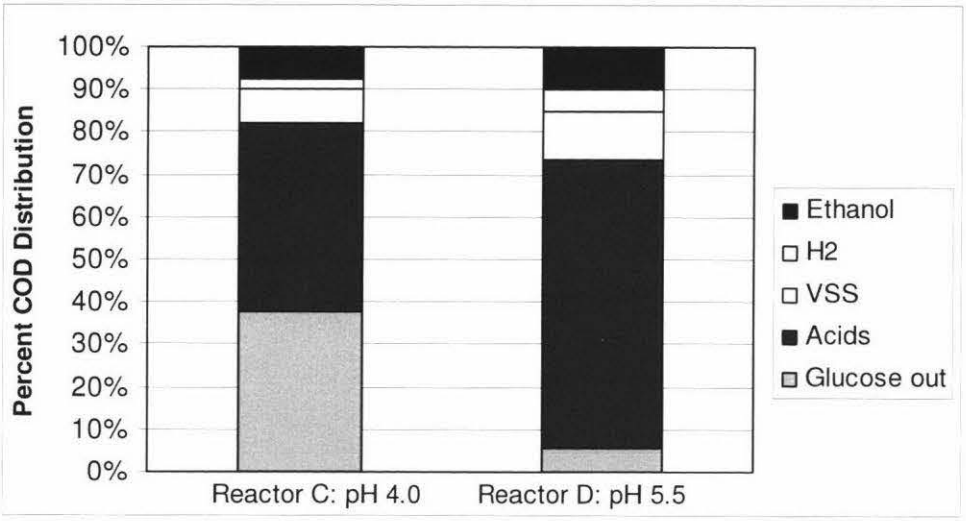


Figure 34. The percent COD distribution at pH 4.0 and 5.5 under nitrogen deficient conditions.

Reactor C had an 83 % COD recovery, while reactor D had an 81 % COD recovery. It is unclear as to why the COD recoveries were far from 100 %, implicating that there may have been unidentified product formation within the fermentation.

4.4 Reaction Stoichiometry and Kinetics.

From the biomass yields in Equation 7, it seems that the productivity is the same with a biomass yield of 9 % at pH 4.0 and at pH 5.5. This suggests that reactors C and D were equally productive at converting carbohydrates to products rather than to biomass. However, a significant amount of the carbohydrate fed to Reactor C remained unconverted, indicating a reduced reaction rate at low pH.

$$\text{Observed Biomass Yield, Y} = \frac{\text{g COD biomass produced}}{\text{g COD removed}} \; or \; \frac{\text{g COD biomass}}{\text{g COD removed}}$$

$$reactorC/_{pH\,4.0} = \frac{323\text{mg VSS COD/L}}{3595\text{mg COD/L}} = 0.09 \qquad \text{Equation 7}$$

$$reactorD/_{pH\,5.5} = \frac{412\text{mg VSS COD/L}}{4720\text{mg COD/L}} = 0.09$$

At pH 4.0 significant levels of unreacted glucose were being washed out suggesting the cells were slower growing than at pH 5.5. Hence, at pH 4.0 it is likely that the cell

growth rate was close to 2 d^{-1} and the SRT was near the minimum required to sustain the biomass as shown in Equation 8.

$$\mu = \frac{\text{mg new cells}}{\text{mg cells} \times \text{day}} = \frac{1}{\text{SRT}} = \frac{1}{12\ \text{hours}} = \frac{1}{0.5\ \text{days}} = 2d^{-1} \qquad \text{Equation 8}$$

Hence a shorter SRT than 12 hours would likely washout the cells.

Table 15. Products expressed as yields (mg COD as product/mg COD from glucose) for reactors A, C and D.

Reactor	% glucose lost to effluent	VFA yield *	VSS yield *	CH_4 yield *	Ethanol yield *	Hydrogen yield *
A: pH 5.5, COD:N ratio of 53 (Control)	0.004	0.61	0.19	0	0.02	0.05
C: pH 4.0, Nitrogen deficient	0.26	0.47	0.09	0	0.08	0.02
D: pH 5.5, Nitrogen deficient	0.01	0.56	0.09	0	0.08	0.04

* The units of yield for these products were $mgCOD/mgCOD_{converted}$

Table 15 shows product yields from the various reactors, including the control reactor A discussed in the previous chapter. It can be seen that reactor C lost far more glucose to effluent than did reactor D. Reactors C and D produced equal amounts of solids and ethanol in terms of yields, but reactor D yielded more VFAs and hydrogen than reactor C. Hence, it seems that reactor D at pH 5.5 performed better than reactor C at pH 4.0, consistent with earlier work which found that pH 5.5 is the optimal pH for CSTR reactor production (Inanc *et al.*, 1996; Fang and Liu, 2002).

These results suggest that it is possible to run a nitrogen deficient reactor at pH 4.0, but that a longer SRT than 12 hours may be desirable so as not to lose glucose in the effluent as waste. According to Zhang et al. (2006), SRT is thought to be helpful in selecting for certain microbes, with fewer species being found at shorter SRTs. Cha and Noike (1997) found that substrate degradation was greater at longer SRTs, suggesting this may be helpful under the conditions here. Hence adjusting some of the environmental reactor conditions at pH 4.0 may help to run the reactor more efficiently.

When comparing reactors A, C and D it is obvious that the glucose was taken up best under reactor A conditions at a low COD:N ratio of 53. It is apparent that the microbial cell growth rate was faster than 2 d^{-1}. It is also clear that the yield for VFAs, VSS, and hydrogen were greatest for reactor A, suggesting that pH 5.5 and a low COD:N ratio were better conditions for anaerobic digestion than were pH 4.0 or pH 5.5 run under nitrogen deficient conditions.

However, ethanol yields were elevated under nitrogen deficient conditions, and there appears to be an unidentified product being formed under these conditions, implicated by the lack of closure of the COD and carbon balances under the low N environment.

4.5 Summary.

pH appears to have a large effect on the type of bacteria present and what percent nitrogen content the microbes have in their cells. As the pH was found to affect the biomass present, it was expected that the products would also differ at different pHs. A similar biomass yield was found at pH 4.0 and at pH 5.5. The total VFA production yield was much greater at pH 5.5 than at pH 4.0, reflecting incomplete substrate removal at this lower pH. Acetic acid was the dominant VFA at pH 4.0 and butyric acid dominated at pH 5.5. Propionic acid and valeric acid production were both similarly low at both pHs. Hydrogen production was greater at pH 5.5 than at pH 4.0 predictable from the higher butyric to acetic acid ratio at the higher pH. No methane was produced at either pH under nitrogen deficient conditions. There was similar ethanol production at pH 4.0 and pH 5.5.

While biomass yield was unaffected by the pH, the reactor operated at pH 4 lost a large portion of glucose to effluent at the low pH. Thus it is concluded that the maximal growth rate of the microbial population at this pH was near the dilution rate of $2d^{-1}$. However, when comparing the products produced at the two pH levels, it seems that the reactor at pH 5.5 produced more useful products including VFAs, lactate and hydrogen. Clearly, it is possible to keep a reactor alive at pH 4.0, but overall reactor productivity is better at pH 5.5 under nitrogen deficient conditions.

5. Conclusions

This study has shown that nitrogen fixation can take place under nitrogen deficient conditions via acidogenic fermentation. This suggests that there is likely to be a bacterial shift from a mixed culture when there is sufficient nitrogen to a more select group of nitrogen fixing microorganisms as nitrogen becomes limited and then insufficient. It was found that a mixed bacterial culture can withstand and adapt to a gradual decrease in available nitrogen and an increase in COD:N ratio.

As the COD:N ratio is increased, ethanol and lactate production were found to increase while VFA and carbon dioxide production remained at steady levels. The amount of hydrogen produced is thought to decrease as COD:N ratio increases but further study is required in this area as methanogen growth may have disrupted these findings. Overall volatile fatty acids were the dominant products followed by biomass production. This suggests that most of the energy input was consumed in biochemical pathways involving volatile fatty acid production, with much less going towards gases and ethanol.

pH was found to have a significant effect on overall acidogenic reactor productivity under nitrogen deficient conditions, though reactors survived at both pH 4.0 and pH 5.5. More VFAs and hydrogen were produced at pH 5.5 than at pH 4.0 and the pH 4.0 reactor was found to have incomplete substrate removal suggesting it was unable to cope well with the amount of carbohydrate input and hence would be a wasteful system long-term.

Reactors run at pH 4.0 or at pH 5.5 run under nitrogen deficient conditions were not as productive in terms of VFA or gas production as compared to the control reactor run at pH 5.5 with a low COD:N ratio of 53. The findings in this study agree with previous findings that running reactors at pH 5.5 with a COD:N ratio around 50 are optimum conditions for hydrogen production. However, it has been shown here that it is possible to operate nitrogen deficient reactors at pH 4.0 and pH 5.5, though productivity is limited at pH 4.0. It is important to consider what the desired products

are in order to determine what operating conditions are optimal when running CSTRs under acidogenic conditions.

6. Further Considerations and Recommendations

Having determined that nitrogen fixation is possible under nitrogen deficient conditions, there are some further details to be investigated. It would be interesting to know what bacterial populations are responsible for the nitrogen fixation under nitrogen deficient conditions and also how the bacterial population changed from a mixed culture at low COD:N ratios to a more specified nitrogen fixing population as the COD:N ratio increased. Detailed microbiological study would be helpful in determining what species were present under what conditions. Perhaps the bacterial cultures would be found to shift as the environmental conditions change, for example from acetogenic bacteria dominating under sufficient nitrogen conditions or nitrogen fixers dominating under nitrogen deficient conditions. Knowing the microbial species present at certain time periods could allow water treatment plants to choose specific treatment conditions in order to maximize the production of desired products.

The determination of what biochemical pathways were involved in the production of the various products such as VFAs and hydrogen could be very useful in helping to inhibit unwanted pathways or products or in enhancing certain pathways. This could be done by labeling certain chemicals and tracing their pathways in the system.

As the COD balance did not close under the nitrogen deficient conditions, it would be interesting to do further study on complete product analysis to determine if other products were being made other than the ones measured in this study. Further studies on COD:N ratio could be performed in order to look more closely at what happens as the COD:N ratio increases from 100 upwards until nitrogen is no longer available. This may clarify what happens at high COD:N ratios.

There were several limitations in this study. It was realized that ensuring good mixing is very important in order to prevent solids building up on the vessel walls and leading to a lengthened SRT. In this study, the GC did not calibrate well for carbon dioxide making the carbon dioxide results over-estimates. Lastly, the acetylene-reduction test

was performed several times without getting any results. It is unclear as to why this was but in future the method should be clarified to try to prevent getting negative results.

7. Literature Cited

Aguilar, A., Casas, C., and J.M. Lema. 1995. Degradation of volatile fatty acids by differently enriched methanogenic cultures: Kinetics and inhibition. *Water Research* **29**(2): 505-509.

Ahring, B. K., Ibrahim, A.A., and Z. Mladenovska. 2001. Effect of temperature increase from 55 to 65 degrees C on performance and microbial population dynamics of an anaerobic reactor treating cattle manure. *Water Research* **35**(10): 2446-2452.

Ammary, B.Y. 2004. Nutrients requirements in biological industrial wastewater treatment. *African Journal of Biotechnology* **3**(4): 236-238.

Annous, B. A., Shieh, J.-S., Shen, G.-J., Jain, M.K., and J.G. Zeikus. 1996. Regulation of hydrogen metabolism in Butyribacterium methylotrophicum by substrate and pH. *Applied Microbiology and Biotechnology* **45**(6): 804-810.

APHA-AWWA-WPCF. 1998. Edited by Clesceri, L.S., Greenberg, A.E., and A.D. Eaton. Standard Methods for the Examination of Water and Wastewater (20^{th} edition) Washington, DC.

Arp, D.J. 1990. Edited by P.M. Gresshoff, Roth, L.E., Stacey, G., and W.E. Newton. H2 cycling in N2 fixation: Past, present, and future outlook, in Nitrogen Fixation: Achievements and Objectives, Proceedings of the 8th International Congress on Nitrogen Fixation. New York: Chapman and Hall.

Banerjee, A., Elefsiniotis, P., and D. Tuhtar. 1998. Effect of HRT and temperature on the acidogenesis of municipal primary sludge and industrial wastewater. *Water Science and Technology, **38***(8-9): 417-423.

Barnett, J. W. 1984. Anaerobic filtration of waste waters arising from the production of baker's yeast: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology at Massey University, Palmerston North, New Zealand.

Beccari, M., Bonemazzi, F., Majone, M., and C. Riccardi. 1996. Interaction between acidogenesis and methanogenesis in the anaerobic treatment of olive oil mill effluents. *Water Research* **30**(1): 183-189.

Blanch, H. W., and D.S. Clark. 1996. Biochemical engineering. New York: Marcel Dekker Inc.

Boone, D.R. 1982. Terminal reactions in the anaerobic digestion of animal waste. *Applied and Environmental Microbiology* **43**(1): 57-64.

Brandberg, T., Gustafsson, L., and C.J. Franzén. 2007. The impact of severe nitrogen limitation and microaerobic conditions on extended continuous cultivations of *Saccharomyces cerevisiae* with cell recirculation. *Enzyme and microbial technology* **40**: 585-593.

Brown, R. C. 2003. Biorenewable Resources: Engineering New Products from Agriculture. Ames, Iowa: Iowa State Press.

Bruce, M. E., and T.A. Clark. 1994. Klebsiella and nitrogen-fixation in pulp and paper-mill effluents and treatment systems. *Appita Journal* **47**(3): 231-237.

Burris, R.H. 1971. Fixation by Free-Living Micro-organisms: Enzymology, in The Chemistry and Biochemistry of Nitrogen Fixation. Edited by J.R. Postgate. New York: Plenum Press.

Bushell, M. E., and J.H. Slater. 1981. Mixed Culture Fermentations. Sydney: Academic Press.

Cha, G. C. and T. Noike. 1997. Effect of rapid temperature change and HRT on anaerobic acidogenesis. *Water Science and Technology* **36**(6-7): 247-253.

Cheong, D.-Y. and C.L. Hansen. 2006. Acidogenesis characteristics of natural, mixed anaerobes converting carbohydrate-rich synthetic wastewater to hydrogen. *Process Biochemistry* **41**: 1736-1745.

Cheong, D.-Y., Hansen, C.L., and D.K. Stevens. 2006. Production of Bio-hydrogen by mesophilic anaerobic fermentation in an acid-phase sequencing batch reactor. Logan, Utah: Utah State University.

Datar, R.P., Shenkman, R.M., Cateni, B.G., Huhnke, R.L., and R.S. Lewis. 2004. Fermentation of biomass-generated producer gas to ethanol. *Biotechnology and Bioengineering* **86**(5): 587-594.

Demirel, B., and O. Yenigün. 2002. Review Two-phase anaerobic digestion processes: a review. *Journal of Chemical Technology & Biotechnology* **77**: 743-755.

Demirel, B., and O. Yenigün. 2004. Anaerobic acidogenesis of dairy wastewater: the effects of variations in hydraulic retention time with no pH control. *Journal of Chemical Technology & Biotechnology* **79**: 755-760.

Droste, R.L. 1997. Theory and Practice of Water and Wastewater Treatment. New York: John Wiley and Sons, Inc.

Elefsiniotis, P., and W.K. Oldham. 1994. Effect of HRT on acidogenic digestion of primary sludge. *Journal of Environmental Engineering-ASCE* **120**(3): 645-660.

Elefsiniotis, P., Wareham, D.G., and M.O. Smith. 2004. Use of volatile fatty acids from an acid-phase digester for denitrification. *Journal of Biotechnology* **114**(3): 289-297.

Erickson, E., and D. Y.-C. Fung. 1988. Handbook on Anaerobic Fermentations. New York: Marcel Dekker.

Fang, H. H. P., and H. Liu. 2002. Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresource Technology* **82**(1): 87-93.

Fothergill, S., and D.S. Mavinic. 2000. VFA production in thermophilic aerobic digestion of municipal sludges. *Journal of Environmental Engineering-ASCE* **126**(5): 389-396.

Gauthier, F., Neufeld, J.D., Driscoll, B.T., and F.S. Archibald. 2000. Coliform bacteria and nitrogen fixation in pulp and paper mill effluent treatment systems. *Applied and Environmental Microbiology* **66**(12): 5155-5160.

Gerardi, M. H. 2002. Nitrification and denitrification in the activated sludge process. New York: John Wiley & Sons, Inc.

Girovich, M. J. 1996. Biosolids treatment and management: processes for beneficial use. New York: Marcel Dekker.

Hafner, S. 2006. Biological hydrogen production from nitrogen-deficient substrates. *Biotechnology and Bioengineering* **97**(2): 435-437.

Hansel, A., and P. Lindblad. 1998. Towards optimization of cyanobacteria as biotechnologically relevant producers of molecular hydrogen, a clean and renewable energy source. *Applied Microbiology and Biotechnology* **50**: 153-160.

Hawkes, F. R., Dinsdale, R., D.L. Hawkes, and I. Hussy. 2002. Sustainable fermentative hydrogen production: challenges for process optimization. *International Journal of Hydrogen Energy* **27**: 1339-1347.

Hearn, C. R. 1994. Anaerobic treatment of thermo-mechanical pulp mill waste water: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Process and Environmental Technology at Massey University, Palmerston North, New Zealand.

Hitte, S. J. 1975. Anaerobic digestion of solid waste and sewage sludge to methane. Washington: Environmental Protection Agency.

Hoa, P.T., Nair, L., and C. Visvanathan. 2003. The effect of nutrients on extracellular polymeric substance production and its influence on sludge properties. *Water SA* **29**(4): 437-442.

Hutnan, M., Drtil, M., and L. Mrafkova. 2000. Anaerobic biodegradation of sugar beet pulp. *Biodegradation* **11**: 203-211.

Hwang, M.H., Jang, N.J., Hyun, S.H., and I.S. Kim. 2004. Anaerobic bio-hydrogen production from ethanol fermentation: the role of pH. *Journal of Biotechnology* **111**(3): 297-309.

Inanc, B., Matsui, S., and S. Ide. 1996. Propionic acid accumulation and controlling factors in anaerobic treatment of carbohydrate: Effects of H-2 and pH. *Water Science and Technology* **34**(5-6): 317-325.

Kapdan, I.K. and F.Kargi. 2006. Bio-hydrogen production from waste materials. *Enzyme and microbial technology* **38**: 569-582.

Kargi, F., and S. Özmihçi. 2002. Improved biological treatment of nitrogen-deficient wastewater by incorporation of N2-fixing bacteria. *Biotechnology Letters* **24**: 1281-1284.

Kessler, P.S., Blank, C., and J.A. Leigh. 1998. The nif gene operon of the methanogenic archaeon *Methanococus maripaludis. Journal of Bacteriology* **180**: 1504-1511.

Khan, A. W., and T.M. Trottier. 1978. Effect of Sulfur-Containing Compounds on Anaerobic Degradation of Cellulose to Methane by Mixed Cultures Obtained from Sewage Sludge. *Applied and Environmental Microbiology* **35**(6): 1027-1034.

Khanal, S.K., Chen, W.-H., Li, L., and S. Sung. 2004. Biological hydrogen production: effects of pH and intermediate products. *International Journal of Hydrogen Energy* **29**: 1123-1131.

1/ Kim, S. H., Han, S.K., and H.S. Shin. 2004. Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. *International Journal of Hydrogen Energy* **29**: 1607-1616.

2/ Kim, I.S., Hwang, M.H., Jang, N.J., Hyun, S.H., and S.T. Lee. 2004. Effect of low pH on the activity of hydrogen utilizing methanogen in bio-hydrogen process. *International Journal of Hydrogen Energy* **29**: 1133-1140.

Kim, J.O., Kim, Y.H., Yeom, S.Y. Song, B.K., and I.H. Kim. 2006. Enhancing continuous hydrogen gas production by the addition of nitrate into an anaerobic reactor. *Process Biochemistry* **41**(5): 1208-1212.

Kisaalita, W. S., Pinder, K.L., and K.V. Lo. 2004. Acidogenic fermentation of lactose. *Biotechnology and Bioengineering* **30**(1): 88-95.

Klass, D. L. 1998. Biomass for Renewable Energy, Fuels, and Chemicals. Sydney: Academic Press.

Kraemer, J.T., and D.M. Bagley. 2005. Continuous fermentative hydrogen production using a two-phase reactor system with recycle. *Environmental Science & Technology* **39**(10): 3819-3825.

Kyazze, G., Martinez-Perez, N., Dinsdale, R., Premier, G.C., Hawkes, F.R., Guwy, A.J., and D.L. Hawkes. 2006. Influence of substrate concentration on the

stability and yield of continuous biohydrogen production. *Biotechnology and Bioengineering* **93**(5): 971-979.

Larsson, C., Nilsson, A., Blomberg, A., and L. Gustafsson. 1997. Glycolytic flux is conditionally correlated with ATP concentration in *Saccharomyces cerevisiae:* a chemostat study under carbon- or nitrogen-limiting conditions. *Journal of Bacteriology* **179**(23): 7243-7250.

Lin, C.-Y. 1993. Effect of heavy-metals on acidogenesis in anaerobic-digestion. *Water Research* **27**(1): 147-152.

Lin, C.-Y., and R.-C. Chang. 1999. Hydrogen production during the anaerobic acidogenic conversion of glucose. *Journal of Chemical Technology & Biotechnology* **74**: 498-500.

1/ Lin, C.-Y., and C.H. Lay. 2004. Carbon/nitrogen ratio effect on fermentative hydrogen production by mixed microflora. *International Journal of Hydrogen Energy* **29**: 41-45.

2/ Lin, C.-Y., and C.H. Lay. 2004. Effects of carbonate and phosphate concentrations on hydrogen production using anaerobic sewage sludge microflora. *International Journal of Hydrogen Energy* **29**: 275-281.

Lin, C.-Y., Hung, C.-H., Chen, C.-H., Chung, W.-T., and L.-H. Cheng. 2006. Effects of initial cultivation pH on fermentative hydrogen production from xylose using natural mixed cultures. *Process Biochemistry* **41**(6): 1383-1390.

Logan, B. E., Oh, S.-E., Kim, I.S., and S.V. Ginkel. 2002. Biological hydrogen production measured in batch anaerobic respirometers. *Environmental Science & Technology* **36**(11): 2530-2535.

Maharaj, I., and P. Elefsiniotis. 2001. The role of HRT and low temperature on the acid-phase anaerobic digestion of municipal and industrial wastewaters. *Bioresource Technology* **76**(3): 191-197.

Maillacheruvu, K. Y., and G.F. Parkin. 1996. Kinetics of growth, substrate utilization and sulfide toxicity for propionate, acetate and hydrogen utilizers in anaerobic systems. *Water Environment Research* **68**(7): 1099-1106.

Marasabessy, A. 1998. Hydrolysis and ethanolic fermentation of cassava starch in a two-stage continuous bioreactor: a thesis presented in partial fulfilment of the requirements for a diploma in Biotechnology at Massey University, Palmerston North, New Zealand.

Marchaim, U., and C. Krause. 1993. Weak acid bases and pH control in anaerobic systems - A review. *water SA* **19**(1): 1-10.

McSweeney, C. S., and G.J. McCrabb. 2002. Inhibition of rumen methanogenesis and its effect on feed intake, digestion, and animal production. Greenhouse Gases and Animal Agriculture: Proceedings of the 1st International Conference on Greenhouse Gases and Animal Agriculture. Gatton, Australia: Elsevier.

Metcalf and Eddy. 2003. Wastewater Engineering: Treatment and Reuse. Sydney: McGray Hill.

Minamisawa, K., Nishioka, K., Miyaki, T., Ye, B., Miyamoto, T., You, M., Saito, A., Saito, M., Barraquio, W.L., Teaumroong, N., Sein, T., and T. Sato. 2004. Anaerobic Nitrogen-Fixing Consortia Consisting of Clostridia Isolated from Gramineous Plants. *Applied and Environmental Microbiology* **70**(5): 3096-3102.

Mitsumori, M., Tajima, K., and H. Itabashi. 2002. Edited by J. Takahashi and B.A. Young. Detection of methanogens from the rumen by PCR-based techniques, in Greenhouse Gases and Animal Agriculture: Proceedings of the 1st

International Conference on Greenhouse Gases and Animal Agriculture. p. 125-128. Gatton, Australia: Elsevier.

1/ Mu, Y., Yu, H.-Q., and Y. Wang. 2006. The role of pH in the fermentative H2 production from an acidogenic granule-based reactor. *Chemosphere* **64**: 350-358.

2/ Mu, Y., Wang, G., and H.-Q. Yu. 2006. Response surface methodological analysis on biohydrogen production by enriched anaerobic cultures. *Enzyme and microbial technology* **38**: 905-913.

Najafpour, G., and H. Younesi. 2006. Ethanol and acetate synthesis from waste gas using batch culture of Clostridium ljungdahlii. *Enzyme and microbial technology* **38**(1-2): 223-228.

Nakamura, M., Kanbe, H., and J.I. Matsumoto. 1993. Fundamental studies on hydrogen production in the acid-forming phase and its bacteria in anaerobic treatment processes- The effects of solids retention time. *Water Science and Technology* **28**(7): 81-88.

Niessen, J., Schröeder, U., Harnisch, F., and F. Scholz. 2005. Gaining electricity from *in situ* oxidation of hydrogen produced by fermentative cellulose degradation. *Letters in Applied Microbiology* **41**(3): 286-290.

O'connor, O.A., and L.Y. Young. 1993. Effect of nitrogen limitation on the biodegradability and toxicity of nitrophenol and aminophenol isomers to methanogenesis. *Archives of Environmental Contamination and Toxicology* **25**(2): 285-291.

Oh, S.-E., Van Ginkel, S., and B.E. Logan. 2003. The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. *Environmental Science & Technology* **37**(22): 5186-5190.

Parawira, W., Murto, M., Read, J.S., and B. Mattiasson. 2004. Volatile fatty acid production during anaerobic mesophilic digestion of solid potato waste. *Journal of Chemical Technology & Biotechnology* **79**(7): 673-677.

Park, W., Hyun, S.H., Oh, S.-E., Logan, B.E., and I.S. Kim. 2005. Removal of headspace CO2 increases biological hydrogen production. *Environmental Science & Technology* **39**(12): 4416-4420.

Penaud, V., Delgenes, J.P., Torrijos, M., Moletta, R., Vanhoutte, B., and P. Cans. 1997. Definition of optimal conditions for the hydrolysis and acidogenesis of a pharmaceutical microbial biomass. *Process Biochemistry* **32**(6): 515-521.

Pometto III, A. L., and D.L. Crawford. 1986. Effects of pH on Lignin and Cellulose Degradation by *Streptomyces viridosporus*. *Applied and Environmental Microbiology* **52**(2): 246-250.

Punal, A., Trevisan, M., Rozzi, A., and J.M. Lema. 2000. Influence of C:N ratio on the start-up of up-flow anaerobic filter reactors. *Water Research* **34**(9): 2614-2619.

Rahmat, H. 1991. Microbiological solid state fermentation of apple pomace by yeast and fungus: a thesis presented in partial fulfilment of the requirements for the master's degree in Biotechnology at Massey University, Palmerston North, New Zealand .

Reid, I. D. 1983. Effects of Nitrogen Sources on Cellulose and Synthetic Lignin Degradation by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **45**(3): 838-842.

Ren, N., Wang, B., and J-C. Huang. 1997. Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor. *Biotechnology and Bioengineering* **54**(5): 428-433.

Rodríguez, J., Kleerebezem, R., Lema, J.M., and M.C.M. van Loosdrecht. 2006. Modeling product formation in anaerobic mixed culture fermentations. *Biotechnology and Bioengineering* **93**(3): 592-606.

Rogers, G. M., Jackson, S.A., Shelver, G.D., and A.A.W. Baecker. 1992. Anaerobic degradation of lignocellulosic substrates by a 1,4-ß-Xylanolytic *Clostridium* species novum. *International Biodeterioration and Biodegradation* **29**: 3-17.

Ruan, W., Chen, J. and S. Lun. 2003. Production of biodegradable polymer by A. *eutrophus* using volatile fatty acids from acidified wastewater. *Process Biotechnology* **39**(3): 295-299.

Ruiz, G., Jeison, D., and R. Chamy. 2006. Development of denitrifying and methanogenic activities in USB reactors for the treatment of wastewater: Effect of COD/N ratio. *Process Biochemistry* **41**: 1338-1342.

Saha, B. C., and J. Woodward. 1997. Fuels and Chemicals from Biomass. Washington, D.C.: American Chemical Society.

Schwarz, W. H. 2001. The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology* **56**(5-6): 634-649.

She, Z., Gao, M., Jin, C., Chen, Y., and J. Yu. 2005. Toxicity and biodegradation of 2,4-dinitrophenol and 3-nitrophenol in anaerobic systems. *Process Biochemistry* **40**(9): 3017-3024.

Sheng, G.-P., and H.-Q. Yu. 2007. Formation of extracellular polymeric substances from acidogenic sludge in H_2-producing process. *Applied Microbiology and Biotechnology* **74**: 208-214.

Shin, H.-S., and J.-H. Youn. 2005. Conversion of food waste into hydrogen by thermophilic acidogenesis. *Biodegradation* **16**: p. 33-44.

Silvester, W.B., and D.R. Musgrave. 1991. Free-Living Diazotrophs, in Biology and Biochemistry of Nitrogen Fixation. p. 162-186. New York: Elsevier.

Stanbury, P. F., and A. Whitaker. 1984. Principles of Fermentation Technology. Sydney: Pergamon Press.

Taguchi, F., Yamada, K., Hasegawa, K., Taki-Saito, T., and K. Hara. 1996. Continuous hydrogen production by *Clostridium* sp. strain no. 2 from cellulose hydrolysate in an aqueous two-phase system. *Journal of Fermentation and Bioengineering* **82**: 80-83.

Tao, Y., Chen, Y., Wu, Y., He, Y., and Z. Zhou. 2007. High hydrogen yield from a two-step process of dark- and photo-fermentation of sucrose. *International Journal of Hydrogen Energy* **32**: 200-206.

Toerien, D. F., Siebert, M.L., and W.H.J. Hattingh. 1967. The bacterial nature of the acid-forming phase of anaerobic digestion. *Water Research* **1**: 497-507.

Ueno, Y., Otsuak, S., and M. Morimoto. 1996. Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. *Journal of Fermentation and Bioengineering* **82**(2): 194-197.

Ueno, Y., Haruta, S., Ishii, M., and Y. Igarashi. 2001. Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. *Applied Microbiology and Biotechnology* **57**: 555-562.

Valdez-Vazquez, I., Sparling, R., Risbey, D., Rinderknecht-Seijas, N., and H.M. Poggi-Varaldo. 2005. Hydrogen generation via anaerobic fermentation of paper mill wastes. *Bioresource Technology* **96**(17): 1907-1913.

van Andel, J. G., and A.M. Breure. 1984. Anaerobic waste water treatment. *Trends in Biotechnology* **2**(1): 16-20.

Van Ginkel, S.W. and B. E. Logan. 2005. Increased biological hydrogen production with reduced organic loading. *Water Research* **39**(16): 3819-3826.

Vavilin, V. A., Rytow, S.V., and L.Y. Lokshina. 1995. Modelling hydrogen partial pressure change as a result of competition between the butyric and propionic groups of acidogenic bacteria. *Bioresource Technology* **54**: 171-177.

Veziroglu, T. N., and F. Barbir. 1992. Hydrogen-The wonder fuel. *International Journal of Hydrogen Energy* **17**(6): 391-404.

von Munch, E., and P.F. Greenfield. 1998. Estimating VFA concentrations in prefermenters by measuring pH. *Water Research* **32**(8): 2431-2441.

Vorobjeva, N.V. 2005. Selective stimulation of the growth of anaerobic microflora in the human intestinal tract by electrolyzed reducing water. *Medical hypotheses* **64**(3): 543-546.

Wang, Y. T., Gabbard, H.D., and P.C. Pai. 1991. Inhibition of acetic acid methanogenesis by phenols. *Journal of Environmental Engineering-ASCE* **117**(4): 487-500.

Wang, G., Mu, Y. and H.Q. Yu. 2005. Response surface analysis to evaluate the influence of pH, temperature and substrate concentration on the acidogenesis of sucrose-rich wastewater. *Biochemical Engineering Journal* **23**(2): 175-184.

Wang, L., Zhou, Q. and F.T. Li. 2006. Avoiding propionic acid accumulation in the anaerobic process for biohydrogen production. *Biomass and Bioenergy* **30**(2): 177-182.

Wise, D. L. 1984. Bioconversion Systems. Boca Raton, Florida: CRC Press Inc.

Yang, K., Oh, C., and S. Hwang. 2004. Optimizing volatile fatty acid production in partial acidogenesis of swine wastewater. *Water Science and Technology* **50**(8): 169-176.

Yenigun, O., Koksal, G., and G. Yilmazer. 1995. Nickel and cadmium inhibition in acidogenesis during anaerobic digestion. *Journal of Environmental Science and Health Part A- Toxic/Hazardous Substances & Environmental Engineering* **30**(5): 981-987.

Yu, H.-Q., and H.H.P. Fang. 2000. Thermophilic acidification of dairy wastewater. *Applied Microbiology and Biotechnology* **54**(3): 439-444.

1/ Yu, Y., Hansen, C.L., and S. Hwang. 2002. Biokinetics in acidogenesis of highly suspended organic wastewater by adenosine 5' triphosphate analysis. *Biotechnology and Bioengineering* **78**(2): 147-156.

Yu, H.-Q., and Y. Mu. 2006. Biological hydrogen production in a UASB reactor with granules. II: Reactor performance in 3-year operation. *Biotechnology and Bioengineering* **94**(5): 988-995.

Zeikus, J. G. 1979. Thermophilic bacteria: Ecology, physiology and technology. *Enzyme and microbial technology* **1**(4): 243-252.

Zhang, Z.-P., Show, K.-Y., Tay, J.-H., Liang, D.T., Lee, D.-J., and W.-J. Jiang. 2006. Effect of hydraulic retention time on biohydrogen production and anaerobic microbial community. *Process Biochemistry* **41**(10): 2118-2123.

Zhang, Z.Y., Jin, B., and J.M. Kelly. 2007. Production of lactic acid and byproducts from waste potato starch by *Rhizopus arrhizus*: role of nitrogen sources. *World Journal of Microbiology and Biotechnology* **23**: 229-236.

Zheng, X.-J., and H.-Q. Yu. 2005. Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. *Journal of Environmental Management* **74:** 65-70.

1/Zoetmeyer, R. J., Arnoldy, P., Cohen, A., and C. Boelhouwer. 1982. Influence of temperature on the anaerobic acidification of glucose in a mixed culture forming part of a two-stage digestion process. *Water Research* **16**: 313-321.

2/Zoetmeyer, R. J., Van den Heuvel, J.C., and A. Cohen. 1982. pH influence on acidogenic dissimilation of glucose in an anaerobic digester. *Water Research* **16**: 303-311.

Zyabreva, N.V., Isakova, E.P., and V.V. Biryukov. 2001. Conditions for ethanol production during bioconversion of cellulose-containing raw material. *Applied Biochemistry and Biotechnology* **37**(5): 566-572.