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A Study of Fatty Acid Production by Clostridium butyricum

By

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Special to my love;

Shariah Bt Shariff

Tajarudin Bin Ahmad

Dayang Hazelinna Bt Abg Ali

Husnul Ariff Bin Tajarudin

Husnul Khuluk Bin Tajarudin

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Abstract

This thesis investigates the fatty acid production from carbohydrates using *C. butyricum*. In nature a common route for the anaerobic degradation of carbohydrate in the environment is via methanogenesis. At the heart of these processes however, is the metabolism of a diversity of carbohydrate materials that produce a few fatty acids (acetate and butyrate) which are then slowly converted to methane. In this context, fatty acids can be considered as a common end-product/intermediate from carbohydrate degradation that could be used to produce chemicals. Already, acetic and butyric acid are important feedstock chemicals in the pharmaceutical, food and industrial sectors and there is potential to expand this further. As a first step to investigate the conversion of waste carbohydrate to fatty acids for chemical production, *C. butyricum*, a strictly anaerobic bacterium, was investigated as a model system for the potential production of acetic and butyric acid. The production efficiency of *C. butyricum* relies on the type of substrate, production methodology, the strain and environmental conditions.

Pure cultures of *C. butyricum* were investigated for fatty acid production from carbohydrates. Initial studies involved medium optimization in test tube culture for high growth rate and maximum biomass production (OD_{max}). In this medium, glucose was selected as the main substrate together with yeast extract, KH_2PO_4 and $NH_4(SO)_4$. The studies were carried out in three types of pH controlled reactors; batch stirred tank (SRT), continuously stirred tank (CSTR) and membrane bioreactor (MBR) A comparison the fatty acid production kinetics and productivity in each reactor was undertaken and the effect of glucose concentration and where appropriate, glucose feed rates, were also investigated.

The results show that fatty acid production could be carried out in all three fermentation systems. A common observation in these systems was that fatty acid production was influenced by the glucose concentration in that at low glucose concentration the ratio of acetate to butyrate was about 30:1 while at higher concentrations the ratio was reduced to about 3:1 on a molar basis.

The detailed kinetic studies generated unique data for this organism and shows that the maintenance coefficient (m_s) increase with increasing glucose concentration (0.02 to 1.1 g substrate/g cell/h), due to mainly to end product inhibition and the true yield ($Y_{x/s}^{max}$) was around 0.2 for all glucose concentrations tested. Meanwhile substrate saturation (K_s) decreased with increasing glucose concentrations (2.06-6.41 g/L). This observation was atypical to that observed in other anaerobic fermentations by previous workers.

A comparison of fatty acid productivities using a 10g/l glucose feed in the 3 reactors for acetic acid were 0.95 g/l/h for STR, 4.41 g/l/h for CSTR and 37.88 g/l/h for MBR and for butyric acid 0.15 g/l/h for STR, 1.27 g/l/h for CSTR and 14.34 g/l/h MBR. Although, previous work in this area is limited the data obtained in this study was also compared with other published work and this suggests that the production of fatty acid, especially acetic and butyric acid in the MBR system is by far the most productive yet reported.

The results are discussed in the context of the waste treatment process for fatty acid production and its application to waste conversion and its further development.

CHAPTER 3: MATERIALS AND METHODS

CHAPTER 4: BATCH CULTURE SYSTEM

CHAPTER 5: CONTINUOUS CULTURE

Abbereviations and symbols

Abbreviations

%	Percentage
°C	Degree centigrade
C.butyricum	*Clostridium butyricum*
CTSR	Continuous tank system reactor
g	gram
GC	Gas chromatography
h	Hour
l	liter
MBR	Membrane bioreactor
mg	milligram
mol	mole
OD	Optical density at 660nm
OD^{max}	Optical density maximum at 660nm
STR	Stirrer tank reactor
UV	Ultra violet light
VFA	Volatile fatty acid
w/w	Concentration weight per weight

Symbols

μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})
D	Dilution rate (h^{-1})
F	Feeding rate/Flow rate (l/h)
K_S	Substrate saturation constant (g/L)
m_s	Maintenance coefficient (g sugar/g cell/h)
A	Area (m^2)
P	Pressure (psi)
q_s	Specific rate of sugar uptake (g sugar/g cell/h)
t_d	Doubling time (h)
V	Volume (L)
x	Cell concentration (g/L)
$Y_{x/s}$	Biomass yield (g cell/g sugar)
$Y_{a/s}$	Acetic yield (g acetic/g sugar)
$Y_{b/s}$	Butyric yield (g butyric/g sugar)
$Y_{x/s}^{max}$	Maximum biomass yield (g cell/g sugar)
S	Substrate concentration (g/L)
S_i	Inlet substrate concentration (g/L)
S_o	Initial substrate concentration (g/L)

Chapter 1: Introduction

1.1 Recycling technologies for organic waste

From an environmental viewpoint the term "organic waste" includes all waste that has one carbon-carbon bonding within the salt. According to Chongrak (1996), organic waste therefore includes human and animal waste, agro-industrial waste and a substantial proportion of municipal solid waste. In many situations organic waste can be considered as a resource and can be recycled into valuable products, chemicals and energy for use in industry agriculture, aquaculture or indirectly reused as fertilizer (Favoro et al., 2012). Generally, the bulk organic waste is easily biodegradable and can be processed in the presence of oxygen in composting or in the absence of oxygen though processes involved in anaerobic digestion. Both methods can be used to produce a soil conditioner, or if prepared correctly, can be used as a valuable source of nutrients for agriculture (Rivas and Arends, 1990; Rivero, 1995). Anaerobic digestion also produces a mixture of methane and carbon dioxide (biogas) as a potential important source of bio-energy (Lusk and Moser, 1996).

1.1.1 Technologies for organic recycling

The technology of organic recycling and organic waste processing is well established being developed over the past 100 years for both wastewater and solid wastes (Mottock,1984 and Monnet, 2003).

The processing of waste water usually involves several operations by which the wastes in water are oxidised to carbon dioxide and the sludge generated by these processes is digested to produce biogas. These processes however, are now considered energy intensive and new technology that reduces energy consumption in these processes is now being considered. For example, more effective and efficient

aeration processes and better integration of energy use within sewage works, are two methods being investigated.

The recycling of solid organic waste also has a long history and traditionally involved landfilling. This practice in the UK and Europe is now being reduced considerably as the environment at impact of these practices can have long term consequences to water supply and land use.

Solid waste handling processes are divided into 3 classes. First is mechanical/physical processing such as combustion or incineration. Second is chemical treatment and the third is biological treatment.

Mechanical processing was quite popular a few decades ago because it was, and remains, easy to implement and operate. In terms of recycling, wastes can be separated or fractionated into useful base materials such as glass, paper, metal and plastic that can then be reused. The remaining material are then burnt to recover the some of the embedded energy. However, because of its impact on the environment – notably its impact on air and water, it is losing popularity. The problem of incineration of these processes is the waste generated – notably ashes that contain heavy metals. These require special treatment for safe disposal, and, as a result, the operating costs are potentially very high.

The recycling of organic waste through chemical routes is potentially more interesting, though the use of chemicals makes this route expensive. Generally the waste is fractionated by physical methods into specific groups of materials and these are reprocessed using chemicals in multi-stage processes that result in a new material. Examples of this include recycling to produce glass, plastic, paper, fertilizer and fuel. This is also a relatively high cost recycling route.

The biological method is another option for the recycling of organic material. Even if quite complicated, it can potentially be quite cheap and safe for the environment. This method can produce a variety of products from organic waste, such as platform chemicals, liquid fuels or energy, new materials such as plastic,

nutrients and feed. Therefore the biological mediated process of recycling organic waste is a powerful method of waste treatment. Normally, biological methods use micro-organisms such as bacteria or algae. The nature of the biological process is that the process provides a diverse set of metabolic pathways by which organic materials can be degraded or converted into useful products. As biological processes are generally autocatalytic in nature, they require very little resources to achieve good conversions. The main consideration in these systems is to maintain environmental conditions conducive to biological activity. Aerobic systems therefore require continuous aeration to ensure that all of the microorganisms can survive, so carrying out complete oxidation of the waste. Thus a major energy cost is incurred when supplying the aeration requirements of such systems. In contrast anaerobic degradation, although relatively slower than aerated systems, requires lower energy and offers the potential for energy and materials recovery in the form of methane (Richardson & Peacock, 1994).

Organic recycling brings a lot of benefits to the environment, community and country. Recycling reduces the burden required for treatment while utilising these wastes as materials, fertilizers and energy, so improving the general economic situation while bringing both environmental and cost benefits.

1.2 Low carbon technologies

Nowadays, due to climate change, the adoption of low carbon technologies is seen to be the best strategy to create a sustainable environment. Most low carbon technologies focus on the production of energy because the bulk of fuel or energy production is based on the combustion of fossil carbon with high levels of carbon emissions as carbon dioxide.

Low carbon technologies are usually divided into two categories. The first comes from environmental or renewable energy while the second is to recycle waste or re-use organic materials without the production of carbon dioxide.

Many methods, are, and have been, used to produce energy from the environment through low carbon technologies. One group of these collect energy from natural sources such as the wind and the sun. In the case of wind generation technologies, suitable sites require steady and strong wind, and these are usually found near coastlines or in hilly areas. Construction and installation of equipment for generation of wind power is quite expensive, but the continuous production of energy from this system is very reliable (given suitable locations for the turbines). Solar technology produces energy from sunshine. Many solar energy production technologies have been documented, and these include solar photovoltaic and solar thermal electricity generation (Houses of Commons Energy and Climate Change, 2010).

Other low carbon technologies capture energy from the environment, and these include geothermal power and hydro. Geothermal technology generates power by tapping the Earth's deep-underground heat energy. Hydro-electricity is generated from waterfalls, rivers and streams (Esposito & Augustine, 2012).

The use of organic recycling techniques to generate useful energy can also be considered a low carbon technology. There is a huge amount of research in this field and this reflects its great potential. Interestingly, many researchers are now studying how to maximize and diversify these means of production (Houses of Commons Energy and Climate Change, 2010).

Biomass technology is a good example. Biomass is typically vegetable materials grown specifically for its energy content and includes wood, grasses and other plant materials. The most common use of biomass is the use of wood as fuel and has been employed for a long time in countries throughout the world. However, lately this technology has become increasingly more attractive because it is safe and the processes are capable of high throughput. The production of energy from biomass requires burning, gasifying and anaerobic digestion of feedstocks like wood, crop waste, municipal solid waste, and industrial organic waste. Biofuels, ethanol and

biodiesel can also be considered a technology for recycling waste, even though in many cases it does not consume waste, rather using plants or microorganisms as the method of production. For example, in the USA, ethanol is produced from sugarcane, whereas, traditionally, ethanol has been produced by the petroleum industry. Brazilian sugarcane derived ethanol is already competitive at today's petroleum prices (Adele et al., 2012). Nevertheless, bio fuel from food or plants will experience difficulties with organisations concerned with world food issues. Another example is the development of other second-generation bio fuels including cellulosic and algal variants. This "not derived from food" bio fuel is already competitive with oil, coal and natural gas (Pienkos et al., 2011 and Pienkos & Zhang., 2009).

This study will consider extending these concepts of biomass utilisation technology by investigating anaerobic fermentations as its focus. The thesis will consider the use of anaerobic technology to produce carbon materials that can be used in chemical synthesis. Although potentially a complex process, it could provide a good source of materials while avoiding release of carbon dioxide to the environment.

1.3 Anaerobic digestion

The first biogas plants which employed anaerobic technology, and have been documented, were established in Bombay (India) in 1859. Biogas generators or digesters are operating throughout Asia. In India alone more than 100,000 have been reported. In Korea 30,000 biogas generators were reported and there are several million in China. The Middle East, Africa, Oceania, Europe and America also have many operating biogas systems (Mottock,1984).

Exeter City Council in England, started the use of anaerobic processes for sewage treatment in 1895, and the biogas produced was used as fuel for street lamps (Monnet, 2003). Anaerobic treatment technology has come a long way since then. The last four decades have seen serious development and research on the use

of anaerobic digestion technology to treat other organic waste such as solid waste from farms, municipalities and industry (David et al., 2001).

Anaerobic digestion is a complex process that requires specific environmental conditions and a series of different types of fermentation, catalysed by a number of distinct bacterial populations. Mixed bacterial populations degrade organic compounds, by hydrolysis and conversion to produce a series of fatty acids. These intermediates are then converted to end products; a valuable high energy mixture of gases (mainly CH_4 and CO_2) termed biogas. Figure 1.1 shows the processes involved anaerobic digestion. The individual stages of the anaerobic digestion process are considered below.

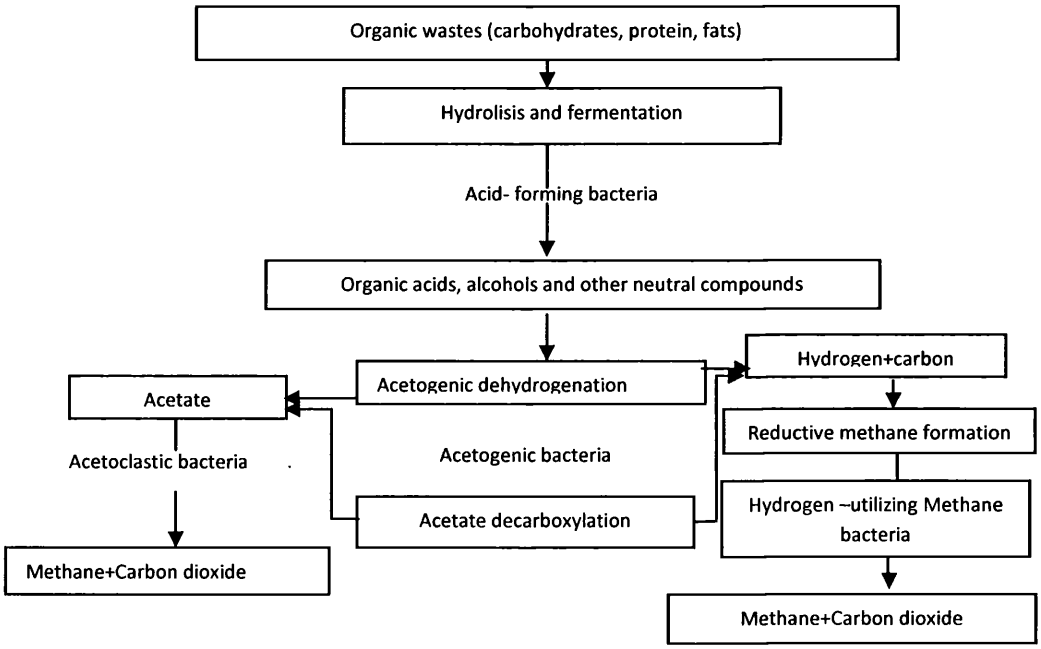

Figure 1.1: Diagram of an anaerobic digestion process (after, Pavlostathasis & Gomez, 1991). Through the use of enzymes produced by different kind of bacteria, the organic waste undergoes four main reactions which are: hydrolysis, fermentation (acidogenesis); acetogenesis; and methanogenesis (Lastella et al., *2002*).

1.3.1 Hydrolysis

In the hydrolysis stage, particulates are made soluble and large monomers are converted to uncomplicated monomers, such as carbohydrate, protein and fat

which are de-polymerized by extra-cellular enzymes. Extra-cellular enzymes produced by fermentative bacteria catalyze the hydrolysis reactions. The conversion of carbohydrate to simple sugars (hexose and pentose sugars) is catalysed by carbohydrases; proteins to amino acid with proteases and fats to fatty acid and alcohol with lipases. As no mineralization of organics is involved, these conversions result in no significant reduction in chemical oxygen demand (COD) (Eckenfelder, 2000). Though most biopolymers are readily degradable, the cellulose of highly lignified plant material (straw, wood, etc) has been shown to be resistant to hydrolysis (Lynd et al., 2002).

The rate of hydrolysis is dependent on many factors and includes pH, temperature, composition and the particle size and crystallinity of the substrate (Veeken & Hamelers, 2000 and Paramsothy et al., 2004). Volatile fatty acid production from the hydrolysis-acidification of coffee pulp was investigated by Houbroun et al., (2003). In that investigation 23% COD based on a hydrolysis process was achieved at an organic loading rate (OLR) of 5 g COD/L. Equation 1.1 shows the hydrolisis process.

$$(C_6H_{10}O_5)^n + 2H_2O \rightarrow nC_6H_{12}O_6 \quad (1.1)$$

1.3.2 Acidogenesis

Acidogenic bacteria will transform the products from the hydrolysis stage to volatile fatty acids, ketones, alcohols, hydrogen and carbon dioxide. Acetic acid, hydrogen and carbon dioxide formed at this stage go directly to methanogenesis. Equations 1.2, 1.3 and 1.4 show the process in this stage (Bilitewski et al., 1994).

$$C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2 \quad (1.2)$$

$$C_6H_{12}O_6 \leftrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1.3)$$

$$C_6H_{12}O_6 \leftrightarrow 3CH_3COOH \quad (1.4)$$

1.3.3 Acetogenesis

Acetogenic bacteria transform long chain fatty acids, ketones and alcohols into acetic acid, molecular hydrogen and carbon dioxide (Lastella et al., 2002). Hydrogen concentration is very important in this stage while the partial pressure is low enough to thermodynamically allow the conversion of all acetic acid and hydrogen. Normally, the lowering of the partial pressure of hydrogen will be done by hydrogen scavenging bacteria (Mata-Alverez, 2003). Equations 1.5, 1.6 and 1.7 show the processes in this stage

$$CH_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + H^+ + HCO_3 + 3H_2 \quad (1.5)$$

$$C_6H_{12}O_6 + 2H_2O \leftrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1.6)$$

$$CH_3CH_2OH + 2H_2O \leftrightarrow CH_3COO^- + 2H_2 + H^+ \quad (1.7)$$

1.3.4 Methanogenesis

Methanogenisis is the final stage where acetic acid is degraded to methane and carbon dioxide. The group of bacteria employed in this process are called methanogenic bacteria or "methanogens". The methanogenic bacteria use acetic acid, formate, methanol (or carbon dioxide) and hydrogen to produce methane. Normally these bacteria are very sensitive to oxygen concentration in the system and in a well-balanced system, the pH ranges between 7 and 8 (Lastella et al, 2002 and Chongrak, 1996). Equation 1.8, 1.9 and 1.10 show the processes in this stage. However, methanogenic activity can be limited below pH 5. For example, high fatty acid concentrations can accumulate in imbalanced systems. Similarly, high concentrations of sulphide and ammonia also cause inhibition of methanogens.

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (1.8)$$

$$2C_2H_5OH + CO_2 \rightarrow CH_4 + 2CH_3COOH \quad (1.9)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (1.10)$$

1.3.5 Problems and Alternatives to Anaerobic Digestion

As indicated in section 1.3.1 to 1.3.4, although anaerobic digestion is a complex, fairly robust process in well controlled systems; it is susceptible to accumulations of many intermediates and end products. Another problem is that this is a relatively slow process requiring up to 20 days residence time within the reactor to achieve complete digestion. To overcome some of these problems the single-stage process can be split into a two stage system that is usually more rapid than a single stage system. In the two stage digestion, the hydrolysis acidification processes are optimised in the first stage and the resultant fatty acids are then fed into the second optimised methanogenic stage, so enhancing the overall process by better control of the process (Ince, 1998; Jeyaseelan & Matsuo,1995; Yilmazer & Yenign, 2002; Banks & Wang, 1999; Bhattacharya et al., 1996; Qi et al., 2003; Bo et al, 2007).

Another problem with methanogenesis is that the primary product of the process is methane and carbon dioxide. Although these gases are easily separated from fermenting liquid, they have limited uses as products. It is generally burnt in engines or furnaces to produce power and/or heat. If it is to be added to mains gas systems the carbon dioxide has to be removed and the gas standardised prior to injection. An alternative process to the methane formation is to consider the initial acidogenic phase of the process where a wide range organic carbon compounds (carbohydrate polymers) are hydrolysed and converted to fatty acids, solvents, carbon dioxide and hydrogen. As such this represents a conversion of a wide range substrates into a few common fatty acids. The range of products formed by these processes are limited to a few fatty acids, acetic and butyric acids, and the solvents ethanol and butanol; and hydrogen and carbon dioxide.

These carbon materials represent potential sources of feedstock platform chemicals for synthesis of a wide range of materials and thus represent a feedstock that is not derived from fossil carbon. Indeed, these chemicals can be substituted directly for those from fossil fuels.

Acetic acid for example has many uses in industry (see figure 1.2).

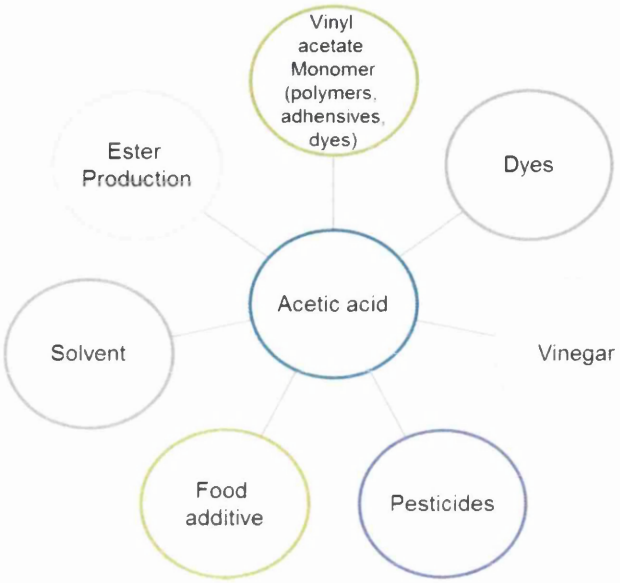

Figure 1.2: The uses of acetic acid

Acetic acid is an important commodity chemical that is typically produced from hydrocarbon feedstocks by methanol carbonylation, acetaldehyde oxidation, ethylene oxidation or by Oxidative/Anaerobic fermentation. The global demand of acetic acid is around 6.5 million tonnes per year (Mt/a), of which approximately 1.5 Mt/a is met by recycling; the remainder is manufactured from petrochemical feedstock. Only 10% of the global acetic acid production is deriving by fermentation so there is scope for direct replacement /substitution of petrochemically derived feedstocks. The benefits of this approach are that both carbon and encapsulated energy are recovered so making overall synthesis more efficient. The current price ranges from $600 to $800 per tonne (Massingham, 2011).

1.4 Problem statement

Methanogenesis is a relative slow process and offers little potential other than for the recovery of energy in the gaseous form, methane. Methane has to be burnt to recovery the energy and if released into the environment methane is a very potent greenhouse gas. As discussed in the introduction, fatty acids are general intermediates associated with the degradation of organic wastes, particularly carbohydrates and could be recovered avoiding carbon release while capturing much of the embedded energy in the form of a commodity chemical. If the waste organic carbon can be recovered as fatty acids and hydrogen, the waste treatment process may be more rapid and productive than that from methanogenesis and much of the energy in the waste would be still captured and in many potentially valuable forms.

The focus of the work in this thesis is therefore to investigate fatty acid production of the anaerobic bacterium *C. butyricum.* The organism produces acetate and butyric acids together with hydrogen and carbon dioxide as the main end products of the bacteria's carbohydrate metabolism. This investigation will be focused on the comparison of reactor technology for fatty acid production, the product yield and relative productivity, key aspects of producing fatty acid economically. In this study the experimental systems will be somewhat simplified from natural enriched fermentations to pure cultures of *C. butyricum.* This approach will allow a clear analysis of the problems by using systems that are reproducible and of lower complexity than fermentations of enriched mixed microbial populations.

1.5 Aims and objectives.

The aim of this study is to investigate the production of fatty acids from wastes as a potential source of commodity chemicals using *Clostridium butyricum.* The work can be divided into the following tasks and objectives.

1. Investigation of the growth of *C. butyricum* in the anaerobic tubes.

The objective of this study is to establish and optimize medium composition for the growth of *C. butyricum*. The results of this study are very important to set the foundation for further systematic study.

2. Investigation of growth in batch cultures in a Stirrer Reactor Tank (STR).

The objective of this work is to determine the optimum growth rate and the growth yield for a variety of common carbohydrates substrates in batch culture. To further define the growth conditions, especially pH. The effects of carbohydrate concentration on product yields included an assessment and analysis of the fermentation products by carbon balances.

3. Culture in continuous system in the (CSTR).

These experiments will: seek to clarify the kinetics of the fermentation process; determine the influence of nutrient feed flow rate on production of fatty acids; and identify the amount of fatty acid produced (acetic and butyric acid) in a CSTR. The analysis will also include the fermentation balances.

4. Membrane bioreactor (MBR).

MBR's offer many potential advantages over STR and CSTR in that they allow the in situ removal of end products from the culture while retaining the cells in the reactor. The main objective of this experiment is to study the growth of the organism in a MBR and to establish its kinetic performance as compared with STR and CSTR. This will be done by analysis of end product formation and carbon balances.

1.6 Description of this study

This research therefore combines studies of microbiology, microbial growth and product kinetics with a comparison of batch, continuous, and membrane bioreactor technology. *C. butyricum*, strict anaerobe bacteria will be used as a model experimental system to investigate the conversion of carbohydrate into fatty acids.

The results and analysis of this study will enable optimisation of the selection of the reactor conditions and operation by comparing the three fermentation systems. The results of this research will enable an evaluation of the production of fatty acid materials that can be used as a feedstock for pharmaceutical, food and environment applications.

The novelty of this research lies in the fact that to date, little research has been documented regarding fermentation processes employing *C. butyricum.* This is especially true of the study of these organisms in CSTR and membrane bioreactors which in theory have higher productivities than batch systems. Most previous work in this area has been concerned with production and optimization of fermentations of *C. butyricum* in batch culture. Van Andel et al., (1985), Vandak et al., (1995) and have studied the effect of pH and temperature on the growth *C. butyricum*. Other researchers have studied the environmental factors that influence the growth of *C. butyricum* (Vandak et al (1995), Wang et al., (2011) and Kawasaki et al., (1998)). Others have been more interested in hydrogen production in the fermentation process (Wang & Jin (2009),Fang et al., (2006)). Due to the ability of *C butyricum* to produce fatty acid some researchers have focussed on fatty acid production in batch culture (Abbad-Andaloussi et al., (1995), Zigova et al., (1999) and Qing et al., (2005)). This study will there seek to contribute to this field by investigating the enhanced performance of *C. butyricum* in continuous cultures and the use of MBR systems to intensify these processes.

Chapter 2: Background to the Study

2.1 Introduction

Chapter two introduces the background to the research and summarises information concerning *C. butyricum*, fermentation processes, bioreactors and membrane filtration.

2.2 A brief history of *C. butyricum*

In 1880, Prazmowski was the person who discovered and reported on *C. Butyricum* (Gruger & Gruger, 1989). According to Murray et al., (1984) it can be found in the soil and the intestines of humans and animals. In some situations *C. butyricum* can be considered a probiotic bacterium (Kong et al., 2004). Generally, species of clostridia come from the group of endospore obligate anaerobic forming and gram-positive rod shaped bacteria and they lack a catalase and a respiratory chain. One familiar hypothesis is that the bacteria lack systems for the of detoxification oxygen compounds (such as superoxide, hydrogen peroxide and hydroxy radicals, allowing a build up of these compounds to levels which cause serious damage to cell components (Storz et al., 1990). Therefore, the process whereby almost any organic waste material can be biologically transformed must be delivered in the controlled absence of oxygen. Strict environmental conditions are therefore required for these bacterial species to degrade organic substrates into a final supernatant (organic acids and solvents) and gaseous end-products (hydrogen and carbon dioxide). Carbon substrates are metabolized by *C.butyricum* via two main reactions, hydrolysis and acidogenesis, thereby transforming the organic macromolecules (carbohydrates, proteins and fats) into fatty acids, solvents and gaseous constituents. The main products are acetic, butyrate, ethanol, butanol, hydrogen and carbon dioxide. Table 2.1 shows properties of *C.butyricum* according to its physiological and biochemical characteristics. This table shows that the

organism is primarily saccharolytic being able to metabolise both hexose and pentose sugars (the major components of vegetable carbohydrate) as well as disaccharides and starch.

Table 2.1: Physiological and biochemical characteristics of *C.butyricum;* from Bergey's Manual of Systematic Bacteriology (Murray et al., 1986).

Characteristics	***C. butyricum***
Physiological characteristics	
Growth type	Strict anaerobe
Growth in NaCl concentration	
3.0% NaCl	NT
4.0% NaCl	NT
6.5% NaCl	NT
Oxidase test	Negative
Catalase test	Negative
Methyl red test	NT
Voges-Proskauer test	NT
H2S production	Negative
Rezasurin reduction	Positive
Indole produced	Negative
Lecithinase produced	Negative
Lipase produced	Negative
Starch hydrolyzed	Positive
Gelatin hydrolyzed	Negative
Esculin hydrolyzed	Positive
Biochemical characteristics	
Amygdalin	Positive/Negative
Cellobiose	Positive
Fructose	Positive
Galactose	Positive
Lactose	Positive
Maltose	Positive
Mannitol	Positive / Negative
Mannose	Positive
Melibiose	Positive
Raffinose	Positive
Rhamnose	Negative
Ribose	Positive
Sorbitol	Negative
Starch	Positive
Sucrose	Positive
Trehalose	Positive
Glycerol	NT
Gluconate	NT

*Positive reaction (90-100%), Negative reaction (90-100%), NT (Not tasted)

2.2.1 *C. butyricum* in the natural environment

Generally, the clostridia can be found and isolated from the natural environment, being found in anaerobic environments, animal sewage, marine sediments, activated sludge and soil. (Gamboa, et al., 2005, Cato, et al., 1986, Nusslein & Tiedje, 1998, Borneman & Triplett, 1997, Miwa, 1975, Kameyama, et al., 1996, Wang & Jin 2009; Wang et al., 2007 Zigova et al. 1999, Michel-Savin et al., 1990).

Most researchers have cultured *C.butyricum* in the mesophilic conditions (20-45 °C) and at controlled pH (around neutral), to support the production of volatile fatty acids, carbon dioxide and hydrogen (Wang & Jin, 2009, Wang et al., 2004). Previous research indicates that the favourable temperature range for hydrogen production and growth was in the range 30-37 °C (Majizat, et al., 1997, Nielsen, el.al., 2001). While others have found that optimum temperatures were up to 45 °C (Wang & Jin, 2009, Wang et al., 2004).

Normally the pH for an anaerobic process is around 5.5-8.0. However, for *C. butyricum* a slightly acidic pH in the range 4.5-7.0 can be tolerated. The organisms can be grown in an atmosphere of pure N_2, or CO_2, but mixtures CO_2 and N_2 in the ratio 1:9, have been shown to be preferable (Zigova et al.,1999, Van Andel et al., 1985).

Earlier researchers stated that *C. butyricum* would not survive aerobic conditions and this agrees with research reported by Kawasaki et al., (1998), who tested growth capability under aerobic, semi-aerobic and pure anaerobic conditions. The results showed, *C. butyricum* could not survive in a totally anaerobic environment, but that under semi-aerobic conditions (between 20 µM and 40 µM concentrations of dissolved oxygen, 10 to 20% saturation) the cell could be considered to be growing, but it could grow at higher oxygen levels (120 µM level 40% saturation of dissolved oxygen). Nevertheless, in sealed liquid culture the concentrations of oxygen are gradually decreased after inoculation, showing that the organism has some capacity

to metabolize oxygen slowly. The rates of oxygen consumption in the 40 µM oxygen were almost the same as that in the 120 µM oxygen medium.

2.3 Fermentation of carbohydrates by *C. butyricum*

As indicated in table 2.1, *C. butyricum* is capable of metabolising a wide variety of carbohydrates most notably hexose sugars and polymers (i.e glucose, fructose sucrose lactose starch), pentose sugars such as xylose and ribose. *C.butyricum* therefore possesses some capability to produce extracellular enzymes such as amylase. However, this organism is not cellulolytic, lipolytic and proteolytic and cannot ferment sugar alcohols like glycerol and mannitol.

These properties mean that they are saccharolytic organisms that can ferment a wide variety of wastes including waste sugar streams such as molasses into acetate, butyrate, carbon dioxide and hydrogen. Many researchers such as Miyake et al., (1984), Yokoi et al.,(1998), and Wang & Jin (2009), have studied the capability of *C. butyricum* in sugar substrates.

The biochemistry of fermentation can be viewed as a process by which sugar is reconfigured within the cell to give a mixture of reduced and oxidised end products and by so doing generates energy for growth. A key aspect of this is the maintenance of the redox balance of electron flow between electron generating reactions and electron consuming reactions as the cell must recycle electron carriers to maintain rapid metabolism. If there is a redox imbalance then the carbon flow (and hence energy generation) through metabolism slows, reducing growth rates and cell yields.

Figure 2.1 shows the decomposition of carbohydrate and its transformation to acetic and butyric acid, carbon dioxide and hydrogen. As shown the organism can ferment a wide variety of carbohydrates and these are taken up by the cell and metabolised to 2 moles of pyruvate plus 2 moles of reducing equivalent (NADH) and 2 moles of ATP via the Emden-Myerhof-Parnas pathway. This is the most common carbohydrate pathway in anaerobic bacteria. From pyruvate the carbon metabolism

is more specialised and restricted to strict anaerobes because of the involvement of electron transfer processes involving ferredoxin (Fd) (Gerhard, 1979). A series of enzymes allow the electrons to flow from NADPH and NADH and thus allows electron transfers between the key parts of metabolism within these organisms. The end result is the generation of Hydrogen derived from electrons being transferred to hydrogenase to form molecular hydrogen and reduced end products such as butyrate. Both ferredoxin and hydrogenase are easily poisoned by oxygen and causes serious metabolic problems within the anaerobes where electron balances are easily disturbed.

The carbon flow from the pyruvate is via a further decarboxylation to release carbon dioxide and form Acetyl Co A. This can then be either metabolised to acetate with the generation of ATP or it is condensed and is further metabolised to 4 carbon compounds that are further reduced to form butyrate plus ATP (figure 2.1). It should be noted that the amount of ATP formed by substrate phosphorylation is regulated by the relative proportions of acetate and butyrate. It is to the advantage of the growth of the organism to produce as much acetate as possible from glucose. In theory if more hydrogen is released then more acetate (and hence more ATP) can be generated per mole of glucose metabolised. For this to occur electron flow has to be very efficient, however, this is dependent on the type and characteristics of the enzymes associated with ferredoxin reduction either directly or indirectly from NADPH and NADH. The extreme situation of acetate, CO_2 and hydrogen production is not observed in reality as the thermodynamics of electron transfer to hydrogen are not favourable. However, external electron acceptors (oxygen and some organic materials) can shift the balance towards acetate away from butyrate.

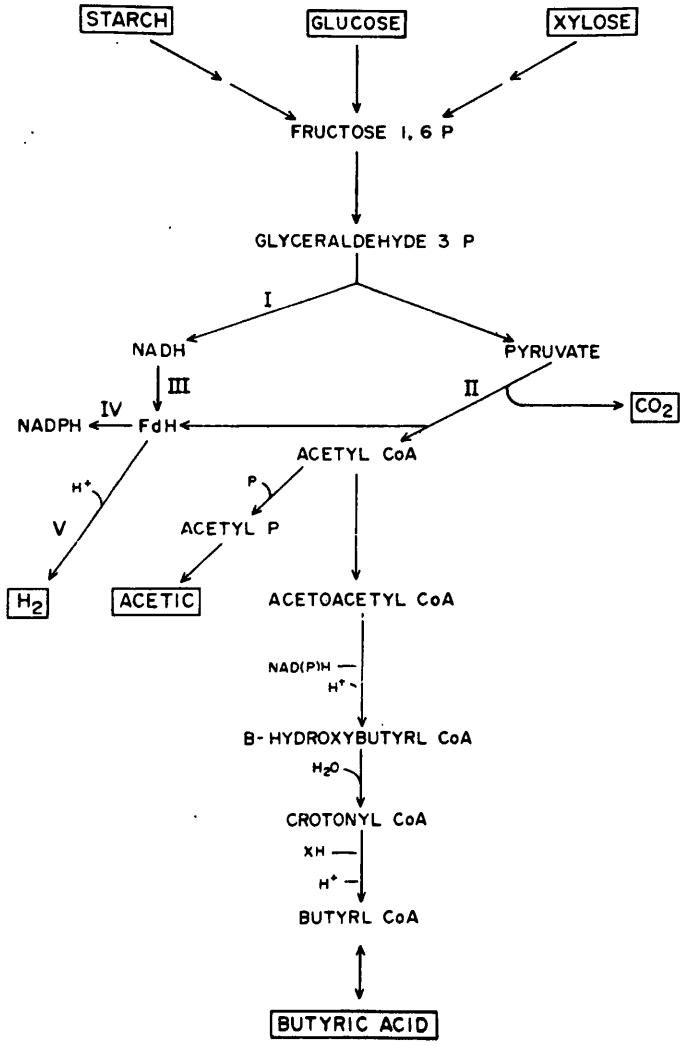

Figure 2.1: Proposed carbon flow pathways for *C.butyricum* in carbohydrate fermentation (After, Petitdemange et al. 1977).

Table 2.2 shows a typical product ratio on glucose for *C. butyricum* published by Wood (1961) and latterly by Gerhard (1979). The balances are incomplete as it does not show the product biomass formed during the fermentation. Typically for anaerobic bacteria the yield is of the order of 0.2 g cell per gram glucose (i.e 36 g/mole glucose). Assuming a yield of 0.2 (Atkinson & Mavituna, 1991) and 50% carbons (Atkinson & Mavituna, 1991) then this account for 22 mMole carbon. Table 2.2, however, does serve to show the proportions of products encountered in typical fermentations.

Table 2.2: Fermentation balances of *C. butyricum* (After, Wood 1961 and Gerhard 1979)

Product	Amount formed in mMole/100 mMol glucose fermented	mMol Carbon/600 mole C as glucose
Butyrate	76	304
Acetate	42	84
Carbon dioxide	188	188
Hydrogen	235	
Cells*		22*
recovery		100%

Assumptions made for the calculation: the cell yield of 0.2 g/g glucose (or 36 g/mole glucose) which is assumed to be the cells carbon is assumed to be 50% of the dry weight of the cells.

2.4 Factors affecting the growth and product formation of *C. butyricum*

Many factors affect the growth of *C. butyricum,* however, according to Gamboa et al., (2005), pH, medium composition and carbon substrate have significant effects.

Carbon substrate concentration will have a significant impact on the production of C. *butyricum*, giving different rates of hydrogen production for 15g/L glucose, starch, xylose or fructose (Fan et al., 2004, Majizat et al., 1997 and Yan et al., 1988).

Many researchers have reported that, the pH for fermentation to efficiently produce hydrogen is within the range of 5.5-6.7 for anaerobic processes (Hawkes et al., 2002, Fang et al., 2002 and Lin & Chang, 1999). However, according to the research by Chen et al., (2005), pH 5 strongly inhibits the production of hydrogen by *C. butyricum*. The relative amounts of hydrogen and butyric acid are significantly affected by environmental conditions and as stated above, high yields of hydrogen will reduce the levels of butyrate production and vice versa (Chen et al., 2005, Chen et al., 2001, Chang et al., 2002, Lee et al., 2003, Wu et al., 2004 and Lee et al., 2004).

One of the key problems in growth is end-product inhibition (Hawkes et al., 2002, Fang et al., 2002 and Lin & Chang, 1999). Organisms such *C. butyricum* produce these inhibitory fatty acids and ultimately they limit the growth of the organism. The sensitivity of the organisms to fatty acids is strain dependent some being able to tolerate far more than others. Environmental conditions also affect tolerance both low temperature and high pH improves fatty acid tolerance considerably. pH control either by effective buffering of the media or directly by alkali addition becomes a critical factor in maximising growth.

Both acetate and butyrate are inhibitors of growth indeed they are used as preservatives and are preservative agents in pickled food stuffs such as silage, sauerkraut and dairy products. The mechanism for inhibition of growth and metabolism is thought to be due to the characteristics of the organic acids being able to cross and interact with cell membranes. Both acetate and butyrate are weak acids with dissociation constants around pH 4.8, meaning that under acid conditions substantial quantities of undissociated organic acids are present. In these conditions, the organic acids are thought to freely pass over the cell membrane causing a severe stress in the cells by raising the internal osmotic pressure of the cell and causing dissipation of membrane charge in the cell.

2.5 Applications of *C. butyricum* in the industrial sector

In the past C. *butyricum* has been used in many industries. This section explains the function of *Clostridium butyricum* in 3 major industries where it finds application - pharmaceutical, food and environmental industries.

2.5.1 Pharmaceutical technologies

Antibiotics effectively control disease; however, their continuous use can create antimicrobials resistance. C. *butyricum* can be considered as a probiotic bacteria. Probiotic being defined as cell preparations that have beneficial effects on

the fitness of the host by harmonizing the intestinal balance of microorganism (Fuller, 1989). Varous strains of *C.butyricum* are used to treat child diarrhoea in the Far East. *C. butyricum* also have a potential to improve the process of digestion of food and increase food transformation effectiveness with health protection because it excretes vitamin B, vitamin K, amylase etc (Song et al, 2006, Wang and Xu, 2006 Ziaei-Nejad et al., 2006, Lara Flores et al., 2003).

2.5.2 Food technologies

C. *butyricum* can produce butyric acid, which makes it important for the food technology sector. Butyric acid can be used to enhance the flavour of butter or, used as an ester for increasing the fragrance of fruit (Sharpell, 1985, Zigova et. al.1999, Jiang et al. 2009, Playne, 1985). *C. butyricum* is also well known as a classic acid producer, and is regularly used to ferment glucose to produce butyrate and acetate (Jungerman et al. 1973). Acetate and butyric acid are used for enhancement of the flavour of food and as preservatives.

In Eastern Asia, especially Japan, Korea and China, clostridia has been used as a probiotic for many years (Ito et al., 1997; Kamiya et al., 1997, Song et al., 2006). Wang & Xu, (2006), Ziaei-Nejad et al., (2006), Lara Flores et al., (2003) and Song et al., (2006), have proposed the use *C. butyricum* as a probiotic to promote shrimp and fish growth.

2.5.3 Environmental technologies

The environmental sector has seen a significant impact through the use of *C. butyricum* based technologies as methods of reducing quantities of waste and cleaning the environment. This organism plays a significant role in the degradation of organic waste in anaerobic systems producing hydrogen and fatty acids as part of the methanogenic process (Chen et al., 2005, Levin et al., 2004, see also section 1.3 in chapter 1). C. *butyricum* from acclimated sewage sludge has been found to be

a very efficient producer of hydrogen (Chen et al., 2001, Chang et al., 2002, Lee et al., 2003, Wu et al., 2004, Lee et al., 2004 and Chen et al., 2005).

2.6 Bioreactor design

The design and operation of bioreactors for anaerobic fermentations are key to maximising productivity and reliability of the fermentation and form a major part of the studies in this thesis

The requirements of a good reactor design are many fold but the key aspects are to:

1. Provide an ideal environment for growth
2. Provide an environment for safe and robust operations
3. Exploit and enhance product formation kinetics and yield
4. Make a simple cost effective system

The reactors for growth of clostridia can be relatively simple and originally were developed from brewing type systems using batch reactors. This is quite appropriate as many of the important products produced are not coupled directly to growth and are either non- (or slow) growing end-product inhibited conditions or are non-growth related secondary products. As such batch systems provide an effective production system. Continuous culture systems in many cases are unsuitable because the maintenance of the cells in such systems is related directly to the yield and growth of the organisms, hence are not suitable for end product inhibition or non growth related product formation.

There is a large body of work investigating reactor designs for *C. acetobutylicum* and related organisms that have the capability to produce solvents, ethanol acetone and butanol under growth inhibitory or non-growing conditions (Zverlov et al, 2006 and Cheng et al, 2012)

However, the downside of a batch process is that it is a relatively low productivity system as compared with continuous systems or systems containing

high biomass concentration such as packed bed reactors or reactors with cell recycle.

Section 2.6.1 will review attempts of using reactor systems for growing anaerobic bacteria and their products.

2.6.1 Bioreactor of anaerobic bacteria fermentation

The industrial sector has many types of bioreactors. These are selected according to the kind of system required, and this section seeks to outline the main types of bioreactors and related systems.

2.6.1.1 Batch operation

In a batch reactor, the reactor is filled with organic matter and microorganisms (a batch) and the process of decomposition is allowed to proceed for a predetermined time or until gas production decreases to a predetermined (low) rate. Normally, in this type of operation, 10 to 20 percent of material is left as seed when the reactor is reloaded and the operation repeats (Chongrak, 1996). Many researchers have reported the application of this operation to culture *C. Butyricum* (Abbad-Andaloussi et al, 1998, Wang & Jin,2009 and Vandak et al, 1997).

In batch cultures, the growth of microorganism will pass through a number of phases and typically starts with the lag phase in which the cells adapt to their new environment. The specific growth rate (μ) then slowly accelerates until it reaches the log or exponential phase. In this phase growth is only limited by the capacity of biomass to grow (the maximum specific growth rate (μ_{max}) and the cell concentration (x)). When the microorganisms have been growing in the exponential phase for some time, nutrient depletion and possible end product inhibition will occur causing the growth rate to slow down and finally stop as the culture enters the stationary phase. Here the growth rate (μ) for the microorganism is equal to the death rate (k_d) of the

microorganism so dx/dt=0. Sometime after the stationary phase the microorganism enters the death phase where there is a net loss of active biomass.

, Figure 2.2 shows the graph for concentration of cell versus time. The shaded area is the cell productivity (g/L/h) and the slope of graph gives $\mu(h^{-1})$

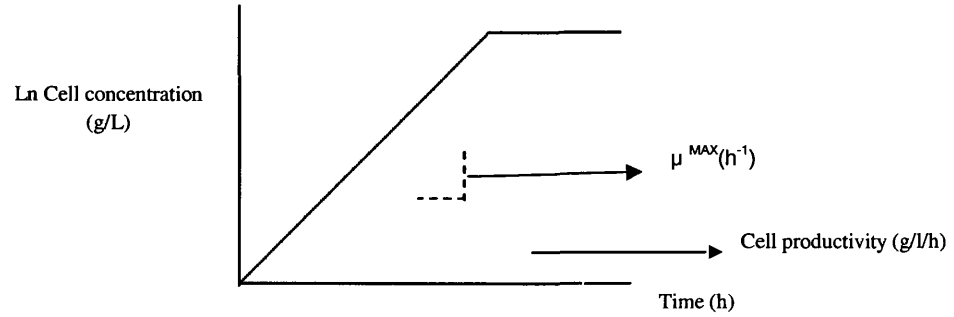

Figure 2.2: Cell concentration versus time in the batch system. Cell productivity will be dependent on the period in which the culture is harvested.

2.6.1.2 Continuous operation

The main concept of the continuous system is that it is an open system where sterile substrate feed is continuously added into the bioreactor. The feeding rate of substrate is equal with the amount of converted nutrient solution with microorganisms that is simultaneously removed from the system. In a continuous process under steady state conditions, cell loss as a result of outflow must be balanced by outgrowth of the organism. Continuously operating reactor systems are very well suited to the treatment of liquid and organic waste (with low solid concentration). These processes typically start as a batch process and once the microbial population establishes itself a feed pump is started and the production stabilizes at steady state where the growth rate of biomass is equal to that leaving the reactor (Chongrak, 1996). The disadvantage of this system is the possibility to contamination due of the fact that it is an open system. The sterility of the feed is of prime

importance. Figure 2.4 shows cell concentration versus time for a continuous system. The shaded area is cell productivity and dilution rate D (h^{-1}) is equal to growth rate, $\mu(h^{-1})$. In these systems productivity is much higher than observed in batch culture as the system is operating at steady state.

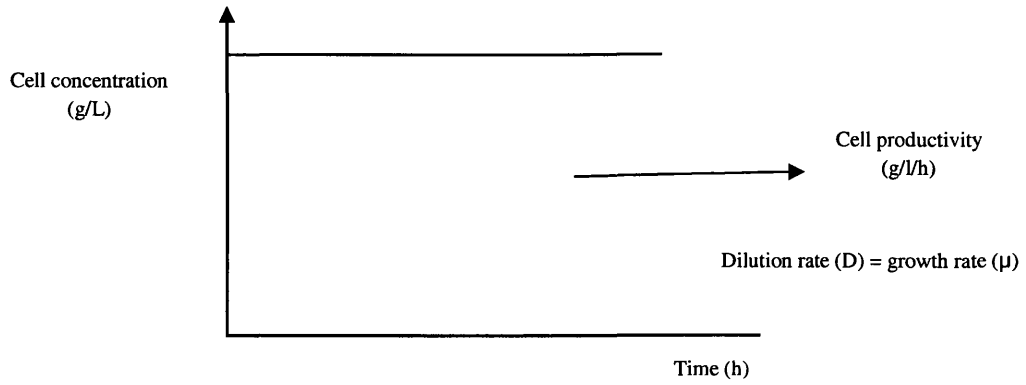

Figure 2.4: Cell concentration versus time in the continuous system

2.6.1.3 Reactors with enhanced performance.

To improve the fermentation system further and avoid disadvantages in the batch and continuous operation, reactors with enhanced performances have been investigated. Two methods of improved operation have been shown to consistently enhance performance. These are immobilized cell reactors or cells recycle reactors.

a) Immobilized cell reactor

In this type of reactor three methods could be applied to immobilize the cells such as covalent bond formation, entrapment and bio film (Qureshi et al, 2005). All of these methods have been applied in different reactors. The types of immobilized cell reactor include;

Fluidised bed reactors-This type of reactor operates with an upward flow of liquid. It is simpler than the packed bed reactor. Fluidised bed reactors are often used in waste treatment with sand or another suitable carrier material which supports the mixed microbial populations inside the fluidised bed. Other applications for this type

of reactor include the commercial production of vinegar. In this process a flocculating organism is applied.

Packed back reactors-This kind of reactor is used commercially with immobilised cells and enzymes for the production of aspirate and fumarate, conversion of penicillin to 6 aminopenicillanic acids and resolution of amino acid isomers. Normally the medium (continuous liquid flow) will feed through a packed bed tube either at the top or bottom of the reactor tube. Inside, the packed bed is equipped with catalyst particles (enzymes or cells). The advantage of this kind of reactor is that damage to the enzymes or cells or fermentation is lower than in a comparable stirred reactor.

Trickle bed reactor-The trickle bed reactor is similar to a fluidised bed reactor. The difference being that the trickle bed reactor is fitted with a spray distributor. Liquid is recycled from the bottom of the reactor to the top of reactor by spraying it back through the spray distributor into the top of the reactor. Zhang et al 2006, applied this type of reactor to produce hydrogen by *C. acetobutylicum.* The result from this study was that the production of hydrogen was enhanced but the reactor was complex and expensive to scale up.

Airlift reactor-The concept of the airlift reactor is similar to that of the bubble column reactor. However it is equipped with a draft tube. Airlift reactors are often chosen for immobilised catalyst, cultural animal and plant cell digestion because shear levels are lower than stirred vessels. The resulting fraction of distributed cultivations is very low.

b) Cells recycle system

If cells are retained in the reactor system then enhanced reactor performance over classic continuous cultures is possible. Fermentation processes whereby cells are retained in the reactor by sedimentation to give very high cell concentrations result in higher volumetric catalytic power leading to high productivity. This allows

higher flow rate through the system well above that for cells in CSTR. This type of enhancement exploits common behaviour in natural environments to form rapidly settling flocks found in fresh and sea water environments. These systems therefore can naturally select for flock communities as they are retained over equivalent free living organisms. There are many variations developed and used especially in waste treatment, from the classic activated sludge plant to Upward-flow anaerobic sludge blanket (USAB) reactors. This type of reactor has not only been applied widely in the waste water treatment, (Schwartz and Keller, 1982) but also has been applied to the investigation of acetic acid production by *C. thermoaceticum*.

MBR (Membrane bioreactor)-The main concept for this reactor is to retain cells in the reactor by filtration. There are two types of basic design in these systems depending on the use of the membrane.

The first type used membranes which filter water by suction through the membrane surface removing clarified water while retaining the particulates. This type of system is commonly used in waste treatment as a replacement for activated sludge processes or flocculation processes where filtration replaces sedimentation to retain biomass within the reactor.

The second type of MBR filters pressurised water to force water out of the system. These systems are easier to engineer especially when sterile environments are required in systems run under positive pressure. They are more often used in reactors with pure cultures.

MBR systems have many advantages over continuous culture or reactors with cell recycle relying on sedimentation, in that cell retention is controlled by a physical separation and so can be generically applied to all types of cells to produce high cell concentrations in the reactor and so allow high productivities. In addition, permeates from MBR's are cell free, thus allowing their further separation and purification. Jung and Lovitt, (2010) studied lactic acid bacteria and found that lactic acid productivity ($P_{x/t}$) and final biomass concentration in the MBR were over 20 times

greater that better than those for stirrer reactor (STR). The use of MBR systems will be clarified further in the section 2.8.

2.7 Membrane filtration

Membrane filtration is a key part of a membrane bioreactor. The area of membrane used and its permeability, control the throughput of a membrane bioreactor and ensure that the output of the bioreactor is continuous, and / or suitable for downstream processes. The following section will review membranes and membrane technology.

2.7.1 A brief history of membrane filtration

The idea of developing membrane filtration came from exploration of the principles of biological membranes, with experiments on osmosis dating from the 18th century. By around 1950, industrial filtration membranes had been developed using synthetic materials. Prior to this, in 1907, ultrafiltration had been introduced, where pressure (at up to several atmospheres) was used to force solutions through membranes prepared by impregnating filter paper with acetic collodion.

The timeline for development of synthetic membranes starts with the exploration of reverse osmosis membranes made from cellulose acetate polymer by Sidney Loeb in 1950. The Loeb membrane is capable of rejecting salt and passing water at particular differential pressures and flow rates. This study has had a great impact for the Middle East and North Africa through its use as a desalination process. From the base in desalination processes many types of membranes have been created to improve water quality such as nanofiltration for partial desalination, through ultrafiltration for virus removal, and finally to microfiltration for the removal of suspended solids. Following this intense period of rapid development of membrane filtration technology the applications have spread widely into sectors which include water treatment, food technology, bioreactors and the pharmaceutical industry.

2.7.2 Functions of the membrane

The functionality of membranes can be considered in four groups: separation, contact, immobilisation and controlled release.

Separation is the process which separates an ingredient from the homogenous solution. The principal of mass transport is applied in this process. The methods of mass transport across the porous membranes depend on factors such as selective absorption, diffusion in membrane pores and diffusion in the membranes.

The use of a membrane for its contact function is to connect two different media such as liquid A with liquid B or a liquid with a gas.

Controlled release is widely used for the delivery of medicines. It is also applied in the agricultural sector to control the content of chemicals. Otherwise, it is employed in fertilisers, pheromones and oxygenation.

Immobilisation is used for culturing tissues and cells and in the electrical and electronic sector.

2.7.3 Mechanisms of membrane filtration

For effective filtration by use of a membrane it is very important that the correct kind of membrane is chosen. To date, five kinds of driving forces for filtration have been reported. Table 2.3 shows the five driving forces of filtration and the suitable membranes for those types.

Table 2.3: Mechanisms of filtration and suitable types of membrane

Process	Driving Force
Micro filtration, ultra filtration, nano filtration, reverse osmosis and gas separation	Pressure driven process
Reverse Osmosis (RO), gas separation, evaporation, membrane extraction and membrane absorption	Diffusion process
Membrane distillation	Heat process
Electro dialysis, electrostatic, pseudo liquid membrane	Electric process
Haemodialysis, Liquid membranes, supported membranes, active transport and facilitated transport.	Chemical process

However, the principal mechanism of separation is size exclusion and thus has been related to the bioreactor types explored in this study, because, only the mechanism of size exclusion will be further explained.

Size exclusion means the separation of media through the use of a specific membrane pore size. For example, in typical microfiltration the specific pore size is around 50nm-5μm and has the capability to retain bacteria, for ultrafiltration the size is 5-10nm and has the capability to separate virus and dissolved substances with molecular weights between 5-5000Da. Lastly, the smallest specific pore size is nanofiltration with a pore size of between 1-10nm, and is capable of separating low molecular weight substances (200 – 5000Da).

2.7.4 Microfiltration

Microfiltration is common in the industrial area for separation processes. It has the capability of removing particles with sizes down to 0.1 to 0.2μm. Normally, membranes of this type are made from hollow fibres that can be operated in the outside-in or inside-out configuration. To ensure that membranes of this type operate efficiently, a pressure differential in the range 5 to 35 psi is maintained across the membrane (normal operating conditions), or -3 to -12 psi under vacuum conditions. For the low cost operation, air or liquid is used for backwashing. The separation by microfiltration is normally based on their shape and molecular weight (Bailey & Meagher, 2000). However for filtration of microbial cells, normally it will work with 0.2-0.45μm and the microbial cell will be retained in surface layer of membrane. In this case often microfiltration used as a first stage of purification (Van Reis & Zydney, 2001).

2.7.5 Ultrafiltration

Usually, ultrafiltration is defined by particles with molecular weights in the range 10^3 to 10^6 or separation sizes in the range 0.1µm-5nm. Generally, ultrafiltration can separate emulsions, colloids, macromolecules and proteins. It is also very efficient for the removal of suspended solids, bacteria, yeast, viruses and other biological contaminants such as Giardia, Lamblia and Cryptosporidium. It uses liquid or air for backwashing and typical fluxes for ultrafiltration range between 50 to 100 gallons per square foot per day. However the productivity and efficiency throughout ultrafiltration relies on the physico-chemical properties of the suspension for example salinity and pH (Belfort et al.1994).

2.7.6 Nanofiltration and RO

Nanofiltration and reverse osmosis have the same basic principles. However, there are differences between RO and NF. While RO relies purely on the diffusion force, NF also involves other forces such as charge. For nanofiltration the separation line is slightly lower and ions dissolved in water are kept back by the membrane filter.

Nanofilters are good membranes for salt rejection. For example, the rate of rejection for calcium salts are in the range 80% to 85%, and such filters can be operated with relatively low pressure differentials, cutting the cost of operation. Normally nanofiltration can be operated for separation through the two methods. The first method is through the molecular weight cut-off (MWCO) and the second method is separation through charge, either positive or negative, depending on the material (Wang et al. 1995).

2.7.7 The structure of membranes

The structure of the membrane is very important for the performance of the membrane. Membrane structures can be categorised as either symmetric or asymmetric. Symmetrical membranes feature a two layer structure with the layers

identified as the dense layer and the porous layer. In the case of an asymmetric membrane the two layers are called the dense skin and the porous skin (Radjenovi´ et al., 2008).

2.7.8 Membrane materials

A range of materials are used in the production of membranes. The material selected depends on the desired function of the membrane.

Ceramic membranes-Ceramic materials are popular for the production of membranes. Normally, such a membrane will have an asymmetric configuration with a skin of a different porosity. The porous skin layer is usually made from some combination of alumina, titania and zirconia. One of the key properties of ceramic membranes is that they do not swell because they do not absorb water. Swelling can be a major problem for membranes as swelling of the pore changes retention characteristics and selectivity. Ceramic membranes are relatively strong and readily withstand the processes of cleaning and cake layer removal (Li, 2007)

Polymeric membranes-Recent decades have seen a lot of research activity into polymeric membranes, particularly in respect of the varieties of polymers that can be used. Work has also been undertaken to develop fabrication processes such as template leaching, stretching, laser ablation, phase inversion, coating of membranes and interfacial polymerisation (Ulbricht, 2006).

Nanocomposite membranes-Most nanofiltration and gas filtration membranes are made from polymeric or inorganic materials, with there being a new trend for the development of new nanocomposite membranes. Nanocomposite membranes will have two or more elements, typically containing different composites, where at least one of these is a nano phase (Van der Brugen, 2012).

2.8 MBR

The first membrane bioreactor was developed by Door-Oliver in the late 1960s and was installed in a ship for waste water treatment (Bemberis et al., 1971, Smith et al., 1969, Hardt et al., 1970). Membrane bioreactors are now widely used for municipal and industrial waste treatment (Judd, 2006).

Anaerobic microbes have a slower growth rate than aerobic microbes because of the relatively long hydraulic retention time (HRT) required to prevent biomass washout in a thoroughly mixed anaerobic digester (Parkin & Owen, 1986; Anderson & Saw, 1984 and Choo & Lee, 1996). To solve this problem, advanced membrane technologies have been developed to improve solid retention time (SRT) and treatment (Chiemchaisri & Yamamoto, 1994; Ross & Strohwold, 1994; Hidenori, 1994 and Choo & Lee, 1996). Improvement of SRT for activated sludge will cause the hydraulic retention time for the process to become faster (Kennedy et al., 1987).

2.9 Type of membrane bioreactor

Membrane bioreactors can be classified into a few groups on the basis of their system and process. Currently, membrane separation is carried out by pressure driven filtration in side stream MBR's. Another method operates with vacuum-driven membranes immersed directly into the bioreactor – this is known as a submerged membrane bioreactor.

2.9.1 Side stream membrane bioreactors

Side stream membrane bioreactors are the most popular for waste water treatment. The process starts with the waste water being pumped into the bioreactor vessel, The water is then pumped through the membrane and returned to the bioreactor. This is repeated until either bioreactor conditions or samples of the permeate indicate that suitable conditions have been reached, and the permeate is run off. This has become popular for treatment of waste water because it is

economical and easy to handle. Shear of the material is developed by the pumping, and is important to generate a permeate flux and minimise membrane fouling. This shear generation does however consume energy. Side stream MBR's are effective because of their high flux permeation levels. The pumping of activated sludge imparts shear to microbial flocs and can cause their degradation or break up. (Wisniewski & Grasmick, 1998). This can also lead to a decrease in particle size and the generation of fouling material from the flocks. Figure 2.5 shows the diagram of a side stream membrane bioreactor.

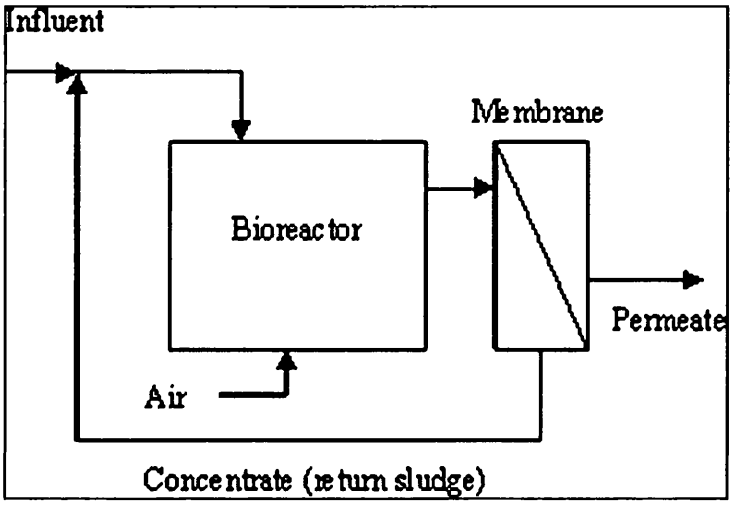

Figure 2.5: A side stream membrane bioreactor (Radjenovic et al., 2008).

2.9.2 Submerged membrane bioreactors

This type of MBR is similar to a side stream bioreactor. It also needs shear to avoid fouling of the membrane surface. However, the advantage of this type of MBR is that the energy required is lower than that for side stream types. Figure 2.6 shows the diagram of a submerged membrane bioreactor.

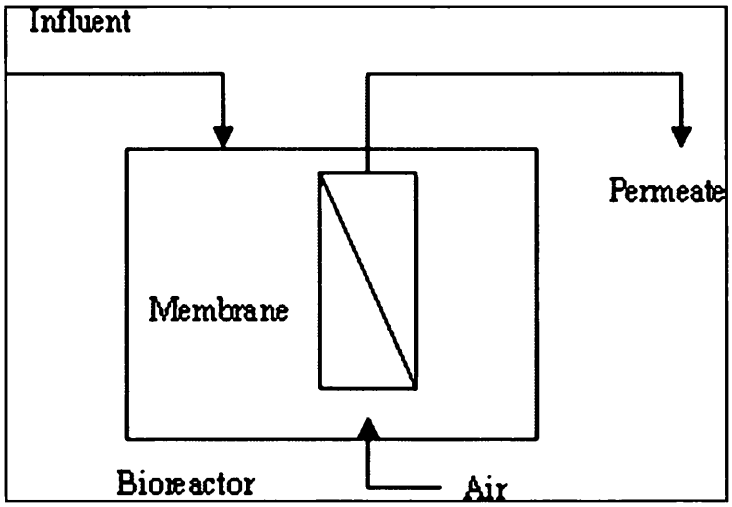

Figure 2.6: A submerged membrane bioreactor (Radjenovic et al., 2008).

2.10 Membrane bioreactor operation

Membrane bioreactors are very complicated systems, and as such correct operation and design of the system is very important to make a robust system.

2.10.1 The hydraulics of MBR

Generally, during the process of separation between bioreactor and membrane a few principles are applied. The first is control of material balance between the feed water permeate out of the reactor into the reactor. The product which passes through the membrane is called permeate. The quantity of feed water (Q_fC_f) is equal to the sum of the concentrate (Q_cC_c) and permeate (Q_pC_p). Figure 2.7 clarifies the explanation and equation 2.1 shows the relationship.

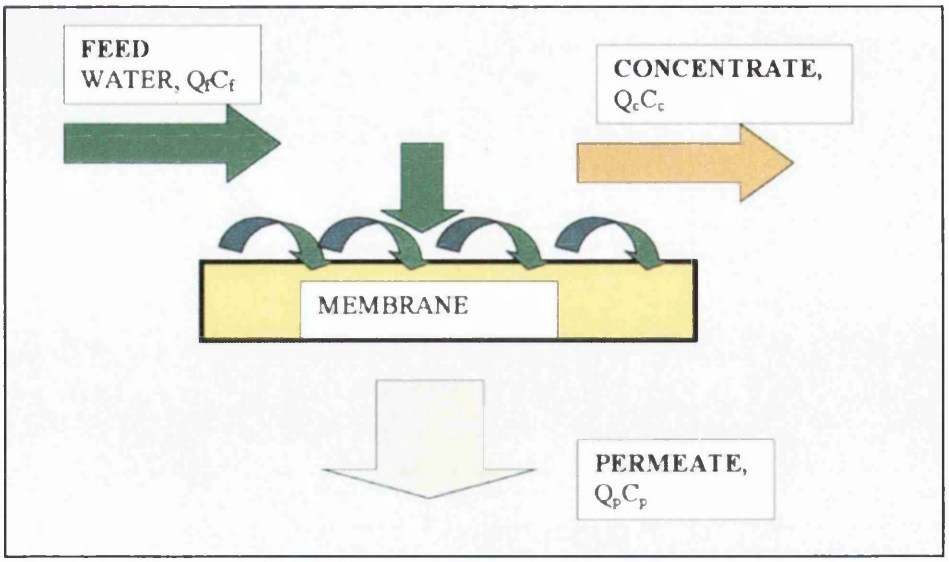

Figure 2.7: Process occurring in the membrane surface (Radjenovic et al., 2008).

$$Q_f C_f = Q_p C_p + Q_c C_c \quad (2.1)$$

where;
Q_f = Feed flow rate
C_f= Solute concentration
Q_p= Permeate flow rate
C_p= Solute concentration in permeate
Q_c = Solute concentration in concentrate
C_c= solute concentration in concentrate

For a MBR system, the feed rate must equal the permeate rate. The product, which is unable to pass through the membrane then accumulates in the reactor.

The amount of membrane rejection (R) also can be calculated from equation 2.2.

$$R = \frac{C_f - C_p}{C_f} \quad (2.2)$$

where;
R= membrane rejection

Therefore, fluid recovery (S) in this process also can be identified using the equation 2.3.

$$S = \frac{Q_p}{Q_f} \quad (2.3)$$

where;
S= Fluid recovery.

2.10.2 Membrane fouling

Fouling of the membrane can be detected by a decrease of the permeating flux levels. It occurs at the interface between the membrane and the mixed liquor. The mechanism of membrane fouling is very complicated, and a lot of research has been conducted in this area (Chua et al., 2002). However, the main reasons include adsorption of macromolecular and colloidal matter; growth of bio films on the membrane surface; precipitation of inorganic matter; and aging of the membrane. Equation 2.4 shows the formula for membrane resistance. Figure 2.8 shows a summary of the mechanism of fouling.

$$R = \frac{\Delta P}{\eta J} \quad (2.4)$$

where;

R = *Resistence*
ΔP = trans membrane pressure ($Lm^{-2}\ h^{-1}\ bar^{-1}$)
η = permeate viscosity in ($kgm^{-1}\ s^{-2}$)
J = Permeate flux ($Lm^{-2}\ day^{-1}$)

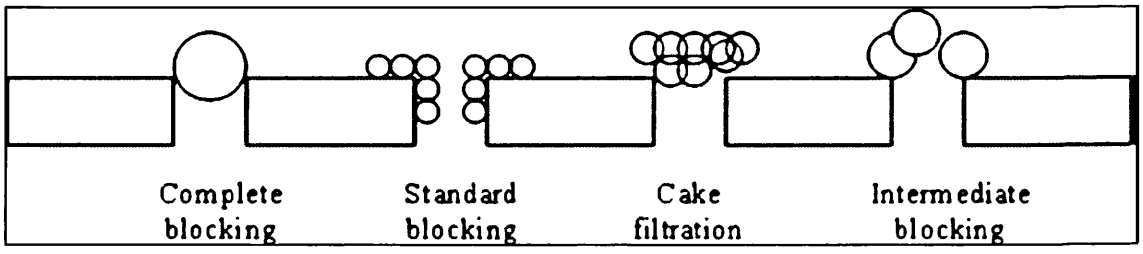

Figure 2.8: Summary of the mechanisms of fouling (Radjenovic et al., 2008)

$$J = \frac{\Delta P}{(R_m + R_c)\eta} \quad (2.5)$$

where;

J = Permeate flux (Lm^{-2} day^{-1})
R_m= Resistence of membrane
R_c = Resistance of cake
ΔP = trans membrane pressure (Lm^{-2} h^{-1} bar^{-1})
η = permeate viscosity in (kgm^{-1} s^{-2})

2.10.3 Prevention of fouling

Many methods have been discussed by previous researchers to prevent or reduce membrane fouling. Both configurations of MBR's (side stream and submerged) use shear at the membrane surface to prevent membrane fouling. Some researchers have mentioned that aeration of the membrane surface can reduce cake formation on the membrane (Ueda et al., 1997; Le-Clech 2003; Howell et al., 2004; Liu et al., 2005). It is likely in the case of side stream MBR's that the shear generated by the pumping will increase cross-flow velocity and thereby supply aeration around the membrane to provide shear stresses. However, the increasing of membrane aeration levels brings an increase in reactor operating costs. To solve this problem, new jet and cycling aeration systems have been produced. When the two systems are employed in membrane bioreactors to control membrane fouling, they are very efficient, and reduce energy demand.

Another method to prevent fouling involves an investigation of properties of liquor and the potential of altering these to minimize fouling. The addition of activated carbon in a mixed liquor has been found to decrease fouling of the membrane whilst at the same time having the potential to reduce organic acid levels in the membrane bioreactor (Ng & Hermanowicz 2005; Kim & Lee 2003).

Another significant factor in the prevention of fouling is the membrane cleaning process. Normally the protocol for membrane cleaning is supplied by the membrane manufacturer. Membrane cleaning has been studied by many

researchers and they have found that it interacts with membrane fouling. Most researchers are interested in investigating the duration and frequency of membrane cleaning and the back-flush flux. Jiang et al., (2005) found that less frequent and longer back flushing is more efficient in avoiding or reducing membrane fouling when compared to more frequent but shorter back flushing

2.10.4 Biological process performance of membrane bioreactor

In the MBR, most of the organic carbon is digested by microorganisms such as bacteria, algae or fungi. All of the microorganisms grow in the media and tend to form flocs due to the formation of micro colonies. This is very important because the ability of microorganisms to form flocs is related to the production of activated sludge. Activated sludge is important to the adsorption of soluble substrate, colloidal matter and macromolecules in waste water (Michael & Fikret., 2002 and Liwarska & Bizukojc., 2005). In activated sludge there will be a complex micro system where the dominant microorganisms will lead the degradation process (Michael & Fikret., 2002).

The MBR will separate and recycle activated sludge to maintain enough microorganisms to ensure ongoing progress of degradation activity. MBR processes can be encouraged by the use of mature sludge or high sludge concentrations. Therefore increasing SRT (solid retention time) and biomass concentration will impact on energy demand (Pirt, 1965). When the maintenance energy requirements of the microorganisms are satisfied it will produce additional biomass. Therefore, the consumption of nutrients has been studied to achieve high biomass and the lowest sludge loading through the concept of food (substrate) to microorganism ratio (F/M - g COD g TSS^{-1} day^{-1}) (Shahalam & Al-Smadi., 1993). Many researchers have studied low or zero sludge production, and they agree that it depends on feed compositions which identify the growth of microbial population (Liu et al., 2003; Rosenberger et al., 2000 and Yoon et al., 2002). Performance of the membrane can

be detrmined through the permeate flux, which is related to the area of the membrane. Equation 2.5 shows the performance of membrane.

2.10.5 Removal and filtration performance of membrane bioreactors

Membrane bioreactors have a known capability to remove and filter certain kinds of chemical, microorganism and degradation products. This section discusses how the MBR works and the necessary interactions required producing the desired material.

2.10.6 Nitrification and phosphorus removal

Normally waste water from industrial, municipal or arable land contains high levels of nitriates and phosphates. These affect both surface and ground water. It also leads to promoting the growth of certain species of weed and algae. Therefore, removal these nutrients is important to maintain water quality standards within levels prescribed by the World Health Organization (WHO).

The contamination of water by nitrate or nitrite is very closely related to several diseases such as gastric cancer, methemoglobinemia (also known as blue baby disease) and induces mutations of DNA (Hansson et al., 1994 and Doyle et al., 1985). Nitrates in waste water are usually removed by bio treatment which reduces the nitrate to nitrogen gas. However, because of the low growth rates and poor cell yield of nitrifying bacteria, nitrification is generally a very limited step in biological nitrogen removal (Barnes & Bliss 1983). MBR technologies offer the next step to the solution of that problem. The application of long SRT in membrane bioreactors will prevent nitrifying bacteria from being washed out and will improve the nitrification capability of the activated sludge.

Phosphorus can be detected in the waste water as a phosphate either arising from precipitation or adsorption. However only small amounts of phosphorus are used for cell metabolism and growth (1-2% of the total suspended solid (TSS)

(Lesjean et al., 2003)). The process for the removal of phosphorous is well known - enhanced biological phosphorus removal (EBPR). EBPR could be improved and more widely established by the adoption of membrane bioreactors in the filtration process. Groups of organisms can capture phosphorus – these groups are called polyp. Polyp is normally larger than 0.5μm and the pores of a microfiltration membrane (~0.2μm). As such, the microfiltration membrane will be a physical barrier to retain the polyp organism, which will then provide sufficient biomass for EBPR (Beun et al., 2001 and Ujang et al., 2002).

2.10.7 Microorganism removal

This is a very interesting achievement for MBR technologies, where the reactor could completely remove microorganisms through filtration. Many researchers have found that MBR treatment has the capability to remove vegetative bacteria (Madaeni et al.,1995; Ottoson et al., 2006 and McGahey & Olivieri 1993). Submerged module MBR's have been found to offer the best configuration for increased rates of bacteria removal over a few weeks through the development of biofilm in the membrane surface (Van Voorthuizen et al., 2001 and Farahbakhsh & Smith 2004). Complete removal of microorganisms, especially viruses using a membrane requires the use of ultrafiltration under appropriate conditions due to the pore size of the membrane (Gander 2000). However, reserve osmosis membranes have been found to be more effective in completely removing viruses from wastewater without any other treatment (Cooper & Straube 1979).

2.10.8 Removal of degradation product

Normally, in the bioreactor system many products will be produced during the complex degradation and biochemistry processes. Most of those products are very serious pollutants which, if exposed directly to the environment, will affect the

Normally, in the bioreactor system many products will be produced during the complex degradation and biochemistry processes. Most of those products are very serious pollutants which, if exposed directly to the environment, will affect the environment, especially that of the aquatic world.Therefore, all of the products resulting from the use of MBR's have to be separated, safely managed and further reprocessed in a controlled manner before being used in the target sectors.

2.10.9 High intensity fatty acid and solvent production using MBR

The combination of *C.butyricum* with MBR technology has the potential to produce useful chemicals as detailed in the foregoing discussion. Even though there is no documented record of the combination of a membrane bioreactor with *C.butyricum,* the characteristics of the microbe and the equipment indicate excellent potential for the production of useful chemicals.

Jung&Lovitt(2010) found the application of MBR has a potential to enhance the production of lactic acid bacteria (LAB) such as lactate, acetate and ethanol. In that study the cell concentrationswere 20 times higher than in a stirrer tank reactor.

Mostafa (2001), compared the immobilized cell and cycle cell (MBR) method for acetic and glycerol production by *Kluyveromycesfragilis.* The MBR's results are much higher than the immobilized cell results.

Chapter 3: Materials and methods

3.1 Introduction

In this chapter an explanation of the methods, chemicals and equipment that were used is presented. All of the chemicals were supplied either from Fisher Scientific or Sigma Aldrich.

3.2 Culture and preserve microbe

Clostridium butyricum NCMB 7432 used in this research was supplied by NCIMB LTD (23 St. Machar Drive, Aberdeen, U.K). *C. butyricum* can be found and isolated from soil, waste water, animal digestive tracts and contaminated dairy products. The general characteristics of clostridia are; gram-positive, heterotrophic, and strictly anaerobic and that it readily forms spores. The cultivation conditions for Clostridia is in the mesophilic range, with a pH from neutral to around 6.5. In this study, investigation of the fermentation of a carbohydrate was undertaken.

3.2.1 Materials and methods for preparation of the microbe culture

Table 3.1 shows the ingredients of the resuscitation media that was used to culture *C. butyricum.* The media were placed in 30ml anaerobe bottles and flushed with nitrogen gas at a pressure of 1.5 bar, closed with a rubber cap and sealed with a aluminium cap. The medium was then autoclaved for approximately 15 minutes at 121°C. The autoclaved anaerobe bottle was then allowed to cool for four hours. The bottle seal integrity ensures that bottle remains free of air or oxygen for several days.

Table 3.1: Recitation media for culture *C.butyricum*

Ingredients	g/L
Soy peptone	2
Yeast extract	5
Glucose	1
Rezurin	0.05%
$(NH)_4SO_4$	0.5

3.2.2 Resuscitation of the freeze dried culture

The tube containing freeze dried bacteria was broken at a pre-scored line. 0.5ml of the prepared sterile medium from the anaerobe bottle was taken in a sterilized syringe and mixed with the freeze dried bacteria using a 1 ml syringe. The resulting solution was then drawn back into the same sterilized syringe and injected through the rubber seal into the medium filled anaerobe bottle. Then the bottle was incubated for 24 hours at 37°C. After 24 hours of incubation, the bottle exhibited a cloudy appearance. Figure 3.1 shows the cloudy appearance as compared with an inoculated culture. For larger batch and continuous cultures, the above process was served as an initial source of seed culture that was scaled up to 500 ml bottles.

Figure 3.1: A comparison between inoculum and not inoculum media bottle

3.2.3 Preserving the microbe

Preservation of the microbe is very important to ensure a stock of microbe remains available over the experimental phase of the investigation. 3ml of sterile 20% w/v glycerol solution was added to a sterile 30mL anaerobic bottle. Next, a 3ml sample was taken from the fresh fast growing culture and injected to the same bottle and the contents mixed. . Finally, the bottle was frozen and stored at -80°C. To revive the microorganism, the preservation vial was removed from the freezer and defrosted at room temperature until the glycerol microorganism suspension had melted (Figure3.2). It was then be transferred to culture in the new growth medium (Table 3.1) and cultured as in section 3.2.2.

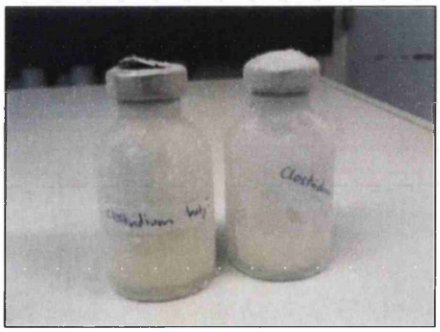

Figure 3.2: Even in the freezer, anaerobic conditions are most important for clostridium bacteria.

3.2.4 Purity of Cultures and illustration of *Clostridium butyricum.*

To confirm the purity of cultures in the early stages inspection using phase contrast optical microscopy was performed (Olympus CX21, Olympus, UK).

SEM (Scanning Electron micrographs) of *C. butyricum* was also investigated. First of all, the sample was dropped on the clean slide, with the slide located in the petri dish. Then, it was air dried at room temperature. After that the sample can go through to SEM. Figure 3.3 shows the size for single cell *C. butyricum* (approximately 1.4 x 0.5μm). Figure 3.4 shows the colonies of *C. butyricum* and the rod shape of the bacterium.

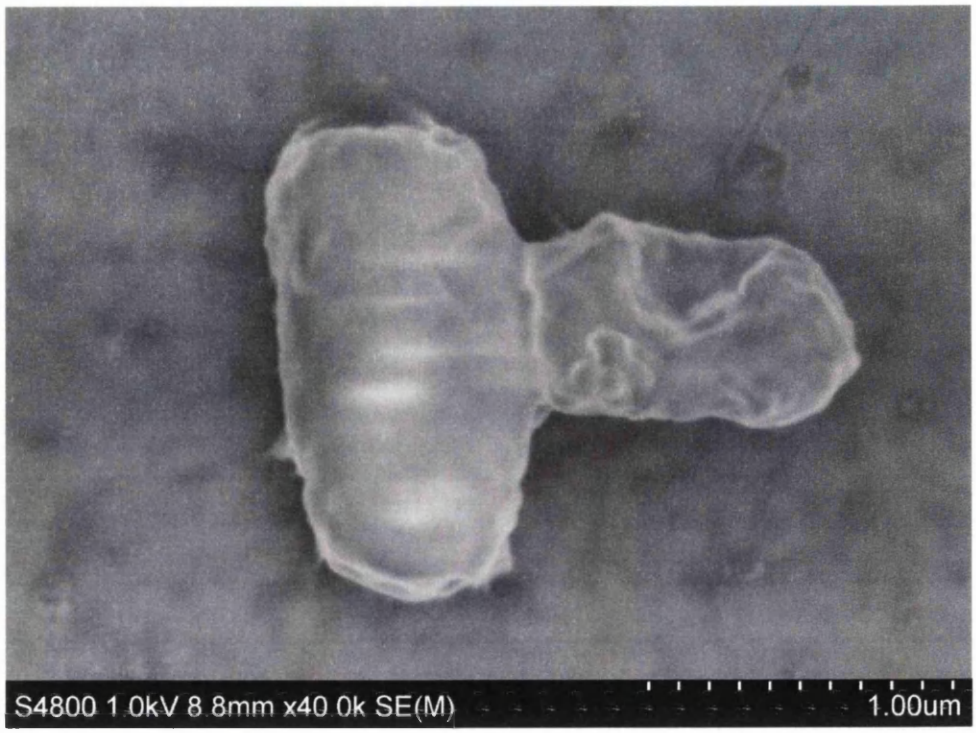

Figure 3.3: Two single cell of *Clostridium butyricum*

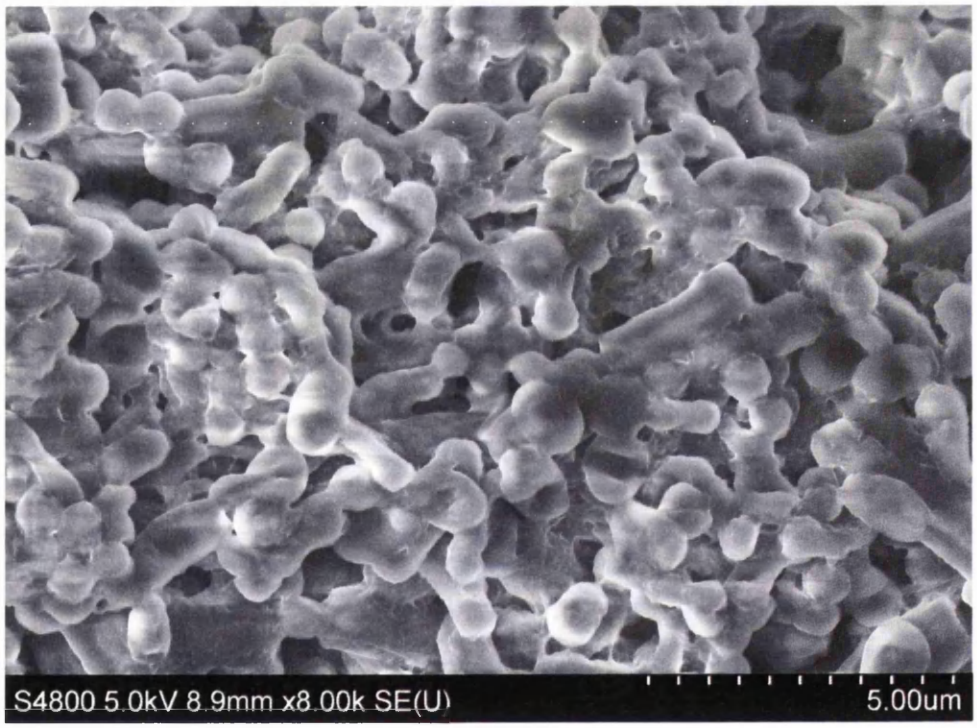

Figure 3.4: Colonies of *Clostridium butyricum*.

3.3 Inoculum preparation

Typically the inoculum consisted of 10% of the fermentation volume. The inoculum was cultured in the anaerobe vials and incubated in the medium at pH 6.5 and 37°C. The inocula were started with 30mL serum vial cultures containing 25mL of growth media. Then, 4mL was taken and transferred to 50mL serum vials containing 40mL of growth media. This was the incubated for 24 hours. The process was repeated to 500ml bottle containing 250ml of growth media where 25ml was taken from the 50mL serum vial and transfer to 500mL. Figure 3.5 shows a diagram of the process of inoculum preparation

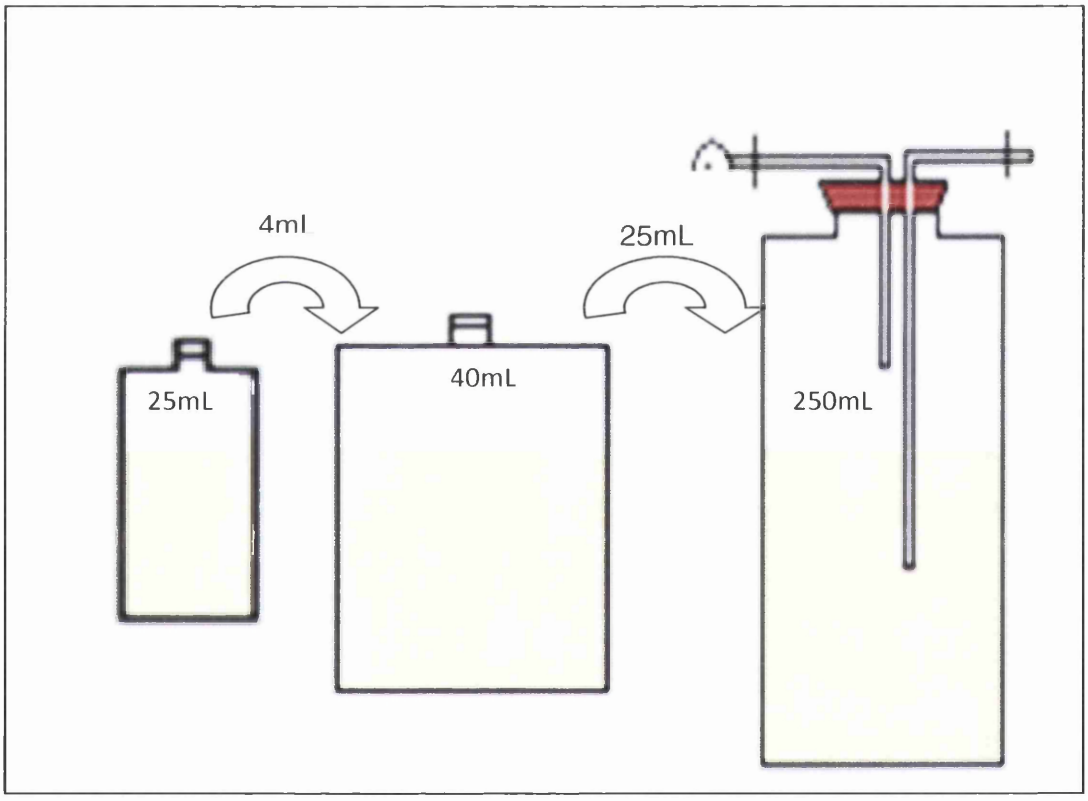

Figure 3.5: Process of inoculums preparation.

3.4 Preparation for anaerobe test tube culture

The use of small test tube cultures was very important to provide information for medium optimisation for growing the microbe and these are documented in chapter 4. Five sets of experiments were carried out using anaerobe tubes. For each experiment 18 inoculated tubes were used and one blank. The medium was first formulated and dispensed to the test tubes as detailed below; they were then gassed with nitrogen, sealed and then sterilised by autoclaving. Further specific additions were made to the medium depending on the variable under investigation. 1 ml of inoculum was added to 9 ml of culture medium. The amount of growth was measured directly by using an optical density UV spectrophotometer within 1.8 mm light path (i.e. the tube diameter) at 660 nm.

3.4.1 Material preparation for the anaerobe test tube

First, the variables and constants were fixed for each experiment. Then, 1L of distilled water was boiled at 70°C to remove all traces of oxygen in the water. Next, the 19 sterilized tubes were prepared for every experiment. The ingredients were mixed with distilled water using a magnetic mixer. The water was allowed to stabilise at 30°C. The ingredients were prepared as detailed in Table 3.2. Resazurin (0.0005%) was added as an indicator to ensure the absence of oxygen in the medium. For each concentration two tubes were prepared. For example, in experiment 1, two tubes were prepared for each of 0, 0.5, 1, 2, 3, 5, 10 and 20g/L and were mixed with the other ingredients detailed such as glucose, KH_2PO_4 and ammonia sulphate. Thereafter, a blank tube containing standard media was prepared. All of the tubes were flushed with nitrogen gas and securely capped to maintain the anaerobic condition. Then, the tubes containing the media were autoclaved at 121°C for 15 minutes. The tubes were then left to stabilise for 2-3 hours. 0.5 ml of inoculum was then injected to every single tube (excluding the

blank). The tubes were then incubated at 37°C. Readings were taken from the tubes with a UV spectrophotometer (wavelength 660 nm) every half hour for 12 hours.

Table 3.2: Variables and constants for anaerobe tube test for optimization of medium

Experiment	Constant	Variable
Experiment 1	Concentration of : Ammonia sulphate (5g/L) KH_2PO_4 (5g/L) Glucose (10g/L) Resazurin (0.05%)	Yeast extract concentrations : *0,0.5,1,2,3,5,10,20 (g/L)*
Experiment 2	Concentration of : Yeast extract (10g/L) KH_2PO_4 (5g/L) Glucose (10g/L) Resazurin (0.05%)	Ammonia sulphate concentrations.: *0,0.5,1,2,3,5,10,20 (g/L)*
Experiment 3	Concentration of : Ammonia sulphate (5g/L) Yeast extract (10g/L) Glucose (10g/L) Resazurin (0.05%)	KH_2PO_4 concentrations.: *0,0.5,1,2,3,5,10,20 g/L (g/L)*
Experiment 4	Concentration of : Ammonia sulphate (5g/L) KH_2PO_4 (5g/L) Yeast extract (10g/L) Resazurin (0.05%)	Glucose concentrations : 0,0.2,0.4,0.8,1.0,1.4,1.6,1.8 (g/L)

3.5 Preparation for the batch system

For this series of experiments, the media formulations were based on the results of the anaerobic tubes tests. Overnight experiments were conducted using varieties of carbohydrate as a variable at 37°C and pH 6.5. A water bath was used to control the temperature and NaOH for the control of pH via a titration system. This is described in section 3.5.1.

3.5.1 pH controlled batch culture.

A 2L jar fermenter with a diameter 12.5cm and a height of 21cm was prepared. The top of the fermenter consisted of a ground glass flange on to which a silicon rubber sheet of 15 cm in diameter was clamped. A series of 12 x 1.2 cm holes were cut to place ports or glass tubing to allow for addition of material and probes to

measure variables within the the reactor. Figure 3.6 shows the side and plan views of the batch fermenter and cap. A metal ring was also prepared with dimensions to suit the rubber for the cap fermenter. The ring was designed so as not to close the holes. Then, rubber was laid on top of the fermenter and attached with the metal ring. This assembly was secured with a special clip.

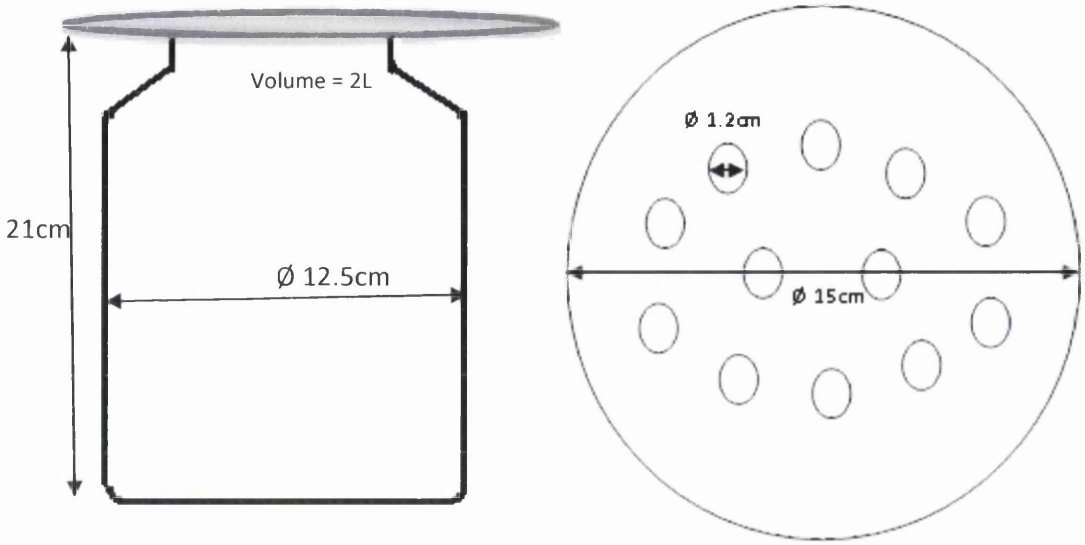

Figure 3.6: Side and plan views of the batch fermenter.

Figure 3.7 shows the fermenter complete with arrangements. The fermenter was connected to other equipment via the ports, for example the alkali addition system including pump and pH meter; gas in and out, and addition ports for inoculations and sample together with temperature control by thermostatic water tank and heat exchanger.

Both gas in and gas out tubes, were fitted with a filter (Polyvent filter, 0.2μm, Whatman filters UK) to avoid air contamination of the fermenter. An autoclavable pH probe (Russell, UK) was fitted. For automatic control of pH, a pH meter and pump unit (Electrolab FerMac 260, Electrolab, UK) were connected to dose the fermenter with NaOH (2M) as required to control the pH. The amount of NaOH (2M) used was

recorded by weighing the alkali reservoir directly on a balance (0.01 g). A liquid port was also included for sampling the process. This port was fitted with silicone tubing which was closed by a pinch valve. To ensure temperature stability a water bath was connected to the water supply and return tubes in the fermenter via a stainless steel heat exchanger.

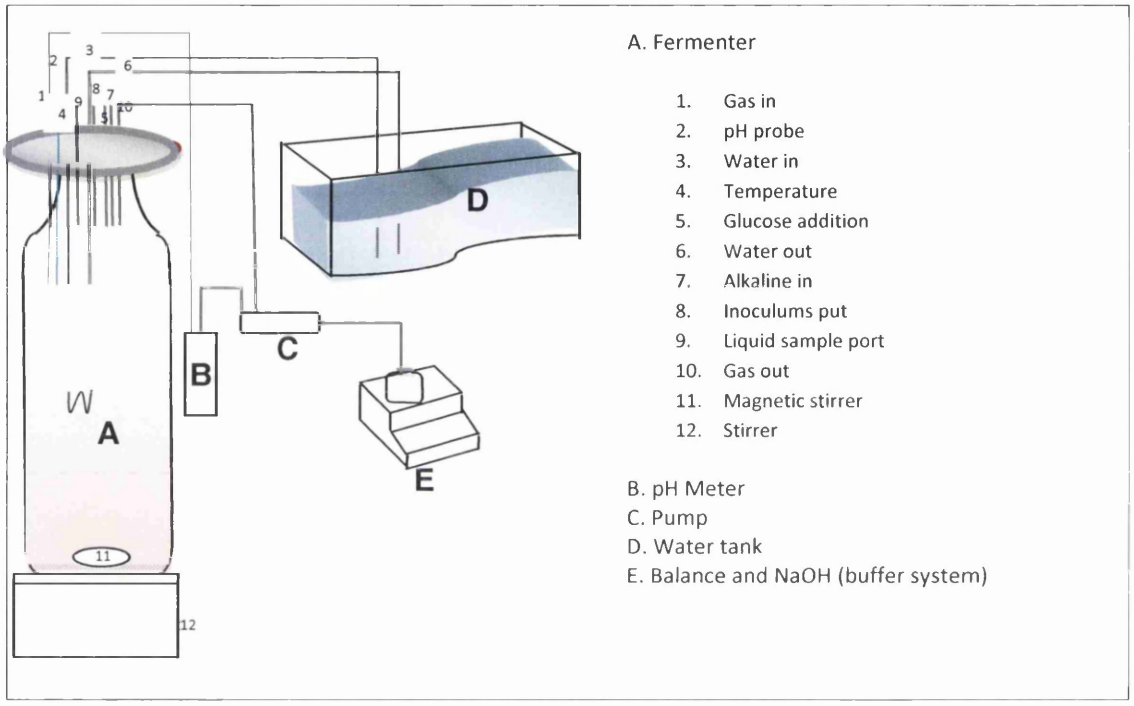

Figure 3.7: The batch process system.

3.5.2 Preparation and sterilisation of the batch fermenter

2L of distilled and boiled water at 70°C was used for every experiment. All of the ingredients were mixed with a magnetic stirrer after the water had cooled to 30°C. The fermenter was mounted on a magnetic stirrer which was coupled to the Teflon stirrer bar within the reactor. Then the pH was adjusted with either 0.5M of sulphuric acid or sodium hydroxide. Table 3.3 shows the ingredients for each experiment. 1.8L of media was poured in the 2L batch fermenter. Then the rubber flange was sandwiched between the ground glass flange and a metal ring and secured by

clamps. Then all ports were sealed with the exception of the gas out port which was fitted with a micro filter to allow gas exchange but maintain sterility. The top of the pH probe was wrapped with cotton and foil. The ends of alkali tubes, liquid sample tubes, gas entry and exit tubes and inoculum port were prepared in similar ways. Next, the fermenter was autoclaved at 121°C for 15 minutes.

After sterilisation the sensor and control equipment were fitted, a thermometer was installed in the thermometer holder tube and the equipment was left to cool and stabilise at 35-30°C. In the next step, the fermenter was connected with the water bath to maintain the temperature at 37°C and the fermenter was flushed with nitrogen through the gas supply port. The system was flushed for 20 minutes with nitrogen. The system was then inoculated by adding 180ml of culture which was added by gravity with a slow nitrogen flow. The slow flowing nitrogen supply was maintained throughout the fermentation process. Readings and samples were taken every hour for 24 to 48 hours, with the main measurements being optical density (see section 3.9) with the UV spectrophotometer and the amount of NaOH (2M) consumed.

Again, information collected from test tube batch system experiments were used to design these experiments. Six experiments were conducted with glucose as the variable source of carbohydrate and other ingredients are maintained at constant levels. Two other experiments were also performed on the pentose sugar, xylose and the polymeric substrate, starch. Table 3.3 shows the medium formulations for these experiments.

Table 3.3: Ingredients for batch culture experiments

Experiment	Ingredients	Source of Carbohydrate
Experiment 1	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	0 g/L of glucose
Experiment 2	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	5 g/L of glucose
Experiment 3	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	10 g/L of glucose
Experiment 4	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	15 g/L of glucose
Experiment 5	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	20 g/L of glucose
Experiment 6	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	28 g/L of glucose
Experiment 7	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	15 g/L of xylose
Experiment 8	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	15 g/L of starch

3.6 Preparation and experimental methods for the 1.8L continuous system

The equipment set up for continuous cultures was as described above with the exception that the culture vessel had an open weir port to allow operation at a constant volume of 1.8 L.

All of experiments in the continuous culture were carried out as described in section 3.6.1 to 3.6.3 in the previous pH controlled batch experiments with a temperature of 37°C and pH 6.5. Each experiment took 400 hours with readings being taken every 5 to 8 hours. At least 5 readings were recorded for each flow rate for each experiment to determine the steady state.

3.6.1 Construction of the continuous culture

The continuous fermenter is quite similar to the batch fermenter with the exception of the weir to allow outflow of liquid. The system included a peristaltic pump which feeds fresh media through a silicon tube, at a constant rate into the vessel. In addition, a 20L aspirator bottle for the storage of fresh medium was also added together with a drain vessel to receive the outflow from the culture. The fresh medium aspirator bottle had tubes for gas supply and exhaust. Again both the input and output gassing lines were fitted with a filter (Polyvent filter, 0.2μm, Whatman Filters UK). Figure 3.8 shows the fermenter and full system diagram.

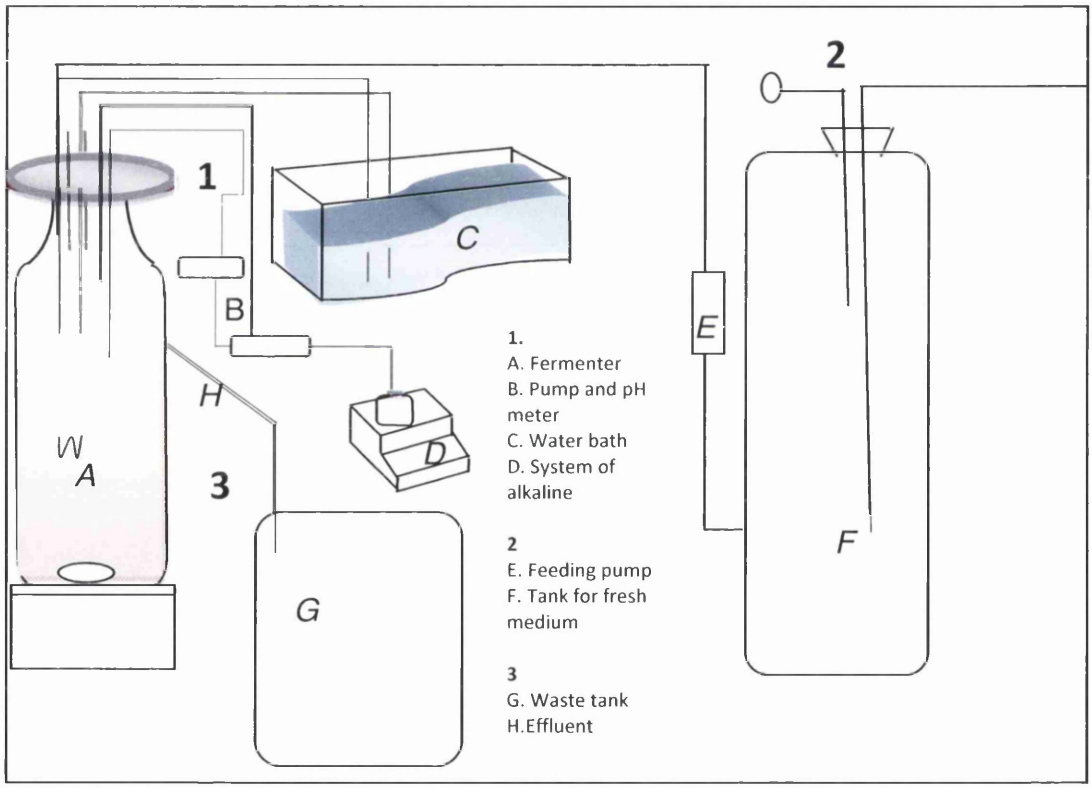

Figure 3.8: Full diagram for continuous system.

3.6.2 Calibration of the feed pump flow rate

An investigation of the feed pump was carried out to characterise the capability of the pump to deliver the medium flow rates required in the continuous

culture experiments. The tube for feeding media to the fermenter was connected to the pump. Using a stopwatch and measuring cylinder the flow rate of the pump at different setting was determined. Appendix 1 shows the calibration results for the pump. The feed flow rate was also influenced by the length of the tube from pump to fermenter. The results detailed in Appendix 1 reflect the flow rate of the complete feed system.

3.6.3 Preparation of medium for the continuous system

The preparation of media for the continuous system is similar to that for the batch system, particularly in respect to preparation of media for the fermenter. The difference being that a total of 20L of fresh medium was prepared with a variety of glucose concentrations. Table 3.4 shows the concentrations of glucose tested with a variety of nutrient feeds. First, 20L of distilled water was prepared. Then the correct proportions of media were mixed and the pH adjusted by the addition of 0.5M sulphuric acid and 0.5M sodium hydroxide. Then, nitrogen gas was flushed through the system for 20-30 minutes. Tube attachment, instrument installation and autoclaving then followed, using the same protocol as the earlier trial.

Table 3.4: Formulated of ingredient and flow rate for every experiment in the continuous system.

Experiment	Ingredients	Source of Carbohydrate	Flow rate of feeding (ml/h)
Experiment 1	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	5 g/L of glucose	70 140 200 252 280
Experiment 2	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	10 g/L of glucose	70 140 200 252 280
Experiment 3	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	15 g/L of glucose	70 140 200 252 280
Experiment 4	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	20 g/L of glucose	70 140 200 252 280
Experiment 5	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L) Rezurin (0.05%)	28 g/L of glucose	70 140 200 252 280

3.7 Preparation of membrane bioreactor

The membrane bioreactor used was developed by with the Centre of Complex Fluids Processing of Swansea University, College of Engineering. This reactor was located and operated in the fermentation laboratory. It was equipped with a fermentation vessel as the main reactor, valves for control of air and fluid delivery and a exhaust and heat exchanger control. A ceramic membrane (0.2 micron, membrelox) was combined with the reactor to incorporate filtration purposes. The reactor has two pumps for pumping and pressurising the fluids within the systems. Figure 3.9 and 3.10 shows a photograph and diagram for membrane bioreactor.

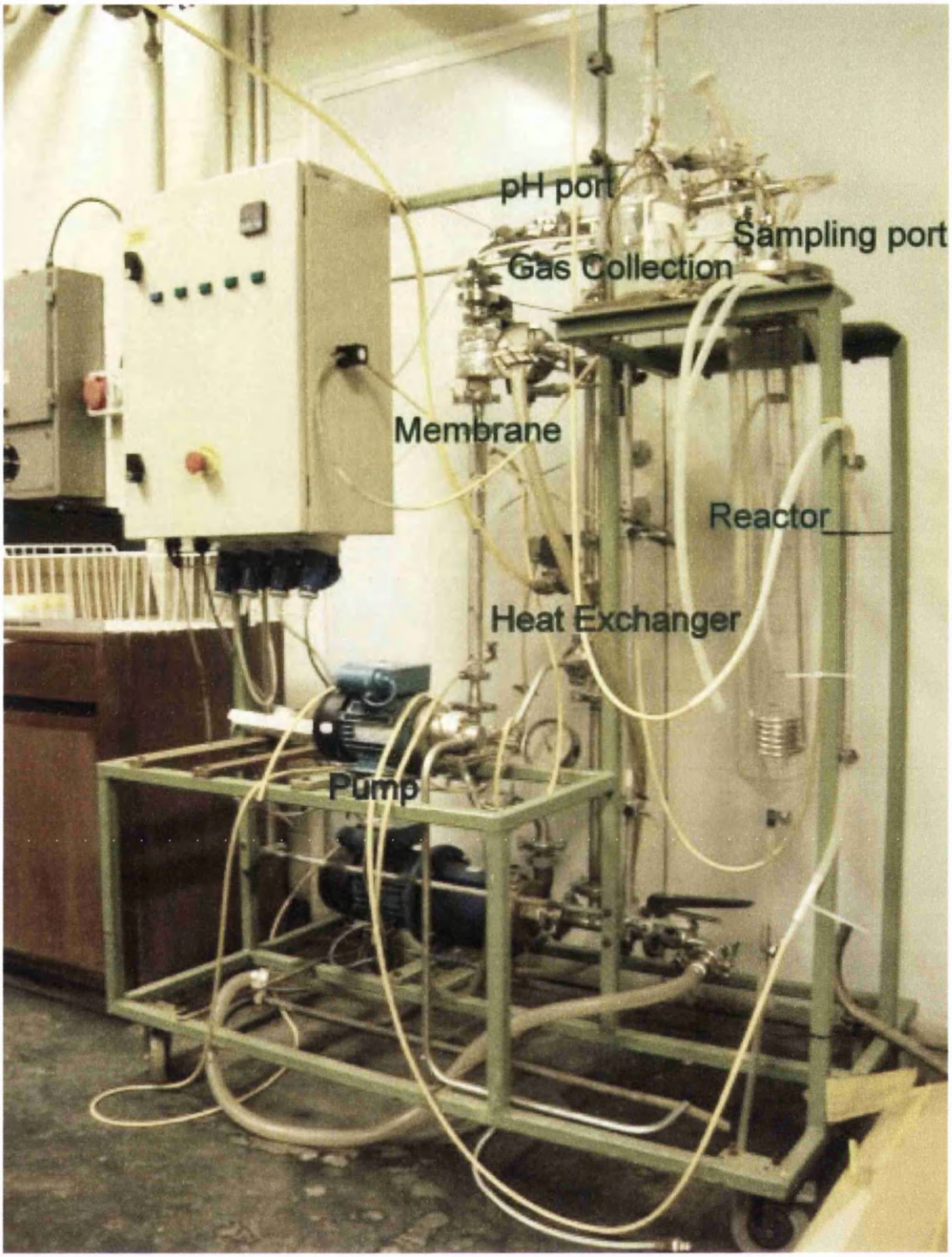

Figure 3.9: Photograph of the MBR used in this study

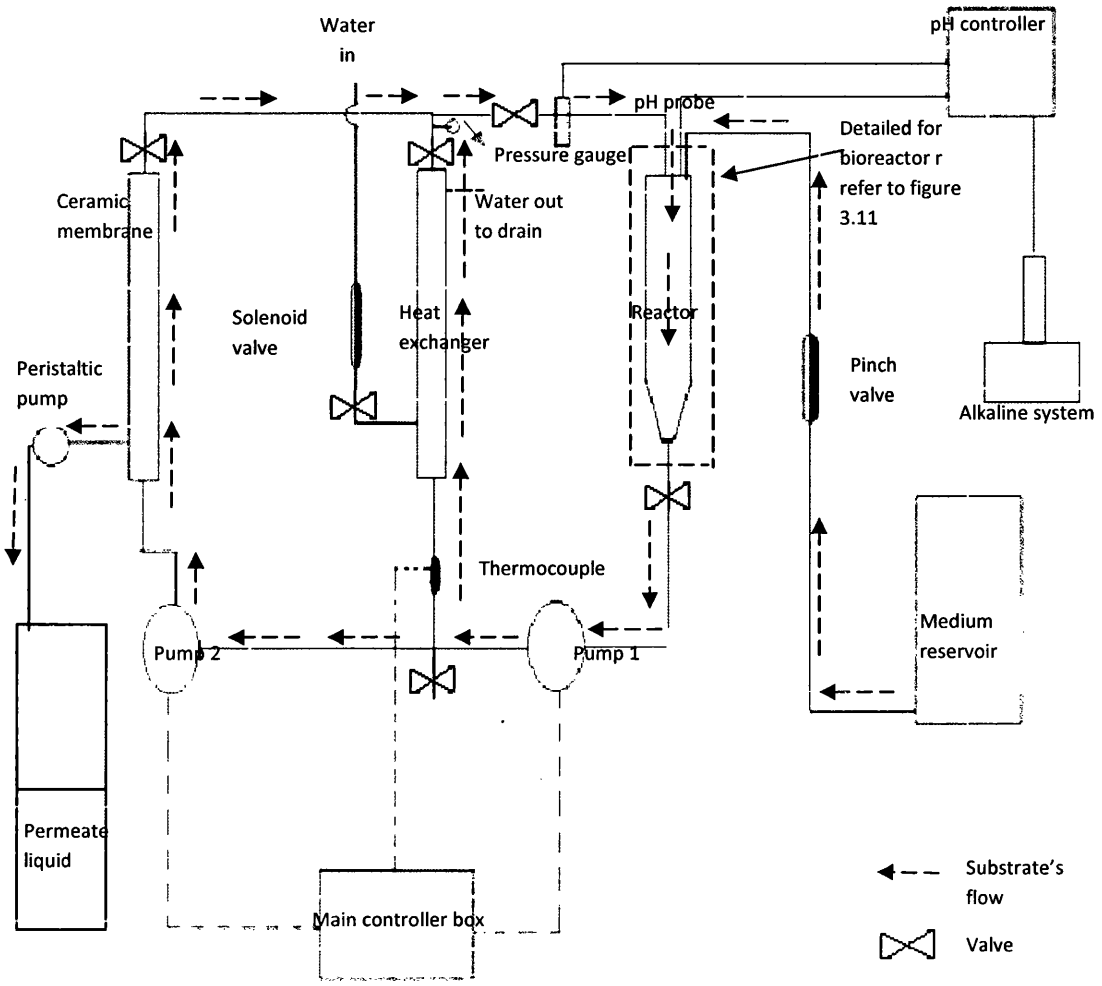

Figure 3.10: Flow sheet of membrane bioreactor

a) Bioreactor

The bioreactor was capable of operation in the fully anaerobic conditions and consisted of five principle components linked together by 1 inch stainless steel tubing, i.e, a reactor vessel, a heat exchanger, the ceramic filter module, pumps and valves.

The main reactor vessel was a 5L glass pipe section with a conned end and fitted with a stainless steel top plate fitted with appropriate ports. The bioreactor was operated in the 5 L volume with 760 mm depth and 120 mm in diameter. Before using the bioreactor, it was sterilized empty, by autoclaving for 30 minutes at 121°C.

The bioreactor has been equipped with a tube for feeding fresh media, recycling substrate and for adding NaOH (4 M). Tubes for the gas inlet and outlet were fixed at the top of the bioreactor. The tube for the gas inlet allowed nitrogen gas to the bioreactor. While the tube for the outlet was fitted with a trap to allow the gas to pass through and prevent diffusion of air containing oxygen back into the bioreactor. Figure 3.11 shows the diagram of bioreactor.

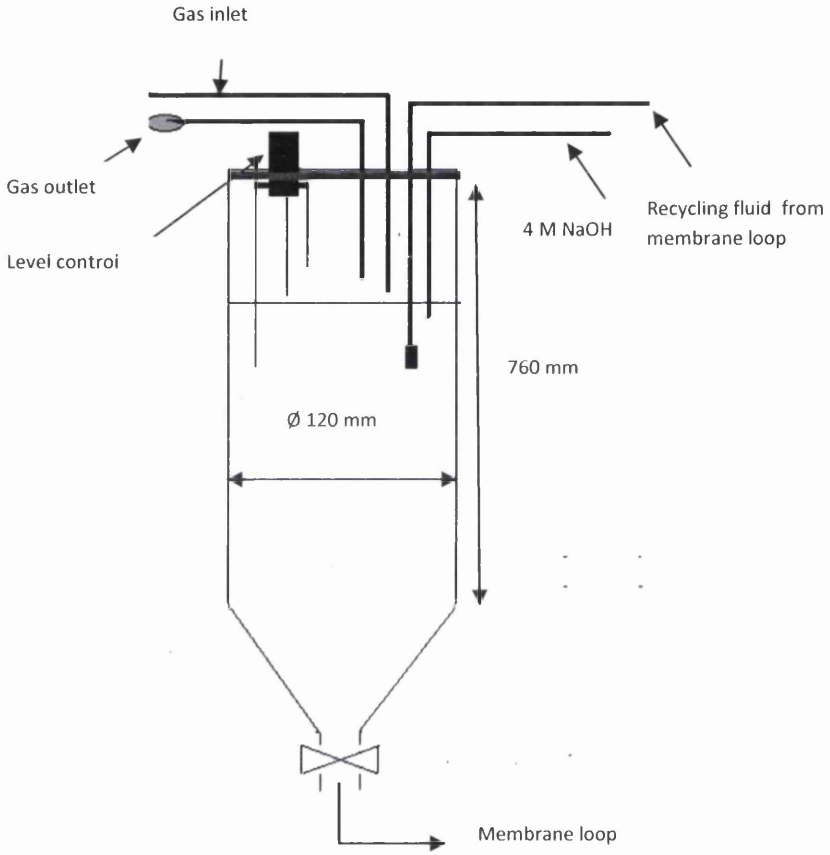

Figure 3.11: Diagram of bioreactor in the MBR system

b) Pumps

In this system, two stainless steel magnetically coupled pumps are installed both were purchased from Michael Smith Engineers. Two other peristaltic pumps were used but were associated with auxiliary functions of pH control and medium feed flow.

1) Pump 1

This pump responsible to circulate fluid around the MBR system. It is smaller than pump 2 and it is a gear pump (Brook Hansen from Micheal Smith Engineer Lid.), type PD 63SFH, 0.12 KW and 2870 r/min. This pump was operated within a flow rate of 3 litre/min up to 3 bar.

2) Pump 2

This pump is very important to avoid fouling of the membrane by providing the scouring action of the process fluid. This gives good heat exchange and cross flow velocity within the membrane system. High flow rate was therefore required (100 L/min). It is large centrifugal liquiflo pump (Brook Hansen from Michel Smith Engineers Ltd.), type ED 80 BD, 0.37KW, 2820 r/min. This pump was capable of delivering about 1 bar pressure but operated much lower than this.

3) Medium feed Peristaltic pump

A peristaltic pump (Watson Marlow Ltd. England) model 501UII10 -0.50Hz, was used in this study to control the flow rate into the fermenter. This function was achieved by attaching the peristalic pump on to the membrane effluent. The flux through the membrane is therefore controlled by the pump. The feed into the system was indirectly controlled by a level controller that actuated gravity flow via a pinch valve. The pinch value was actuated by a conductivity level sensor within the bioreactor. The circuit is broken when the liquid level is low and the pinch valve opens allowing medium to enter the system. As the level rises the conductive circuit is remade so closing the pinch valve. Thus the flow through the membrane flux controls the feed into the system but this is an indirect method.

The peristaltic pump can also be used to clean the membrane creating a back flush through the membrane. Back flushing is very important to unblock the pores of the ceramic membrane and reduce fouling.

Figure 3.12 shows the flow of fluid in the reactor as influenced by the two pumps. The flow path is from the bioreactor to pump 1. Then, when pump 2 is well primed the fluid goes to the filter module and the heat exchanger to maintain their temperature at 37°C, at the same time, some of fluid will also be returned to the reactor via the diaphragm valve. This flows back into the bioreactor and controls the pressure within the membrane loop of the system.

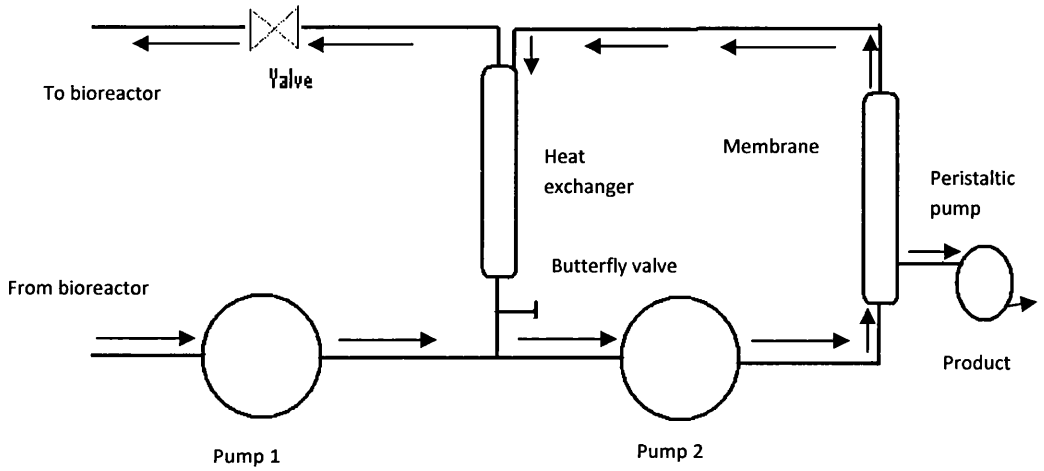

Figure 3.12: Arrangement of pumps. Pump 2 provides high velocity flow, pump 1 provides pressure working against the diaphragm valve.

c) Membrane module

In this study, a ceramic microfiltration membrane had been used for separation of cells and product. The membrane used in this study was a membrelox ceramic filter in stainless steel housing with a surface area of 1.4sq.ft, (0.13m^2) and pore size 0.02μm. The stainless steel membrane housing was purchased from PCI-Memtec, Swansea with a hieight of 340 mm. The maximum operating conditions for this membrane are 130°C, pH between 2 to 13 and pressure between 5-40psi. The fluid from the bioreactor passed through this membrane where the cells are retained so allowing cell recycle. The fluids containing the fatty acids were able to pass through the pores of this membrane and so leave the system as permeate via a

peristaltic pump. This arrangement allows the flow of permeate to be controlled. Figure 3.13 shows a diagram of fluid flow in the membrane housing.

Before the growth experiments in the MBR were carried out, calibration and capability of the membrane filtration module were investigated. Section 6.3 shows the result and analysis of the flux characteristics of the membrane as a function of operating pressure. The membrane and cake cake resistance and flux rate of the permeate are indicators of the status of the membranes and give a good gauge to membrane cleaning operations.

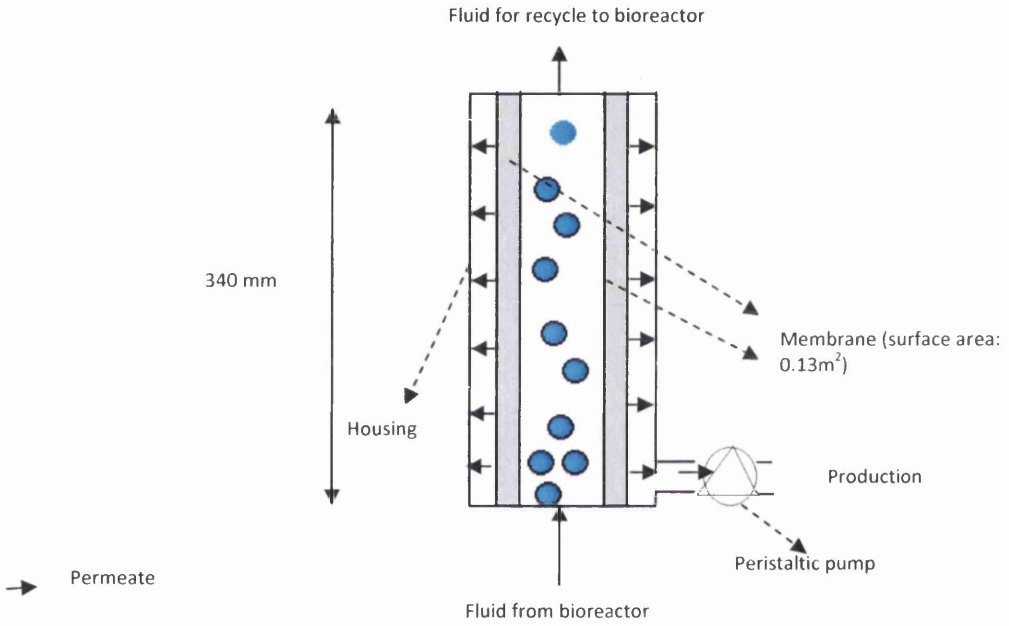

Figure 3.13: Flow of fluid in the ceramic membrane for separation.

d) Reactor Instrumentation

The reactor was fitted with the following instrumentation to set and control key environmental parameters.

1) Heat exchanger

Two pumps were used to run this system and because of this, the heat generation by the pumps needed to to be removed. A heat exchanger was used to

solve this problem and to control the temperature at 37 °C. A concentric tube heat exchanger used with the following dimension: inside diameter of the inner tube 22 mm, outer shell 435 mm by 50 mm. A thermocouple sensor and linked solenoid valve were used to control the flow of coolant to the the shell of the heat exchanger to ensure the temperature of the system was maintain at 37°C.

2) pH controller

The pH controller (Model 260-Electrolab Ltd.England, 115/230 V, 50/60 Hz, 50 W) is very important in this system to ensure that the pH in this system was stable. The function and calibration of this pH controller is similar to a standard bench pH meter. Calibration of the pH controller was done using buffers at pH 4, 7 and 9 at 37° C .The pH controller was fitted with a peristaltic pump (4 RPM 50 Hz motor, 0.87 ml/min) and was used to add alkali solution using a silicone tubing (bore 1.6 mm). The alkali used was sodium hydroxide (4 M). The balance was used to determine the amount of sodium hydroxide required to maintain the set pH. The alkaline dosing system was most important due to the production of organic acids in the system which reduce the pH in this system.

3) Level control

The level control was added to this system to ensure that the system maintained a constant volume. The level control consists of three electrodes and was located inside the bioreactor. The three electrodes are arranged vertically in the vessel, one 30 cm uninsulated electrode serves as a common part of the circuit while the two insulted electrodes, one of them is 5 mm longer than other one are the upper and lower level indicators. As the circuits are made or broken when the electrodes enter or leave the liquid. Thus when the fluid drops, below the longer electrode (low level) the pinch valve will be opened. This situation will allow the fluid to pass through

and enter the bioreactor until the level reaches the upper electrode. At this point, the circuit is made and the pinch valve will be closed.

4) Pressure gauge

The pressure gauge was installed in this system to monitor the transmembrane pressure (TPM). The pressure gauge was situated in the flow after the membrane.

5) Valves

Two kinds of valves were installed in this system and they have different functions. The first kind of valve is a diaphragm valve. It was located before the liquid entry into the main bioreactor vessel and pH probe (figure 3.10). The main function for this valve is to control the transmembrane pressure. 3 butterfly valves were also installed in this system and were used during the sterilisation, cleaning and draining processes. By opening and closing these butterfly valves the steam and fluids may easily be directed to ensure good sterilisation and cleaning.

3.7.1 Sterilisation

Before operation of the membrane bioreactor commenced, sterilisation of the equipment was necessary to avoid contamination. The first step in the sterilisation process was to detach the glass vessel (or bioreactor) and top steel plate from the stainless steel pipe work of the MBR. All the openings and flexible pipes were plugged with cotton wool and covered with aluminium foil. The vessel was then autoclaved at 121°C for 30 minutes.

Then, a steam line was attached to the top of the stainless steel pipe work. It was steamed for around ½ to 1 hours. Importantly, the steam line allowed steam for around 5 minutes to remove any dirt prior to attachment to the MBR. Three sterilisation routes were addressed in this way. The first route was from the top of stainless steel line through to the heat exchanger and the drain. The second was

from the top of stainless steel piping through the membrane and out to drain, and the third through the membrane or heat exchanger, and Pump 2 to drain (fig 3.10).

After completion of these three sterilisation routes the glass vessel (bioreactor) was connected with flexible tubing to the associated equipment. The membrane bioreactor was now sterilised and ready to use.

3.7.2 Cleaning of the membrane bioreactor

Cleaning methods are crucial to maximise the life expectancy of the membrane bioreactor so after completing each MBR experiment, a cleaning process was carried out.

First the system was rinsed with cold distilled water and drained. The MBR was then rinsed with warm water at a temperature of around 50°C. When the system appeared clean the water was allowed to drain from the membrane and stainless steel pipe work. Next, the drain was closed and the MBR was filled with warm water. Then sodium hydroxide was added to the system to make the system pH around 11. Then the system was drained and rinsed with cold water until the pH of the system becomes 7. This was used because dishwasher cleaner is suitable for cleaning complex polyphosphates. The MBR was then operated for 30 minutes with the output from the membrane recycled into the glass vessel, and with the membrane peristaltic pump (Pump 3) being used to back-flush the system.

3.7.3 Operation of the membrane bioreactor

Clostridium butyricum was cultured in the growth medium in large quantities (20L) and were used as inoculums for the experiments. This was added to the glass bioreactor vessel via the pinch valve and stainless steel top plate. Nitrogen gas (3psi) was continuously flushed into the bioreactor system. The level control system avoided overfilling or the emptying of fresh media. Fresh medium and inoculum were

left in the reactor until they had grown. At this time, the feed material was then added to the bioreactor and Pump 1 pumped the complex mixture round the reactor loop at a high velocity. The high velocity was designed to avoid membrane fouling. Some of the complex of fluids was passed through pump 3 and this is the final product. The remainder of the complex fluid is recycled to the bioreactor because it does not pass through pump 3. The processes then continued until it ran out of fresh medium.

3.7.4 Preparation of medium for MBR

In this stage 3 experiments were done. At the early stage, for every single experiment, 20 L culture was prepared and was pumped to the bioreactor. Then, pump 1 and pump 2 were switched on to allow the culture go to the membrane. The cells were retained on the membrane surface and recycled to multiply with the new substrate. Nutrients were used in this stage as shown in Table 3.5.

Table 3.5: Medium for substrate in MBR

Experiment	Ingredients (g/L)	Source of Carbohydrate (g/L)	Flow rate of production (L/h)
Experiment 1	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L)	5 g/L of glucose	4 8 16 32
Experiment 2	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L)	10 g/L of glucose	4 8 16 32
Experiment 3	Ammonia sulphate (5g/L) KH_2PO_4 (2.5g/L) Yeast extract (10g/L)	15 g/L of glucose	4 8 16 32

3.8 Gas Chromatography for volatile fatty acids

Volatile fatty acids were separated and determined by gas chomatography (GC) with FID detection. The GC was used here for the measurement of concentration of the production of acetic and butyric acid.

3.8.1 Apparatus

A VARAIN ProStar GC-3800 (USA) fitted with flame ionization detector (FID) was used. Hydrogen gas for the FID was produced in situ by a Hydrogen generator: UHP-20H NITROX (Swan Hunter, UK). While the air and the carrier gas, helium, were supplied as compressed gases from cylinders (BOC, UK). A 15m x 0.25mm x 0.25μm fused silica capillary column coated high-quality polyimide was fitted (Sigma, UK).

3.8.2 Operation condition and procedure

The standard operation for GC had been applied in this stage. Pressure 10.0 psi; Hold time (total) 15 min; carrier gas helium with 30mL/min; detector FID with temperature 220°C;

3.8.3 Headspace analysis of sample

A headspace method was used to analyse the concentration of acetic and butyric acid by GC. A 10 mL sample was taken and was added to 5g NaCl in the 30mL serum bottle. The bottle was sealed and placed in the water bath at a temperature of 50°C for 10 minutes. Once equilibrated, 0.5 mL of gas was taken with a glass gas-tight syringe from the headspace of bottle and injected into the GC for analysis. The responses of the GC were recorded and the concentrations of the fatty acids were calculated from a standard calibration.

3.8.4 Standard and calibration graph

To prepare a calibration, six concentrations for acetic acid and butyric acid were prepared and the headspace method was applied. The software of the GC was able to integrate the peak and give a value for the peak area. Using these data a graph of peak area verses fatty acid concentration could be constructed. Appendix 2 shows the calibration graph for acetic and butyric acid.

3.9 Spectrophotometry

3.9.1 Dry weight and biomass

Absorbance by UV spectrophotometer was used to measure in-directly the cell concentration expressed as dry weight. According to Willis, (1977), Demain &Davies, (2001), Avonts et al., (2004), optical density (O.D) can used to measure biomass or dry weight (g/L).

A calibration of absorbance for correction factor at 660 nm, 1cm light path verses cell concentration was carried out. Appendix 3 shows the results for calibration. OD(actual) versus OD(measured) was plotted. The relationship was slightly non-linear fitting the equation $y = 0.1977x^2 + 0.7935x + 0.0366$ with a R^2 value of 0.9987. All readings made with the spectrophotometer were converted by this correlation factor. The high R^2 value shows that the equation is valid.

To obtain the dry weight of the culture, 50 ml of fermentation broth was centrifuged at 4°C and 10000 rpm (MSE, SANYO) for 20 minutes to produce a cell pellet. The cells were then washed three times using distilled water and weighed after drying at 105°C for 24 hours. Appendix 4 shows the result for dry weight experiments. From Appendix 4, the relationship between dry weight and absorbance at 660 nm is $y = 0.0959\ x - 0.0006$.

3.10 The measurement of alkaline added by weight.

In the batch and continuous system experiments pH was controlled by alkali addition. A weighing scale was used to monitor the of amount of alkali addition. A container holding sodium hydroxide solution (2M) was placed on the scale pan, and the weight was recorded. Then further readings were recorded as material was used during the fermentation. The consumption of material was recorded in SI units (g). Though for standardization of results, the alkali addition results were converted to

concentration and thereby the amounts and rates of acid produced by the culture can be calculated and expressed as mMoles/litre/h.

3.11 Glucose assay

Glucose assays were conducted using the following technique. The assay was supplied as a kit from Sigma (GACO-20). This kit is based upon enzymes and is a sensitive, quantitative, method to determine glucose. Equations 3.1, 3.2 and 3.3 show the enzymes catalysed steps to determine the glucose. Glucose is reduced to gluconic acid with the production of hydrogen peroxide. In the presence of peroxidase this colour reagent o-Dianisidine will be oxidised and then stabilised in acid conditions

$$\text{D-glucose} + H_2O + O_2 \xrightarrow[\text{oxidise}]{\text{Glucose}} \text{D-gluconic acid} + H_2O_2 \qquad (3.1)$$

$$H_2O_2 + \text{Reduced o-Dianisidine} \xrightarrow[\text{Oxidized}]{\text{Peroxidase}} \text{o-Dianisidine (brown)} \qquad (3.2)$$

$$\text{Oxidized o-Dianisidine (brown)} \xrightarrow[\text{Oxidized}]{H_2SO_4} \text{o-Dianisidine (pink)} \qquad (3.3)$$

3.11.1 Preparation of reagent glucose assay

First glucose oxidise/peroxidise (G3660) reagent was prepared. It was supplied in a capsule containing 500 units of glucose oxidise from Aspergillus niger, 100 purpurogallin units of peroxidise from horseradish and buffer salts. 39.2 ml of deionised water was used to dissolve a capsule of glucose. Next, o-dianisidine reagent (D 2679) was prepared. It was supplied as 5 mg inside a sealed vial. 1.0ml of deionised water was added into the sealed vial and it was inverted several times to dissolve o-dianisidine reagent. All of these processes were done in amber bottles to minimize exposure to light. After that, 0.8 ml of o-dianisidine was added to the amber

bottle which containing 39.2 ml of glucose oxidise. Any turbidity were discarded and inverted were done for several times during the all of adding process. Then, sulphuric acid with a concentration of 5M was prepared.

3.11.2 Glucose analysis

a) Calibration graph for glucose assay

A standard curve was developed before measuring the samples. Appendix 5 shows the calibration graph for the glucose assay kit. For the calibration curve, 5 tubes were prepared with reagent: a blank and 4 standards. For the blank reagent tube 1 ml of distilled water was dropped in the tube. Next, for standard 1, 0.98 ml of distilled water was mixed with 0.002ml of glucose standard solution, (standard 2, 0.096 with 0.004ml glucose standard solution, standard 3, 0.094 with 0.006 ml glucose standard solution and standard 4, 0.092 with 0.008 ml glucose standard solution were prepared). After that, 2ml of assay reagent had been added to each tube. Then, 30-60 seconds was allowed for each subsequent tube. Then the tubes were placed in a water bath at 37°C for exactly 30 minutes. After 30 minutes, all of the tubes were taken out of the water bath and 2ml sulphuric acid solution was added to stop the reaction with intervals of 30-60 seconds for each tube. The spectrophotometer (wave length 540 nm) was used to measure the quantity of colour and the calibration graph was plotted.

b) Sample preparation

Samples were prepared in a similar way to the production of samples for generation of the calibration graph. Dilution of the samples was done with a ratio of 20 to 1. Then 1mL of diluted sample material was taken and poured into labelled tubes. 2mL of assay reagent was then added, reacted in the water bath and finally 2mL sulphuric acid was added to each sample tube to stop the reaction. Again,

readings were taken with the spectrophotometer at a 540 nm wavelength. The calibration graph was then used to determine the amount of glucose.

3.12 Xylose analysis

Xylose was determined using 3-5 dinitrosalicylic acid (DNS) reagent. The basis of this method is that it measures reducing sugars by reacting with the sugar to form a coloured complex that can be measured by the spectrophotometer at a wavelength of 550 nm. The concentration of xylose was determined by measurement against standard curves.

3.12.1 Preparation of reagent xylose assay

10.6g of 3-5 dinitrosalycylic acid were mixed in the 1.416L of distilled water with 19.5g NaOH in a 80°C water bath until all of the chemicals dissolved. Then, 300 g of sodium potassium tartrate was added slowly, followed with 7.5ml of phenol. After that 8.3g of sodium meta bisulphate was added.

3.12.2 Method and analysis of the xylose assay

a) Calibration graph for xylose assay

Eight different concentrations of xylose were prepared (0 g/L as blank, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 g/L). 2ml of each concentration were added to the test tubes. This was followed with 2ml DNS reagent. The test tubes were kept in the water bath with boiling water for around 5 minutes. Next, absorbance was determined with wave length 550 nm and a calibration graph was produced, as shown in the Appendix 6.

b) Sample preparation

Samples were diluted to 10 to 1. After that, 2 ml of the dilute samples were added in the test tubes. Then, 2 ml of DNS reagent were added in the same test

tubes. The reactions between DNS reagent and samples were done in the boiling water bath for around 5 minutes.

3.13 Starch analysis

An assay kit (STA 20) from Sigma was used to determine starch. The assay was based on the enzymatic hydrolysis of starch to glucose. The glucose was then converted to gluconate and hydrogen peroxide and then reacted with o-dianisidine (colour agent) to produce a brown colour. To form a more stable coloured product, oxidized o- dianisidine had been reacted with sulphuric acid and this can be detected spectrophotometrically at 540 nm. The assay was completed with α-amylase for the hydrolysis process and oxidized as a peroxidise reagent. Equation 3.4 shows the complete process for hydrolysis.

$$\text{Starch} + (n-1)\,H_2O \xrightarrow[\text{amyglucosidase}]{\alpha\text{-amylase,}} n\,(\text{-glucose}) \qquad (3.4)$$

After the process of hydrolysis was complete, the processes described above and in equations 3.1, 3.2 and 3.3 were then followed to give a colour reaction.

3.13.1 Preparation of reagent starch assay

In the starch assay kit α-amylase (A4582) was supplied in 25% propylene glycol and it was ready for use. For the starch reagent (S9144), this was supplied in the powder form which contains 50unit/ml of amyloglycosidase from *Aspergillus niger* and buffer salts in the sealed vial. 20 ml of water was added to the vial. The next reagents are glucose oxidise and o-dianisidine. The preparations for these reagents were explained in the section 3.8.6.1. Sulphuric acid at 12 N concentrations had been prepared.

3.13.2 Method and analysis of the starch assay

a) Sample preparation

5 ml samples were poured into the labelled tubes. 5 ml of 80% ethanol was added to the same tubes. Tubes were then mixed and incubated in the water bath at a temperature between 80-85 °C for 5 minutes. Then, a further 5 ml of ethanol was added. The tubes were then centrifuged for 10 minutes at 1000 x g. The supernatant was discarded. The pellets were suspended and mixed in the 10ml of the 80% ethanol solution. The centrifugation process was then repeated and the supernatant again discarded. The starch sample was then ready for assay

b) Starch digestion

2 ml of ethanol solution (80%) was added into each tube of sample and an empty tube for a blank. Then, 3 ml of water and 0.02 ml of α- amylase were added into each tube and then reacted in a boiling water bath for around 5 minutes. All of the tubes were then cooled to room temperature and 10 ml of water was added. 1ml was taken from each tube and this was mixed with 1 ml of starch assay reagent. The reaction mixture was then incubated at 60°C for around 15 minutes in a water bath. The tubes were then cooled to room temperature and 1 ml of each sample and the blank were diluted with 10ml water. Then, the amount of glucose was determined as described in section 3.8.6.2 (b).

c) Calculation measurement

$$\text{ØA}_{\text{standard}} = S_{\text{standard}} - S_{\text{standard blank}} \quad (3.5)$$

$$\text{ØA}_{\text{test}} = S_{\text{test}} - S_{\text{reagent blank}} \quad (3.6)$$

$$\%\ \text{Starch} = \frac{(\text{ØA}_{\text{standard}} \times F \times V \times SF \times SDF \times VGA \times MWF \times 100)}{(\text{Converstion Factor for µg to mg}) \times (\text{Sample weight in mg})} \quad (3.7)$$

$$\%\ \text{Starch} = \frac{\left(\text{ØA}_{\text{standard}}\right)\left(\frac{50}{\text{ØA}_{\text{standard}}}\right)(10)(2)(10)(1.0)(0.9)(100)}{(1000) \times (\text{Sample weight in mg})} \quad (3.8)$$

$$\% \text{ Starch} = \frac{(\text{ØA}_{\text{standard}})(900)}{(\text{ØA}_{\text{standard}}) \times (\text{Sample weight in mg})} \quad (3.9)$$

where;

F= µg glucose in standard / $\text{ØA}_{\text{STANDART}}$ at 540nm = 50/ØA_{540}
V=Initial Sample Volume (From sample preparation)
SF = Total assay Volume from Starch Assay/Sample Volume from starch assay
SDF = Dilution factor from end of starch assay
VGA = Initial sample volume from glucose assay
MWF = Molecular Weight of Starch monomer/ Molecular Weight of Glucose = 162/180 =0.9

3.14 Calculation of growth rate and doubling time

The specific growth rate is a fundamental measurement for assessing kinetic performance. This was obtained from an analysis of the growth kinetics, and was usually performed by monitoring the biomass concentration through analyzing the light scattering properties of the culture where there is a linear relationship between light scattering and biomass concentration. In this experiment, the concentration of cells, N (number of cells per unit volume) and t (time for growth) are recorded. This was used to get the amount of k (specific growth). Equation 3.10 shows the relationship between k (specific growth rate), N (number of cells per unit volume), and t (time for growth).

$$\frac{dN}{dt} = kN \quad (3.10)$$

Rearranging equation 3.10 to the form of equation 3.11

$$\frac{dN}{dt} = kdt \quad (3.11)$$

Integration of equation 3.11 between the limits of 0 to t and N_0 and N, giving.

$$\ln N - \ln N_O = kt \quad (3.12)$$

Equation 3.12 is converted to a common logarithm;

$$\log_{10}N = \frac{kt}{2.303} + \log_{10}N_O \qquad (3.13)$$

From equation 3.13, and taking the antilogarithm, equation 3.13 reduces to

$$\frac{N}{N_O} = 10^{kt/2.303} \qquad (3.14)$$

This equation can be linearised, where the numbers of cells always increase by the same factor in a given time interval. Commonly, a factor of 2 is used to characterize the growth rate and is called Doubling Time, or t_d. It is relatively straightforward to show that the Doubling Time, t becomes t_d when N_0 has increased to $2N_0$, and the kinetic growth rate, μ, is given by

$$\mu = {}^{1}/_{t_d} = {}^{k}/_{0.693} \qquad (3.15)$$

Chapter 4: Batch Culture

4.1 Introduction

In this section the results for the batch culture fermentation are presented. As a first step in the investigation the organism and its growth were investigated. The aims being to establish techniques to give reliable and reproducible growth and to gather basic but important information on growth of the organism in batch cultures, both in experiment tubes and pH controlled 2L batch cultures. The main methods of cultures are detailed in the previous chapter (section 3.2). The experiments were conducted at 37°C and pH 6.5 unless otherwise stated. Prior to starting, preliminary tests were carried out. This is important to optimise the nutrient content of the media employed.

Generally, for the anaerobic process the optimal pH is around neutral i.e. pH 6-8. Research by Wang & Jin, (2009), identifies that the best pH for *Clostridium butyricum* is pH 6.5, and as such pH 6.5 has been selected as the fixed pH in this process with it being reported that at pH 6.5, the product of fermentation will be increased compared to pH's 7.5, 7.0 and 6 (Wang & Jin, 2009).

The optimum temperature for cell growth of *C butyricum* was reported as being between 37°C and 45°C by Baek et al., (2006) and Wang & Jin (2009). However 37°C had widely use by previous researchers into the fermentation processes and its high efficiency for the production of product (Wang et al., 2004; Wang, et al., 2003). For this research 37°C degree was selected, being in the mesophilic range (30-37°C).

4.2 Preliminary growth tests

The first stage, to optimize medium for growth, was done by test tube experiments. The first experiments were conducted by observing the growth of *C.butyricum* in the recitation medium (table 3.1) by the methods given in section

3.2.2. Then, the optimum concentrations of nutrients for microbial growth were investigated to ensure the proportions of nutrients in table 3.2 were acceptable. Figure 4.1 shows the growth of *C.butyricum* in the early stage investigation of nutrient optimization. The growth of the culture was measured by the optical density at 660 nm. Obviously from figure 4.1 there was a 7h lag phase and from 8-12 h an exponential phase and from 11-24 h a stationary phase.

To identify the optimum concentrations of nutrient, two parameters were determined the growth rate (μ) and the maximum optical density (OD) the culture attained in the experiment. The method for determining growth rate is attached in Appendix 7, section 7A.2.

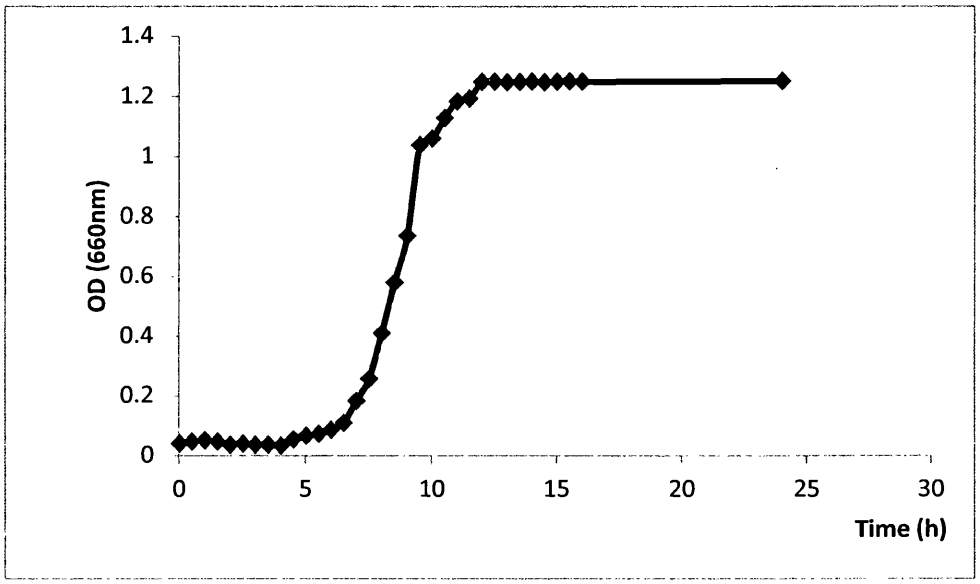

Figure 4.1: The kinetics of growth of *C. butyricum* on resuscitation medium (see section 3.2.1)

4.2.1 The effect of yeast extract on growth rate

The fastidious nutritional requirements, where the organisms require vitamins and other complex organics, yeast extract was added to the medium as a general source of these materials. According to previous researchers, yeast extract contains amino acids, water soluble vitamins, peptides, minerals and carbohydrates. All of which are very good nutrients for bacteria (Peppler, 1982). These experiments were

carried out using a medium formulated in table 3.2. Note that this does not contain peptone as this was seen not to effect growth when it was absent from the medium formation. It was therefore removed to make the medium simpler. The experiments were carried out in test tube culture as described in section 3.4. The growths of these cultures were monitored directly via a spectrophotometer adapted to take the pressure tubes. Figure 4.2 shows the effect of yeast extract on the growth rate and growth yield of *C. butyricum*. Clearly, the results show that the amount of yeast increases the rate of growth. However, when the yeast concentration is 0g/L there is little or no growth of the *C. butyricum*. Figure 4.2 also shows if the concentration is greater than 10g/L the growth rate decreases slightly, showing that the optimum growth rate is 10g/L, at which level the growth rate is 0.823μ. Similar work for OD maximum shows that the amount of OD will increase proportionately to the yeast concentration from 7.5 to 20g/L. This was though due to the fact that the yeast extract contains fermentable carbohydrate and other materials on which the organism can grow. However, as the primary purpose of adding yeast was to stimulate growth rate then 10g/L concentration of yeast has been selected for ongoing experiments.

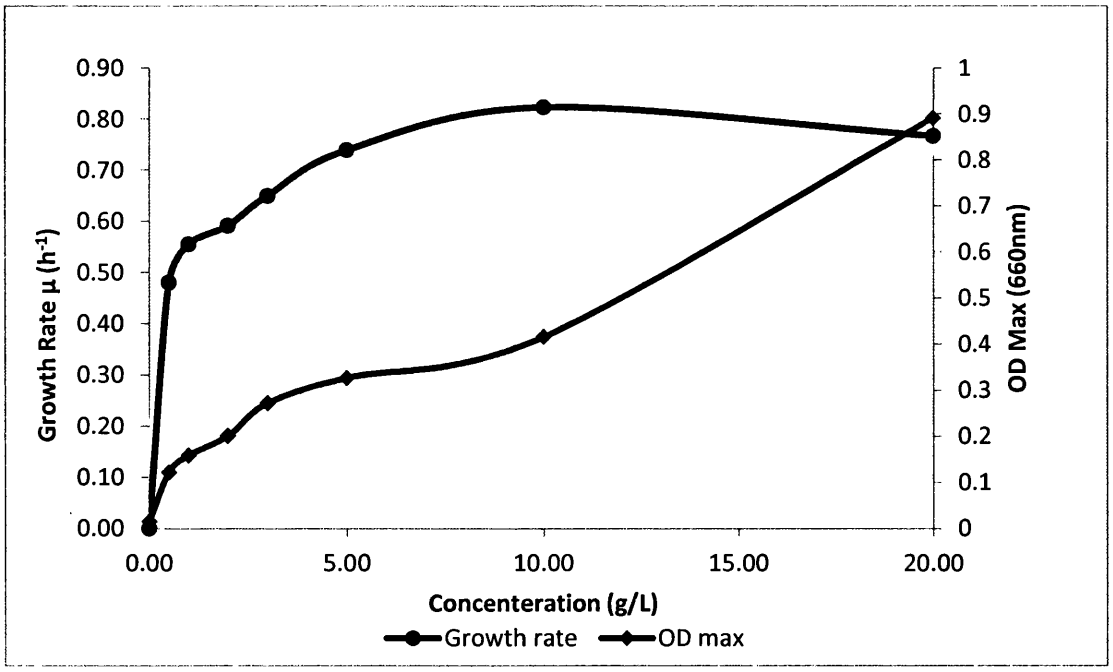

Figure 4.2: The effect of yeast extract concentration on the growth rate and the amount of growth of *C. butyricum* in test tube batch culture (refer to table 3.2).

4.2.2 The effect of dipotassium hydrogen phosphate on growth rate

All organisms require phosphate for growth; however biological systems are well adapted to sequester phosphate at very low concentrations from the environment. In addition phosphate is a very good buffer and is used as a medium component for controlling pH. Most anaerobic systems best operate around neutral pH. However, production of fatty acids can be carried out at pH levels as low as 5.5 whilst the optimum pH for methanogens ranges from 6.8 to 7.2. Methanogens can tolerate wider range of pH – from 6.6 to 7.6. Many products of anaerobic carbohydrate metabolism are acidic and cause growth inhibition when found at high concentrations, phosphate addition thus attempts to arrest pH change caused by acidic end product formation. In this study, K_2HPO_4 was chosen as a buffer because it has the capability to buffer in this system – as reported by Potter and Nelson (1953). The phosphate solution was adjusted to pH 6.5 with HCl during preparation of sterile solutions. As such the phosphate solution was a mixture of KH_2PO_4 and

K_2HPO_4 i.e. a phosphate buffer solution rather than pure solution K_2HPO_4 . The phosphate buffer was then added to the test tube culture to give the specified phosphate concentrations. The tubes were then inoculated with a mid-log culture and the growth was observed. Figure 4.3 shows the graph for K_2HPO_4 being totally at odds with the results for yeast concentrations reported in Figure 4.2. At concentrations between 0g/L and 3g/L of K_2HPO_4, the specific growth rate is $0.19h^{-1}$. However, the range of kinetic growth decreased if the amount of K_2HPO_4 was more than 3g/L. Therefore 2.5g/L was selected for the amount of K_2HPO_4. This corresponds with the highest level of OD maximum.

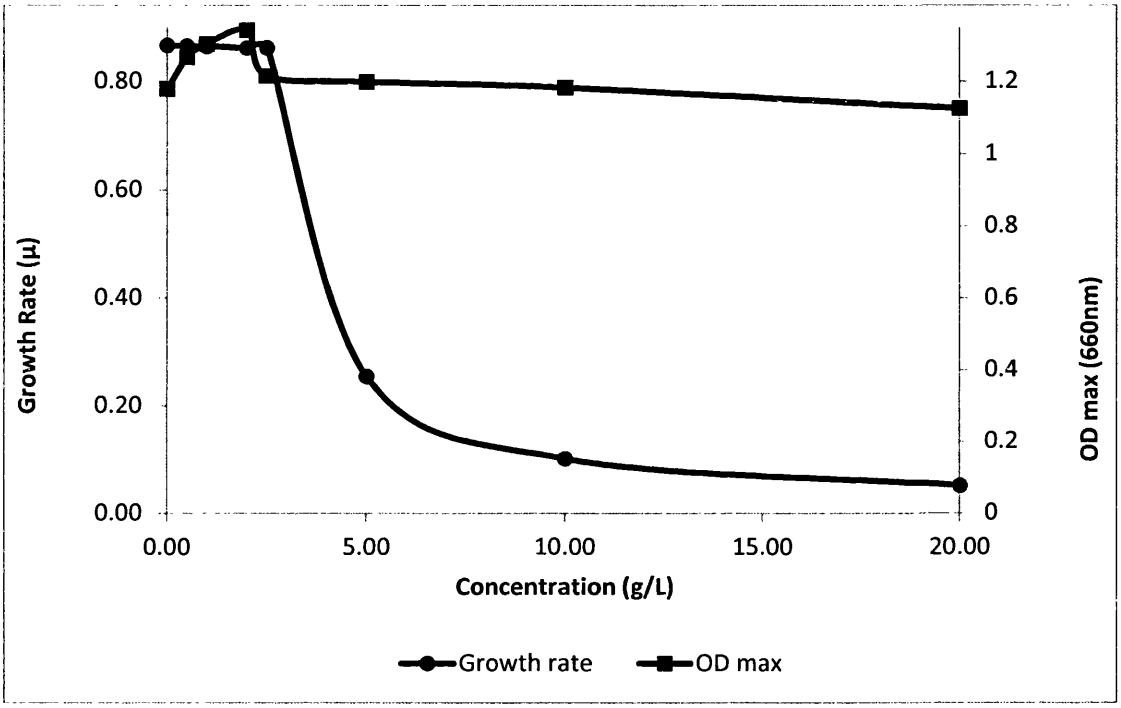

Figure 4.3: The effect of phosphate buffer the growth *C.butyricum* (refer to table 3.2).

4.2.3 The effect of ammonia sulphate on growth rate and amount of growth.

Another important nutrient for *C.butyricum* is ammonia sulphate as they require ammonia as a source of nitrogen for structure. The growth was formulated as described in table 3.2. Sterile concentrated ammonium sulphate solution was prepared and added the medium to give the specified ammonium sulphate concentration (table 3.2). All the concentrations were tested 5 times and the

averages plotted in Figure 4.4. Figure 4.4 shows the results of this experiment where both the growth rate and OD max of *C. butyricum* are recorded.

Initially, the growth rate increases as the concentration of ammonia sulphate increases from 0g/L to 5g/L. At levels above 5g/L increased concentration of ammonia sulphate did not affect growth rate or the amount growth. The specific growth rate at 0g/L of *C. butyricum* is 0.25 h^{-1} and at 5g/L the growth rate increased to 0.84 h^{-1}. Therefore 5g/L ammonia sulphate is optimal for maximisation of the growth rate of *C. butyricum*.

The maximum OD observed, also shows the same pattern as the growth rate trend. It increases from 0g/L to 5g/L. Then, when levels are increased above 5g/L, there is no further increase in OD max levels. At 0g/L the amount of OD max is 0.081 and at 5 g/L it is 0.627.

Undoubtedly, 5g/L was the best concentration for *C. butyricum* to achieve maximum OD and growth rate in these conditions

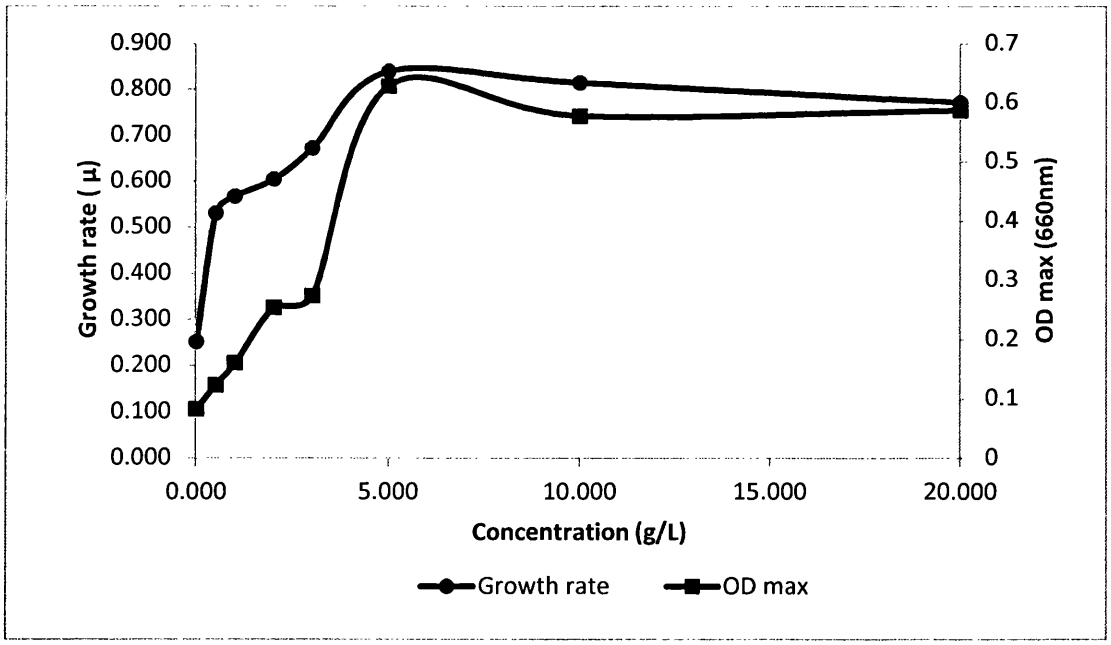

Figure 4.4: The effect of ammonia sulphate concentration on the growth of *C. butyricum* (refer to table 3.2).

4.2.4 The effect of glucose on growth rate

Figure 4.5 shows the pattern of kinetic growth against the amount of glucose. Clearly, glucose has a profound effect on the growth of *C. butyricum*. It can be seen that there is very slow growth when the glucose level is 0g/L indicating a strong requirement for this material. In the presence of increasing glucose concentration growth rates increase with increasing sugar concentration. However, relative low levels of glucose were used here because high sugar would cause acidification of the medium. Further investigations using external pH control are needed (see section 4.3) to investigate this in more detail. At 0g/L concentration the amount of OD maximum is 1.39 but continues to increase as concentration levels increase, even when at 1.8 g/L the amount of OD maximum is 1.658.

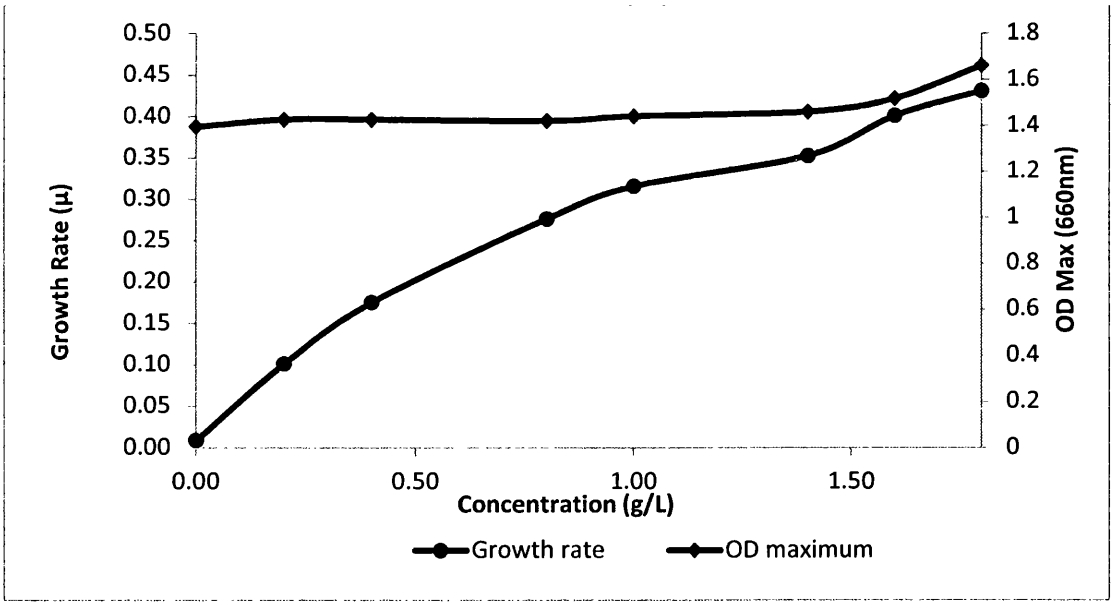

Figure 4.5: The effect of glucose concentration on the growth and growth rate of *C. butyricum* (refer to table 3.2).

4.2.5 Brief conclusions from preliminary tests

From the results of the premilinary test, the standard medium composition was determined. This medium (Table 3.3), was then used for pH controlled batch cultures. From the results, it is concluded that all of the ingredients tested must be

included. From these results it is highly likely that glucose is required as carbon as well as energy sources as there is a significant stimulation of growth rate. Similary, although yeast extract can supply carbon in the form of amino acids and sugars, ammonia is required also to stimulate growth because it is used in the transamination reaction to form amino acids.

4.3 Growth experiments in batch culture with pH control.

From the results of the preliminary tests in section 4.2, pH controlled batch system experiments were designed. Due of the nature of acid product formation, pH control was required to accurately assess the effect of carbohydrate concentration of growth and growth rates of *C. butyricum.* Therefore, a series of batch experiments were carried out using media described in table 3.3 using a 2L pH controlled system that is described in detail in section 3.5.2.

In the batch experiments a range of glucose concentrations were investigated from 0, to 28 g/L. Xylose (15g/L) and Starch (15g/L) were also were tested. All of these experiments were conducted at 37°C and at pH 6.5. The 2L reactor was used and it was filled with 1.8L of medium and 180mL of inoculum as describe in section 3.5.2. To ensure the correct anaerobic environment was in place, the reactor was continuously flushed with nitrogen during the fermentation process. The system was also equipped with a pH control system to stabilise the pH at 6.5. Sodium hydroxide (2M) was used to control the pH and its quantity measured using a weighing balance as described in chapter 3 section 3.10. During each experiment samples were taken to measure the optical density of the culture and these were converted to biomass dry weight as described in section 3.9.1. Periodic samples were also taken to determine the substrate and fatty acid concentration. Figure 4.6 shows the results for all of the experiments was for 10g/L glucose. Results for other glucose concentrations are shown in the Appendix 7.

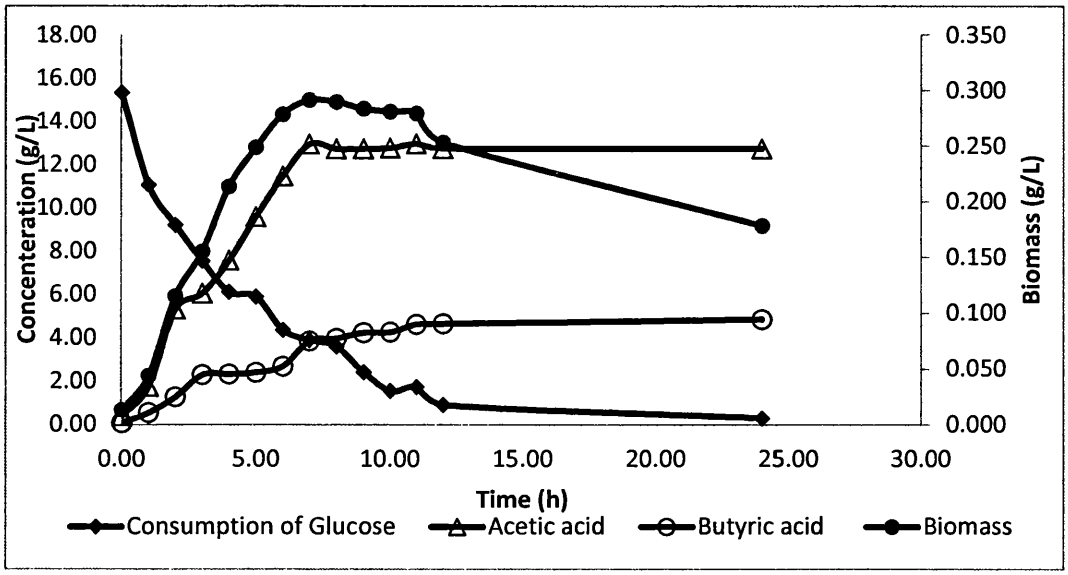

Figure 4.6: The growth of *C butyricum* in pH controlled batch culture at pH 6.5 on 10g/L glucose. The concentrations of biomass and end products are also shown.

4.3.1 The effect of glucose concentration on growth rate

Glucose is almost a universal carbohydrate source and it can supply both energy and carbon to microorganisms. Similarly, glucose is present in many types of waste and the common suitable substrate for industrial scale fermentation. Therefore the study of the effect of glucose concentration on the growth kinetics of *C.butyricum* was carried out. For each individual batch experiment investigated the data was plotted and the specific growth rate calculated. An example calculation of growth rate is attached in Appendix 7, section 7A.2. Figure 4.7 shows the specific growth rate as a function of glucose concentration. The growth rate is lowest at 0g/L and increases up to 15g/L but further increases in glucose reduce the growth rate. The results thus show the peak growth rate to be 0.263μ at 15g/L and the lowest growth rate at 0g/L is 0.094 μ.

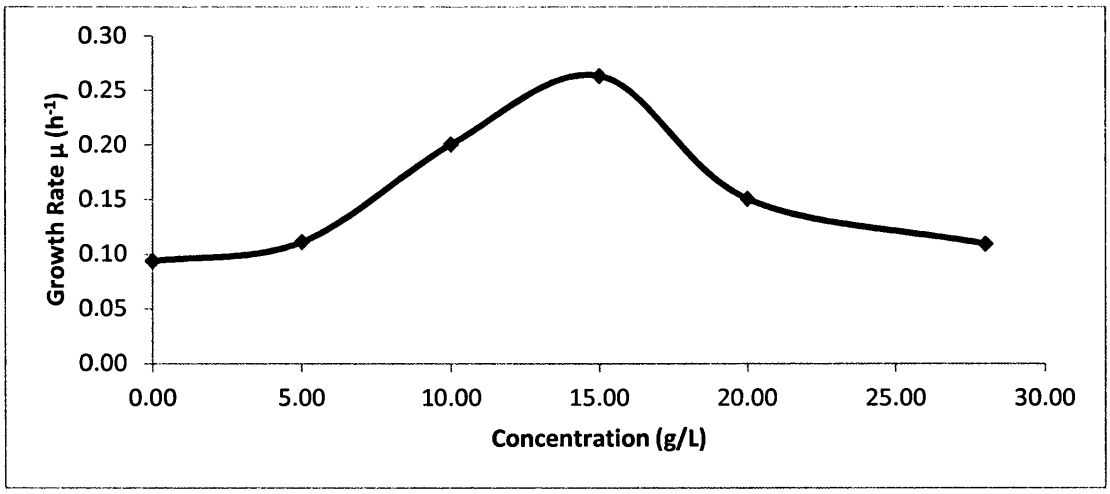

Figure 4.7: The effect of glucose concentration to growth rate of *C. butyricum*.

4.3.2 The effect of glucose concentration of yield

Many factors have to be considered when seeking to identify optimum glucose levels in the batch system, not only to maximise the growth rate of microbe, but also the cell yield must be considered. The biomass maximum levels were recorded from all concentrations of glucose tested in the batch system. Table 4.1 shows that the concentration of biomass maximum at 0g/L is 0.099g/L and that it increases as further glucose is added until 0.374 g/L, at 28g/L. However beyond 20g/L and up to 28g/L the concentration of biomass showed little further increase.

Table 4.1 also shows a pattern for doubling time, this being the time required for the quantity of microbe to double. Here, Table 4.1 shows the time decreases as glucose concentration increases from 0g/L to 15g/L. Table 4.1 shows that at 0g/L the time required is 7.37 hours and at 15g/L the time required is just 2.62 hours. Nevertheless, the times required increase when the concentrations of glucose are more than 20g/L. The time required to double the quantity at a concentration of 20g/L is nearly twice that at 15g/L, being only 2.62 hours at 15g/L but at 20g/L it is 4.59 hours. At 28g/L the doubling time is around 6.32 hours.

Considering the growth rate data, maximum biomass formed and doubling time indicate an optimal glucose concentration for the batch system is 15 g/L.

Table 4.1: The effect of glucose concentration on specific growth rate (μ), OD (max) and doubling time of *C. butyricum* batch cultures at pH 6.5 and 37°C.

Concentration g/L	μ (h^{-1})	Biomass(max) (g/L)	Td h
0	0.094	0.099	7.37
5	0.111	0.187	6.21
10	0.200	0.243	3.43
15	0.263	0.291	2.62
20	0.150	0.366	4.59
28	0.119	0.374	6.32

4.3.3 Concentration of acetic and butyric acid formation

Figure 4.8 shows the graph of concentration for acetic and butyric acid formed during the fermentation. For the acetic acid, the concentrations increase from 0g/L to 15g/L of initial glucose. At 0g/L of initial glucose the production of acetic acid is 0.002 mol/L and it further increases to 0.20 mol/L at 15g/L. From 15g/L to 28g/L the production of acetic acid decreases, probably due to changing the spectrum of fermentation products such as solvents or other non-volatile products. At 28g/L of initial glucose the amount of acetic production is 0.104 mol/L.

Figure 4.8 also shows the pattern of butyric acid concentration. Butyric acid concentrations increase from 0g/L to 28g/L of initial glucose concentration. However the increase in butyric acid concentration is stimulated between 5g/L to 15g/L glucose concentration. However, from 15g/L to 28g/L the increase slows. Presumably high glucose concentrations alter the end products spectrum or the products themselves shift the fermentation away from the acetate and butyrate.

Figure 4.8 also shows the ratio for acetic and butyric acid levels for each concentration of glucose. The ratios have been calculated by dividing the amount of acetic acid (mol/L) by the amount of butyric acid (mol/L). 0g/L glucose concentration produces 14 mol of acetic and 1 mol butyric acid. Meanwhile, 5g/L of glucose will produce 21 mol acetic acid and 1 mol butyric acid. Then, 10g/L of glucose will produce 6 mol of acetic acid and 1mol of butyric acid. Next, 15g/L will produce 3 mol

of acetic acid and 1 mol butyric acid. Lastly for 20g/L and 28g/L glucose will produce 2.4 mol and 2.2 mol acetic to 1mol butyric acid. The explanation for the shift in the ratio could be due to the presence of an excess of electron acceptors at low glucose concentration (from the yeast extract for example) while it shifts to low ratios at high glucose concentration where the relative electron acceptor concentrations are lower. An alternative explanation may be the high glucose concentrations force the production of butyrate with a shift away from hydrogen formation. Unfortunately, hydrogen production could not be measured.

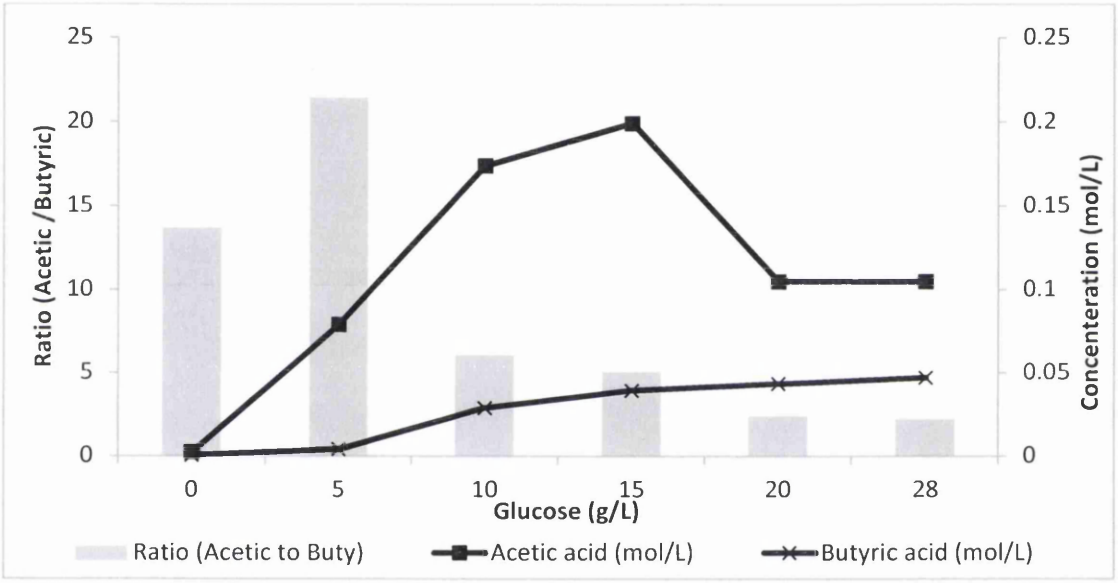

Figure 4.8: The effect of glucose concentration on acetic and butyric acid production and the acetate butyrate ratio in 2l batch culture at pH 6.5 and 37°C.

4.3.4 The relationship between total acid production and initial glucose concentration

Figure 4.9 shows the effect glucose concentration on the ratio between total fatty acid production and glucose consumption. The ratio was identified by dividing the amount of total production by the amount of glucose consumption. Again, the changes ratio can be described by the following examples:

For a 0g/L glucose feed the ratio between fatty acid production to glucose consumption is 3.69; while at 5g/L glucose, 4.04 mol of acid are produced per mol of glucose; at 10g/L glucose, 4.75 mol of total acid are produced per mole of glucose; at 15 g/L glucose, 4.08 mol of total acids were produced per mole of glucose; 20g/L glucose produced 1.92 mol of total acid. Finally 28g/L glucose feed will produce 1.31 mol per mole of glucose. Figure 4.9 shows that the maximum ratio of total production to glucose was at 10g/L. However, for initial glucose concentrations from 15g/L to 28g/L, the total fatty acid ratio declines. Probably, at these higher ratios, other fatty acids and solvent products, that have not been detected, have been produced.

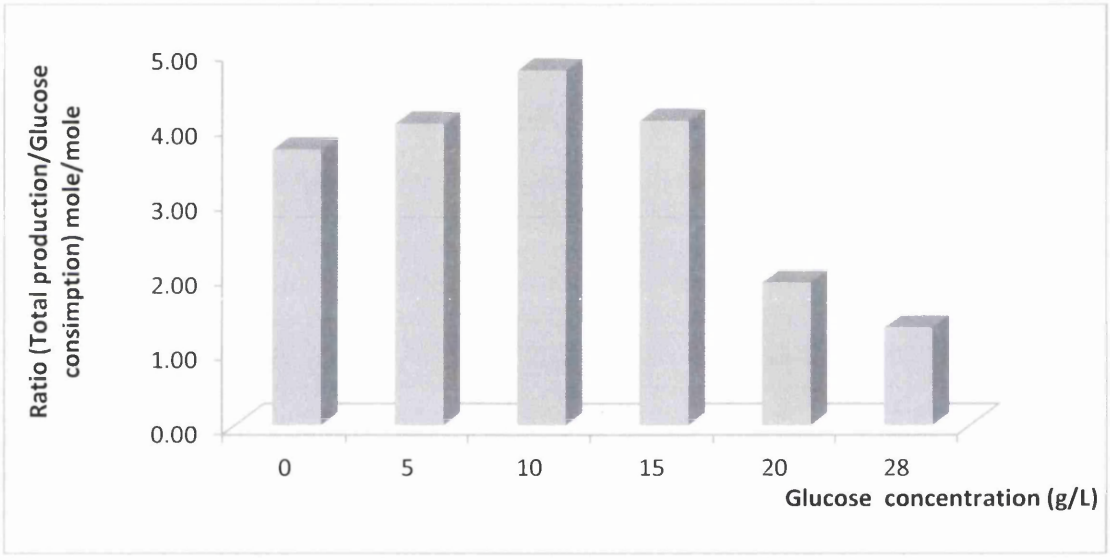

Figure 4.9: Ratio between total production and glucose concentration

4.3.5 The percentage of glucose converted to acetic and butyric acid

Figure 4.10 expresses the data in terms of the percentages of glucose converted to acetic acid and butyric acid and how this is related to biomass production.

. Appendix 7, section 7A.2 shows the calculation about percentages glucose converted to acetic or butyric acid.

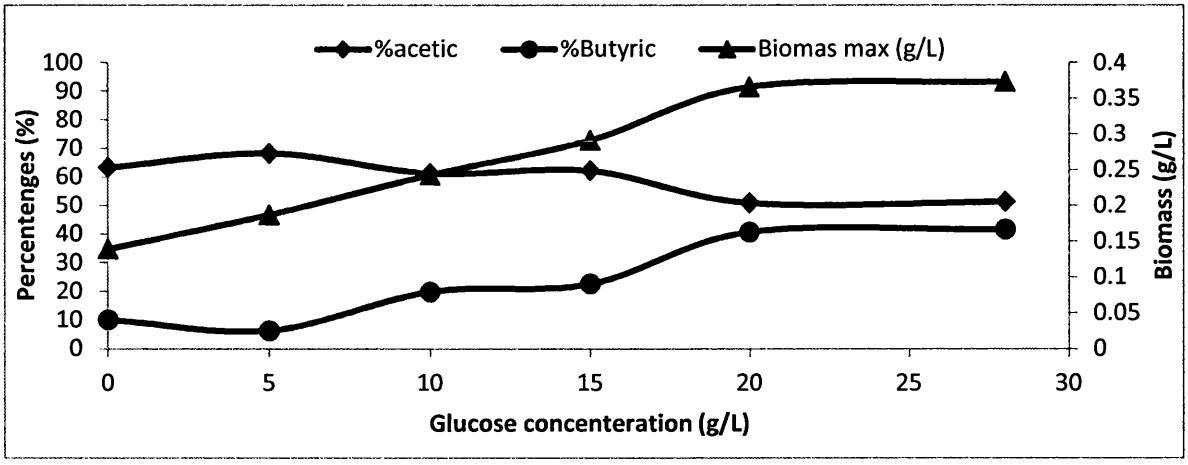

Figure 4.10: The effect of glucose concentration on percentages of acetic and butyric acid produced by *C.butyricum.*

Figure 4.11 shows the relationship between biomass and total production of acetic and butyric acid.

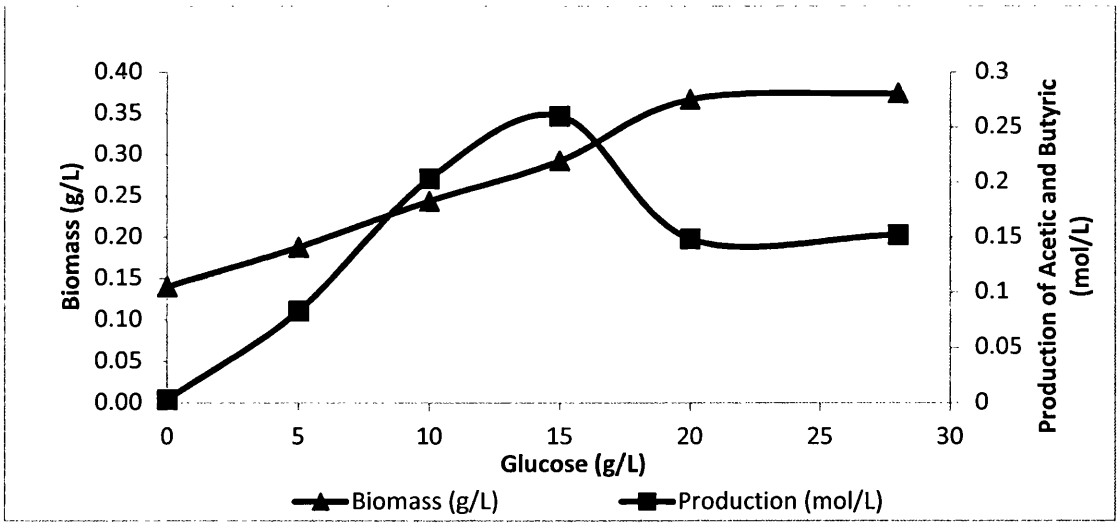

Figure 4.11: The efect of glucose concentration on biomass and total acid production in the batch culures at pH 6.5 and 37°C.

These results reiterate the fact that the best fatty production and is observed at 10 to 15 g/L. At higher concentrations there was a shift away from fatty acid production to undetected end products.

4.3.6 Effect of glucose concentration on the requirement for sodium hydroxide

Figure 4.12 shows the requirement for sodium hydroxide (by pH controller) increased as concentrations of glucose increased up to 20g/L. This correlates very well with the amounts of fatty acid produced. However, above this level the requirement for sodium hydroxide did not increase further and its thought that solvent production increases. The details of the production of fatty acid will be clarified in the next section.

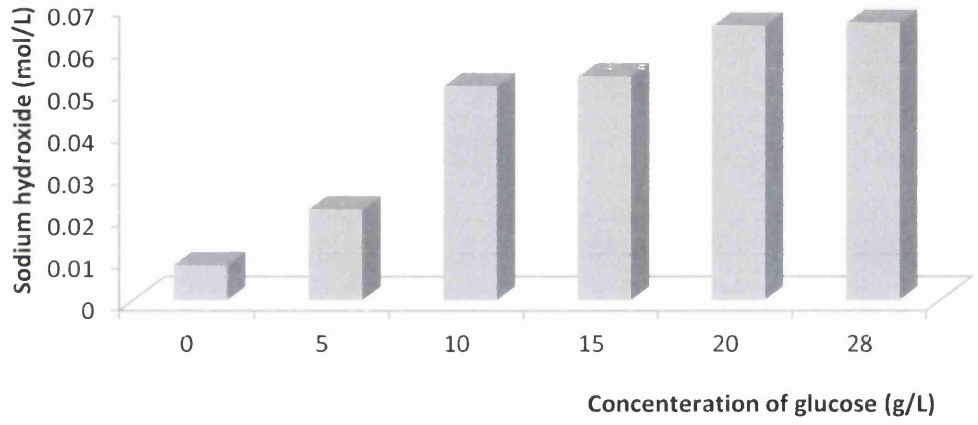

Figure 4.12: The effect of glucose concentration on the cumulative of requirement for sodium hydroxide while maintaining pH at 6.5 at 37°C.

4.3.7 Relationship between cumulative requirement for sodium hydroxide and fatty acid production

Figure 4.13 shows the relationship between total acid production and cumulative consumption of NaOH. The relationship is approximately linear up 15 g/L but falls at high glucose concentrations. For industrial scale applications it is important to study and understand these profiles for early prediction of the amount of fatty acid production and relate this to activity of cultures.

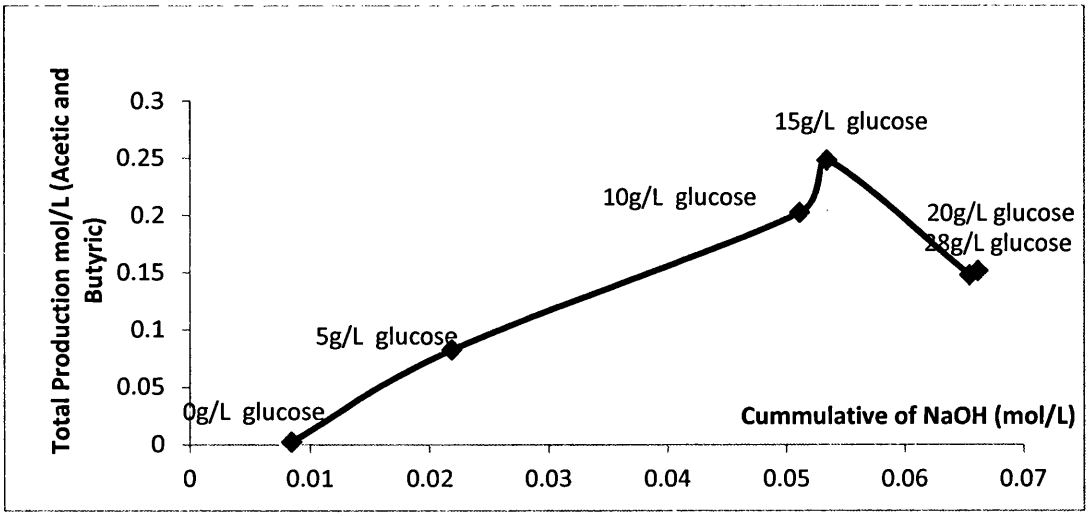

Figure 4.13: The relationship between cumulative of consumption of sodium hydroxide and total acid production.

4.3.8. Fermentation balances

Fermentation balances on the glucose fermentations of *C butyricum* were carried to account for the carbon that can be found in the end products of the fermentation. The assumptions made for these calculations were that all the carbon in yeast extract should be considered, that for every mole of acetate, 1 mole of carbon dioxide is formed. For every mole of butyrate two moles of CO_2 are formed. Biomass was assumed to be 50% C.

Table 4.2: Carbon fermentation balances for a range of glucose concentrations.

Initial Glucose (mM)	ΔS in C (mM)	Yeast extract in C (mM)	Total C input	Cell in C	Acetic acid +CO_2 (mM) in C	Butyric acid +2CO_2 (mM) in C	Total C output	% C recovery
0.00 (0 g/L)	0.00	410.00	410.00	5.79	5.47	0.63	11.89	2.90
27.80 (5g/L)	122.40	410.00	532.40	7.79	214.90	20.12	242.81	45.61
55.50 (10g/L)	255.66	410.00	665.66	10.11	487.50	129.32	626.94	94.18
83.30 (15g/L)	381.30	410.00	791.30	12.13	542.73	234.57	789.42	99.76
111.00 (20g/L)	461.04	410.00	871.04	15.25	285.01	258.70	558.95	64.17
155.40 (28g/L)	690.12	410.00	1100.12	15.58	284.55	280.30	580.42	52.76

*Note

ΔS= Initial glucose-final glucose in the reactor

A few assumptions were used in this calculation especially content of carbon in yeast extract and cell and CO_2 production between acetic and butyric acid production. These assumptions have been detailed in section 5.5.3.

Table 4.2 shows that good results for carbon balances are found for 10 and 15 g/L while only 50% can be identified in 5, 20 and 28 g/L. Clearly other fermentation products are being produced or yeast extract is not utilised as effectively in these conditions. The most probable additional products are solvents; however, in this study no analysis of solvents is considered. The most probable products are diols, however this has to be confirmed.

4.4 Comparison the fermentation of glucose, xylose and starch in the batch system by *C.butyricum*.

This section concerns the comparison of growth and fatty acid production on glucose to that on xylose and starch; three kinds of carbohydrate have been used in the batch fermentation system. In terms of growth rate, according to Figure 4.7, 15 g/L initial of glucose gave the fastest of growth rate (μ). Therefore 15g/L for glucose, starch and xylose were selected. pH and temperature in this study were again fixed at 6.5 and 37°C respectively. Periodic samples were taken through the fermentation and these were analysed for biomass and fatty acid end products.

4.4.1 Biomass yield

Figure 4.14 shows the relationship between biomass and carbohydrate consumption for glucose, starch and xylose. Figure 4.14 shows glucose reached a higher level of maximum biomass quicker than starch and xylose. Glucose achieved a maximum biomass at 7 hours with an amount of 0.291g/L. The starch culture reached its maximum biomass at 11 hours with 0.077 g/L. The xylose culture achieved its maximum biomass slightly faster than starch, but at a lower level, achieving its maximum biomass at 10 hours with 0.051g/L. In both starch and xylose the substrate consumption was not complete.

4.4.2 Growth rate and maximum biomass production

Table 4.3 shows the comparisons of growth rate (μ), doubling time (t_d) and biomass (max) from the data in figure 4.14. The glucose specific growth rate (0.263 h^{-1}) is faster than starch (0.192 h^{-1}) and xylose (0.123 h^{-1}). Presumably starch hydrolysis reduced the specific growth rate while xylose was most probably low because xylose uptake and metabolism are fundamentally slower than that for hexose sugar, glucose. Biomass (max) shows the same pattern as the growth rate. The higher biomass (max) is produced by glucose at 0.291g/L, followed by starch at 0.178g/L and xylose 0.151g/L.

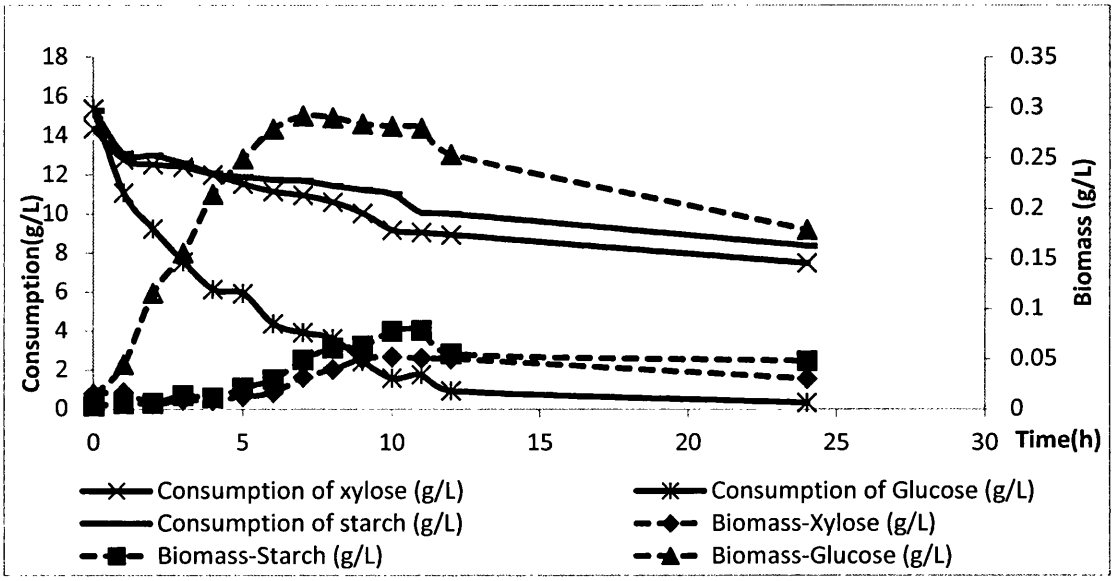

Figure 4.14: A comparison of three cultures grown of glucose, starch and xylose in 2L batch cultures at pH 6.5 and 37°C. The substrate consumption and biomass formation are also shown.

Table 4.3: The growth rate (μ), t_d(h) and OD max for starch, xylose and glucose

Carbohydrate	Starch (15g/L)	Xylose (15g/L)	Glucose (15g/L)
Growth rate μ (h^{-1})	0.192	0.123	0.263
t_d(h)	3.59	5.61	2.62
Biomass (max) (g/L)	0.178	0.151	0.291

4.4.3 Fatty acid production.

Figure 4.15 shows the pattern of acetic and butyric acids that were produced by glucose, starch and xylose. For all fermentations acetate was the major end product with some production of butyrate. Final acetate levels were 0.2 mol/L for glucose, 0.038 mol/L for starch and 0.075 mol/L for xylose. While final butyrate concentrations were 0.040 mol/L for glucose, 0.02 mol/L for starch and 0.015 mol/L for xylose.

From comparison between figures 4.15 and 4.14, all of the substrates were recorded for their maximum production of acetic and butyric acids at the same time as biomass concentration was maximum.

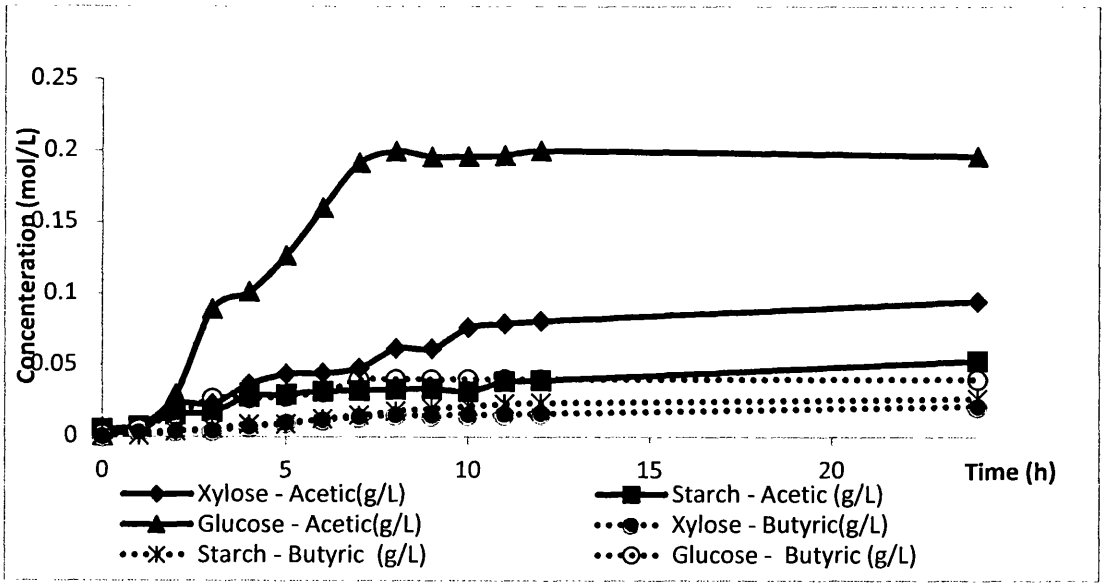

Figure 4.15: Production of acetic and butyric acids in the variety of carbohydrates.

4.4.4 Comparison end product formation on glucose, xylose and starch.

Table 4.4 shows the comparison data derived for the three different fermentations of *C.butyricum* using glucose, xylose and starch. A similar analysis to that conducted when investigating the glucose concentration was performed, in that total acid production, percentage of acid produced, product ratios and substrate:product ratios are shown in Table 4.4.

The most notable in this comparison is the difference between starch and the monsacharides glucose and xylose, is that butrate production was enhanced on starch and that the acetate:butyrate ratio was much lower than that observed with xylose or glucose

Table 4.4: A comparison of fatty acid on glucose, xylose and starch.

Type of Carbon (15g/L)	Carbohydrate consumed (mol/L)	Acetic acid (mol/L)	Butyric acid (mol/L)	Total acid Production (mol/L)	% total acid production		Ratio (acetic/butyric) (mol/mol)	Ratio(total acid production to carbohydrate) (mol/mol)
					Acetic	Butyric		
Glucose (15g/L)	0.064	0.20	0.04	0.24	82.2	17.8	4.94	3.75
Xylose (15g/L)	0.034	0.075	0.015	0.09	83.6	16.4	5.10	2.65
Starch (15g/L)	0.032	0.038	0.022	0.06	63.1	36.9	1.71	1.88

4.5 Conclusions

From the above presentation and discussion of the results of the growth in pH controlled batch cultures, a number of conclusions can be made.

The handling techniques for routinely growing and preserving *C. butyricum* were established.

A simple media was investigated and optimised in test tube cultures. This work showed that yeast extract was required for growth and that in the absence of glucose, growth was very poor.

The growth on glucose in pH controlled batch cultures was established giving good growth and product formation. Certainly the fermentation using high concentrations of glucose were possible because of pH control of the fermentation.

Investigation of the effect of glucose concentration on fatty acid product showed that optimum glucose concentration occurs at 10 and 15 g/L (table 4.2). These concentrations give the highest yields of fatty acid and that the ratio of fatty acid is about 3:1 (figure 4.8) and that these levels correlate well with the highest

biomass concentrations. Above or below these concentrations of glucose a substantial amount of carbon cannot be accounted for in the fermentation balances, probably because of new fermentation products being formed especially at high glucose concentrations (20-28 g/L). Although a search was made for additional products using GC analysis no additional products could be found. These studies showed that ethanol and butanol could not be detected.

The comparative investigation of the carbohydrates showed that important carbohydrates, starch and xylose as well glucose can be utilised to produce fatty acids. This confirms the information published in Bergey's Manual (see figure 2.1 in chapter 2 and Murray et al., 1984) and means that this organism has the potential for converting a wide variety of waste carbohydrate materials to fatty acids. Although growth on xylose and starch are not as good as glucose, good concentrations of fatty acid could be obtained even though the amounts of growth and growth rates were lower. One interesting observation was the difference between the starch and the two monosaccharide, was the high butyrate concentrations. The explanations for this may be that rates of hydrolysis limit the glucose uptake so altering the fermentation end-product profile.

The results especially those with glucose, demonstrate the greatest potential for the production of high levels of acetic and butyric acid. However the work here has not established the detailed kinetics required to precisely assess the kinetic performance of fatty acid production. The next chapter therefore focusses on glucose fermentation in continuous cultures.

Chapter 5: The Continuous culture of *C. butyricum*

5.1 Introduction

This chapter describes the results of the continuous culture experiments. Having established growth in batch culture and investigated the growth performance to optimise growth media and growth conditions in the previous chapter, continuous culture was used here to further define the growth performance and establish detailed kinetic data for growth. Continuous culture is a very good tool to investigate these aspects of growth as the systems allow steady state conditions to develop where, unlike batch culture, the environmental conditions are set and unchanging (Richardson & Peacock, 1994). The effect of growth and feed rate may be investigated to determine fundamental parameters for growth including the saturation constants for nutrient uptake and the maintenance coefficients.

The aim of this chapter was to investigate the growth of *C. butyricum* in continuous culture to establish the kinetic performance under these conditions and to determine the productivity of these systems. This chapter therefore, first outlines the theory of continuous cultures and then describes the results of experiments on the effects of growth rate and substrate concentrations in continuous culture.

5.2 The theory of continuous cultivation

Generally, continuous cultivation can be categorized into four general types according to the ways in which parameters are controlled and modes of operation.

The types of continuous cultivation:

a) Chemostat; A chemostat is a bioreactor to which fresh medium is continuously added, while the culture liquid is continuously removed to keep the culture volume constant;

b) Auxostat; An auxostat is a continuous culture device which, while in operation, uses feedback from a measurement taken on the growth chamber to control the media flow rate, maintaining the measurement at a constant level.

c) Cell recycle; this is an unsteady state system, in which the main objective is that growth equals the feed rate.

d) Multi-stage continuous culture, this system will be operated in several vessels and mimics tubular flow reactor systems.

All of the continuous cultivation mechanisms have a common feature in that they are constantly fed with fresh medium. The flow rate is normally defined as F, expressed in litres per hour (L/h), the culture volume is determined as V and expressed in litres (L) which are kept constant in the continuous cultivation.

In this research, chemostat continuous cultivation was carried out. It was started with the batch culture and the before nutrient becomes a limiting factor, the feed to the continuous system was started. Normally the concentration of cells can be determined from a material balance as shown:

$$net\ increase\ in\ biomass = biomass\ in\ incoming\ medium + growth - output - death$$

This can be arranged into the mathematical form of equation 5.1;

$$\frac{dx}{dt} = x_i \frac{F}{V} + \mu x - x\frac{F}{V} - kdx \qquad (5.1)$$

where;

dx/dt = accumulation of biomass per unit time and per unit volume (g/cell/l/h);

x_i *= biomass concentration (g cell dry weight/l) in the incoming medium;*

x *= biomass concentration (g cell dry weight/l)*

kd *=Specific death rate of cell (h^{-1})*

Then, if the dilution rate, D, is introduced, being F/V (flow rate/volume) equation 5.2 can apply to give equation 5.3 (Richardson & Peacock,1994) ;

$$D = \frac{F}{V} \qquad (5.2)$$

$$\mu x = Dx \qquad (5.3)$$

Then, for a chemostat in steady state, with sterile feed and negligible cell death, the specific growth rate μ can be determined as being equal to the dilution rate (D) as described in equation 5.4. The relationship is useful to identify parameters and kinetic activity in the cell culture. Previous researchers have described this principle (Pirt, 1975 and Doran, 1995).

$$\mu = D \qquad (5.4)$$

The specific growth rate of a culture for a range up to the maximum specific growth rate (μ_{max}) can be controlled by nutrient feed rate if the volume of the reactor remains constant.

5.2.1 Actual biomass yield ($Y_{x/s}^{max}$) and maintenance coefficient (m_s)

Pirt (1975) and Doran (1995) described the observed yield of a culture and its relationship to maintenance and growth, by the following equation.

$$Y_{x/s} = \frac{\mu}{(\mu / Y_{x/s}\,\text{max})} + m_s \qquad (5.5)$$

where;

$Y_{x/s}$ = observed biomass yield from substrate (g/g)

$Y_{x/s}^{max}$ = true biomass yield from substrate (g/g)

m_s = maintenance coefficient (g/substrate/g cell/h)

The maintenance coefficient is thus related to the yield. Rearranging equation (5.5) gives

$$1/Y_{x/s} = 1/Y_{x/s}^{\max} + m_s/\mu \quad (5.6)$$

$$\mu/Y_{x/s} = (\mu/Y_{x/s}^{\max}) + m_s \quad (5.7)$$

Further, the definition of the specific rate of substrate uptake q_s with unit of dimensions is h^{-1} is elaborated in equation 5.8.

$$q_s = \frac{\mu}{Y_{x/s}^{\max}} + m_s \quad (5.8)$$

Given that this continuous system is a chemostat, then; μ=D, then equation 5.8 can be rearranged to become equation 5.9.

$$q_s = \frac{D}{Y_{x/s}^{\max}} + m_s \quad (5.9)$$

Therefore, if $Y_{x/s}^{max}$ and m_s are constant, then a straight line will be produced from a plot of q_s versus D. The slope being 1/ $Y_{x/s}^{max}$ and the intercept m_s.

In a chemostat with sterile feed the observed biomass yield from substrate $Y_{x/s}$ is defined as

$$Y_{x/s} = \frac{x}{(S_i - S)} \quad (5.10)$$

where;

x = *steady state cell concentration(g/L)*

S = *steady substrate concentration (g/L)*

S_i = *inlet substrate concentration (g/L)*

5.2.2 Maximum specific growth rate (μ_{max}) and substrate saturation constant (K_s)

According to the Monod kinetic equation, the culture in a chemostat could be modelled

$$D = \mu_{max} S/(K_s + S) \qquad (5.11)$$

where;

μ_{max} = maximum specific growth rate (h^{-1})

K_s = substrate saturation constant (g/L)

S = steady state substrate concentration in the reactor (g/L)

Equation 5.11 relates substrate concentration to growth rate and saturation constant.

To determine the K_s value, equation 5.11 can be rearranged to become

$$K_s = \frac{\mu_{max} S}{D} - S \qquad (5.12)$$

Alternatively data from the chemostat may be plotted in terms of steady state substrate in the reactor (1/S) vs. dilution rate (1/D). A straight line relationship will be produced with an intercept of $1/\mu_{max}$ and slope of K_s/μ_{max}. So allowing the K_s to be evaluated.

5.2.3 Theoretical calculation of cell concentration

Cell concentration can be predicted if parameters such as dilution rates (D), initial substrate concentration (S_i), substrate level at steady state in the reactor (S), $Y_{x/s}^{max}$, q_p, $Y_{p/s}^{max}$ and m_s are known Equation 5.13 shows the relationship between cell concentration and these parameters.

$$x = D(S_i - S)/(\frac{D}{Y_{x/s}^{max}}) + (q_p/Y_{P/s}) + (m_s) \qquad (5.13)$$

Then, equation 5.13 can be simplified to give:

$$x = D(S_i - S)/(\frac{D}{Y_{x/s}{}^{max}}) + (m_s) \quad (5.14)$$

Further, if maintenance effects can be ignored, then equation 5.14 can be simplified to:

$$x = (S_i - S)/Y_{x/s}{}^{max} \quad (5.15)$$

Specific growth rate has been related to the concentration of growth during the balanced growth phase. The limiting substrate is described in the Monod equation.

$$\mu = \frac{\mu_{max} S}{K_S + S} \quad (5.16)$$

Then, in the Chemostat μ=D, so equation 5.16 can be rearranged to:

$$S = DK_S/(\mu_{max} - D) \quad (5.17)$$

Next, in terms of dilution rate, kinetic and yield parameters, equation 5.18 can be developed, (for steady state cell concentration in chemostat culture) as:

$$x = (S_i - DK_s/(\mu_{max} - D)\, Yx/s^{max} \quad (5.18)$$

5.2.4 Biomass productivity (Q_x)

In a chemostat system, the rate of biomass production is equal to the rate with which cells leave the reactor. If F_x is the rate at which cells leave the reactor and the volumetric productivity Q_x is equal to F_x divided by V, then equation 5.20 developed:

$$Q_x = \frac{F_x}{V} = D_X \quad (5.19)$$

where, Q_x is the volumetric rate of biomass production (g/h). Then, when the maintenance requirement is neglected and the product formation is energy associated or absent, then x could be replaced in equation 5.18 to give equation 5.21.

$$Q_x = DY_{x/s} \ (S_i - DK_s /(\mu_{max} - D) \quad (5.20)$$

5.3 The effect of dilution rate to the growth of *C. butyricum*

The continuous experiments required long periods of operation (weeks) during which the bioreactor was continuously flushed with nitrogen at a pressure of 0.5 bar, and operated at 37°C and pH 6.5. Glucose was selected as the main carbohydrate because of its higher production levels than those of xylose and starch. As with the batch experiments, feed glucose compositions of, 5, 10, 15, 20, and 28g/l were used. For the continuous system fresh medium was pumped to the bioreactor at each of 5 different flow rates. These rates were selected to match the capacity of the pump, viz: 70, 140, 200, 252 and 280 ml/h. This gave a good range of dilution rates so that growth rates from $\mu = 0.0583\ h^{-1}$ to $\mu = 0.233\ h^{-1}$ could be investigated.

In these experiments, the culture was started as a batch system, then once good growth was obtained, then medium was pumped continuously at the specified dilution rates. The continuous system was started at the lowest rate, i.e. 70 ml/h and the rates then increased up to a maximum of 280 ml/h. In each condition a steady state was established as measured by 5 stable points after 5 volume changes of the reactor.

The effects of dilution rate on the amount of biomass production were investigated. In these experiments 5 dilution rates and a range of glucose concentrations were tested.

The average concentration of biomass was calculated for the period during which the biomass was in steady state. Five or more data points were selected to calculate the average of biomass for every single dilution rate. The dilution rates were calculated using equation 5.2. Table 5.1 shows the effect dilution rate and glucose concentrations on biomass concentration in chemostat cultures. Six concentrations of glucose were studied with five dilution rates. In general, the biomass concentrations in the continuous system were higher than observed in equivalent batch cultures (table 4.1).

Table 5.1: The effect of dilution rate on the average of biomass concentration for 5 different glucose concentrations

TD (time doubling) :	**11.90**	**5.91**	**4.14**	**3.29**	**2.96**
Dilution rate (h^{-1}):	**0.058**	**0.1167**	**0.1667**	**0.21**	**0.233**
Concentration (g/L)	**Biomass (g/L)**				
5 g/L glucose	0.33	0.30	0.28	0.23	0.22
10 g/L glucose	0.37	0.45	0.64	0.72	0.88
15 g/L glucose	0.49	0.77	1.04	1.38	1.06
20 g/L glucose	0.53	0.96	1.53	1.43	1.25
28 g/L glucose	0.75	1.64	1.52	1.43	1.24

Figure 5.1 shows the amount of biomass concentration at different dilution rates and glucose concentrations. It clearly shows that the level of biomass produced for three concentrations of 15, 20 and 28 g/L glucose looks like a bell graph, increasing initially, then decreasing continually as the concentration increased. For 28g/L, it started to increase from 0.058h^{-1} with biomass levels from 0.75g/L to 1.64g/L at 0.117h^{-1}, then decreased to 1.24 g/L at 0.233h^{-1}.

For 20g/L glucose biomass concentration increased from 0.53g/L at 0.058h^{-1} to 1.53 g/L at 0.1667h^{-1}, (cell higher growth rates than for the 28g/L concentration). Thereafter the concentrations of biomass decreased to 1.25g/L at 0.233h^{-1}.

Similarly, for 15g/L glucose, it increased from 0.49g/L at 0.058h^{-1} to 1.38 g/L at 0.21h^{-1}, again, later than for the previous 20g/L of glucose then decreased to 1.38 g/L at 0.233h^{-1}.

At a 10g/L glucose level, the results are totally different for the range of glucose concentrations adopted (15, 20 and 28g/L). It shows an increasing pattern for every dilution rate. At 0.058h^{-1} the amount of biomass was 0.37 g/L increasing to 0.88 g/L at 0.233h^{-1}.

For 5g/L, in contrast with 10g/L, the concentration biomass decreases while the dilution rates increased. Probably, in this case, there is poor uptake of glucose to support growth. At 0.05h^{-1} the amount of biomass is 0.33g/L, falling to 0.22 g/L at 0.233h^{-1}.

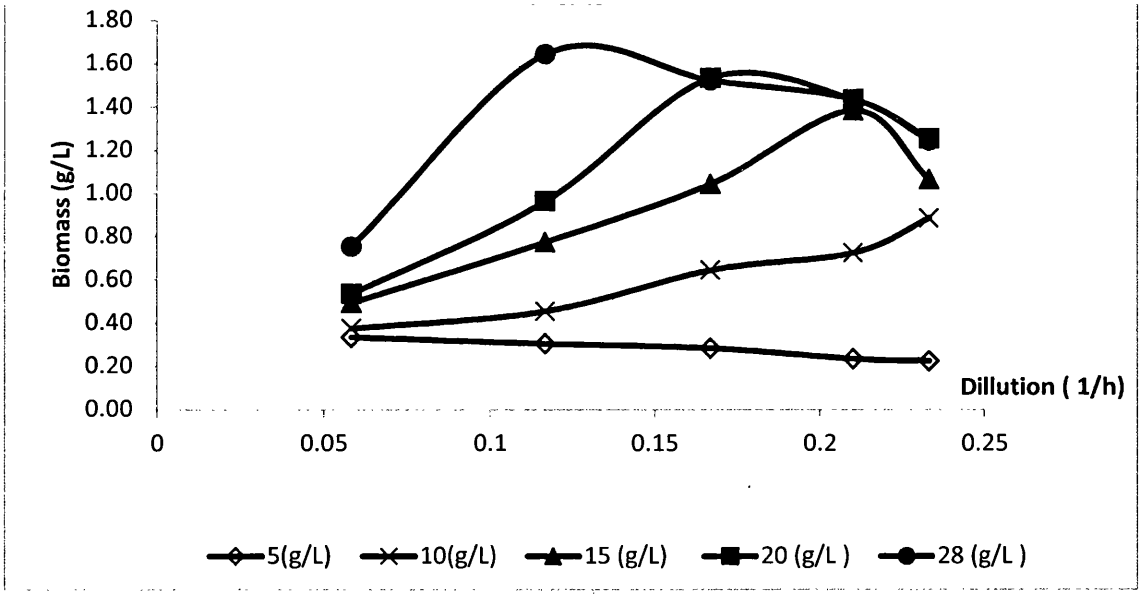

Figure 5.1: The effect of dilution rates and glucose concentrations on the biomass concentration at steady state.

5.4 Evaluation of kinetic and yield parameters

The yield and kinetic parameters for the growth in the chemostat were evaluated by the data obtained above and the relevant equations given by theory in section 5.2. Table 5.2 shows the data for a range of glucose concentrations and

dilution rates for glucose consumption, yield coefficient, specific substrate uptake rate and maximum yield coefficient.

5.4.1 Maximum yield and maintenance coefficient

Section 5.1 describes the terms maintenance coefficient and the concept of the true growth yield and derivation of equation 5.9, $q_s = D/Y_{x/s}^{max} + m_s$, which describes the relationship between the true growth yields and growth maintenance and substrate consumption rates. This can be used to determine maximum biomass yield and maintenance coefficient. The equation showed $D/Y_{x/s}$ as a rate of substrate consumption for growth and m_s is the rate of consumption for maintenance purposes. If the value of $Y_{x/s}$ and m_s are constant, then a graph of q_s against the dilution rate (D), gives a straight line with an intercept of m_s being produced. Figure 5.2 shows an example graph of q_s against the dilution rate for a range of glucose concentrations investigated.

Table 5.2: The effect of dilution and glucose concentration on the kinetic parameters of growth in the continuous culture system.

D (h^{-1})	S (g/L)	S_i (g/L)	$S-S_i$ (g/L)	x (g/L)	$Y_{x/s}$ (g cell/g sugar)	q_s (g sugar/g cell/h)	$Y_{x/s}^{max}$ (g cell/g sugar)	m_s (g cell/g sugar/h)
0.058	5	3.60	1.40	0.34	0.24	0.24	0.26	0.02
0.117		3.80	1.20	0.30	0.25	0.47		
0.167		3.90	1.10	0.29	0.26	0.63		
0.21		4.00	1.00	0.23	0.23	0.91		
0.233		4.20	0.80	0.22	0.28	0.85		
0.058	10	4.90	5.10	0.48	0.09	0.62	0.20	0.33
0.117		4.42	5.58	0.56	0.10	1.17		
0.167		2.81	7.19	0.90	0.13	1.33		
0.21		2.14	7.86	1.08	0.14	1.53		
0.233		1.90	8.10	1.25	0.15	1.51		
0.058	15	6.59	8.41	0.50	0.06	0.98	0.18	0.64
0.117		6.04	8.96	0.78	0.09	1.34		
0.167		5.55	9.45	1.06	0.11	1.49		
0.21		4.80	10.20	1.40	0.14	1.53		
0.233		5.00	10.00	1.05	0.11	2.22		
0.058	20	6.50	13.50	0.54	0.04	1.45	0.21	1.05
0.117		6.21	13.79	0.98	0.07	1.65		
0.167		5.96	14.04	1.56	0.11	1.50		
0.21		6.32	13.68	1.45	0.11	1.98		
0.233		6.68	13.32	1.27	0.10	2.44		
0.058	28	10.35	17.65	0.77	0.04	1.33	0.22	0.83
0.117		13.51	14.49	1.67	0.12	1.01		
0.167		14.95	13.05	1.55	0.12	1.41		
0.21		15.51	12.49	1.46	0.12	1.80		
0.233		16.97	11.03	1.27	0.12	2.02		

*The nomenclature used in this table is defined in the theory (section 5.2).

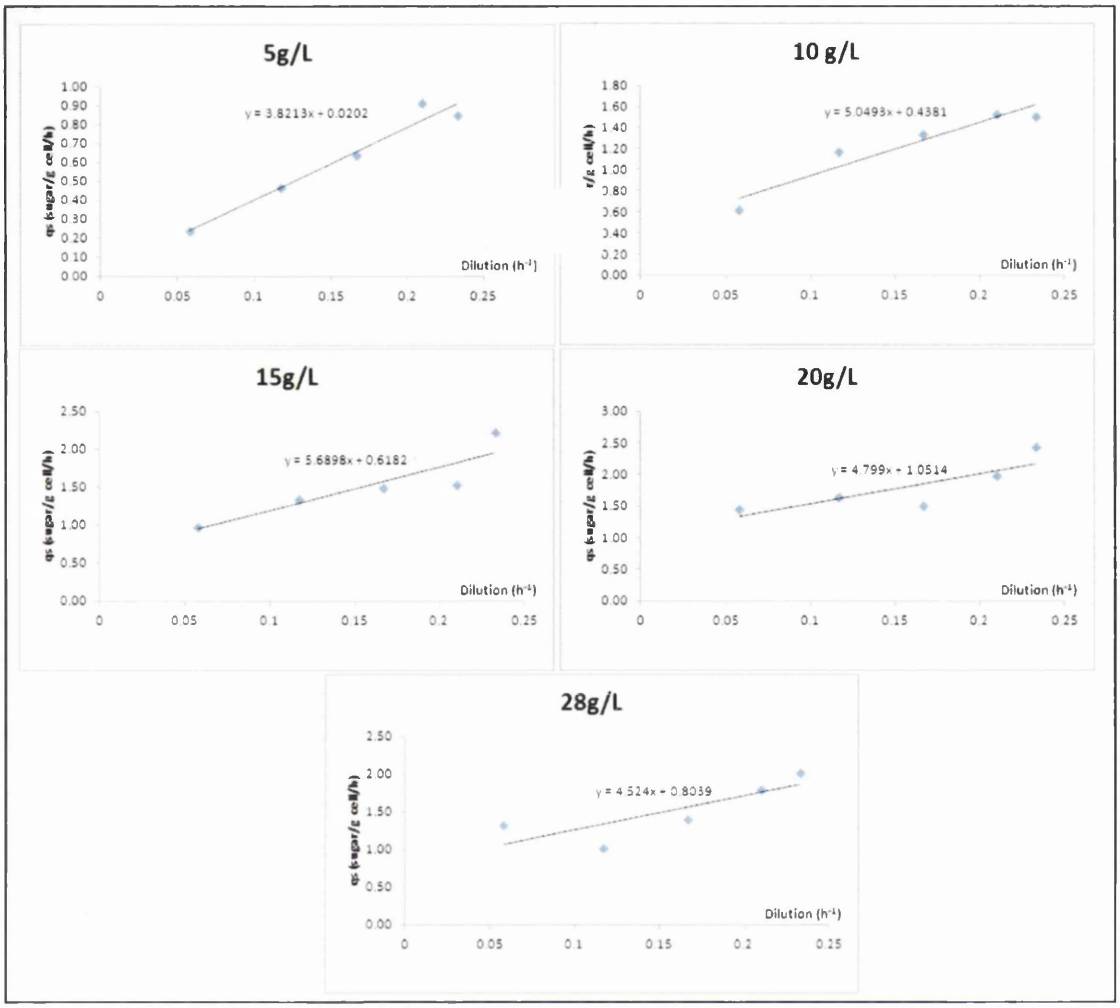

Figure 5.2: Plots of substrate uptake as function of dilution rate in chemostat culture of *C. butyricum* for 5 glucose concentrations. The straight relationships obtained were used to determine the maximum biomass yield ($Y_{x/s}^{max}$) and maintenance coefficient (m_s).

For example; from figure 5.2, the slope of the straight line for 10g/L glucose is 5.049 and the intercept of 0.4381, respectively, hence:

$1/Y_{x/s}^{max}$	5.049
$Y_{x/s}^{max}$	0.20
m_s	0.43

True yields (or maximum yield) represent the yield obtained in the absence of competing reactions. Thus the maximum possible biomass yield was $Y_{x/s}$ max = 0.20 g cell/g sugar uptake.

Figure 5.3 shows the effect of glucose concentration to maintenance coefficient. Figure 5.3 shows that m_s increased from 0.02 to 1.05 g/substrate/g/cell/h (5-20g/L). This then decreased from 1.05 to 0.80/substrate/g/cell/h, for glucose concentrations between 15 to 20 g/L.

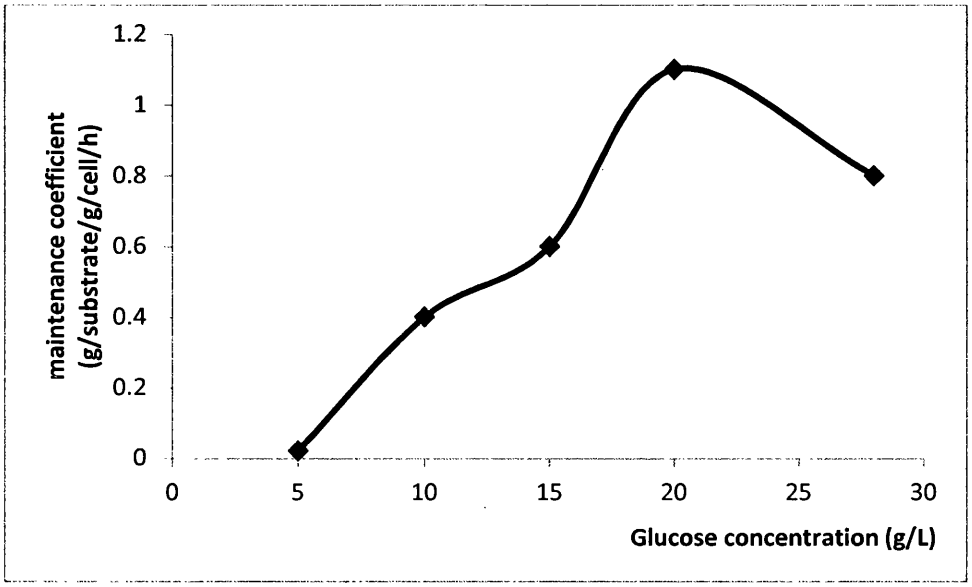

Figure 5.3: The effect of glucose concentration of the maintenance coefficient (m_s).

5.4.2 Substrate saturation coefficients (K_s)

The standard method for obtain K_s in continuous cultures could not be used as the standard double reciprocal plot was not linear due to the fact that growth rates apparently affected the saturation constant. Thus the substrate saturation (K_s) for each individual steady state was determined by applying equation 5.12. The value for μ^{max} was taken from the batch experiments in table 4.1, while the substrate concentration was measured directly for samples from experiments (refer to section 3.11).

Figure 5.4 shows a plot of the K_s values as a function of dilution rate and shows that the substrate saturation was influenced by dilution rate. These are

unusual results as typically the value for K_s increases with higher dilution rate and substrate concentration (Pirt, 1975).

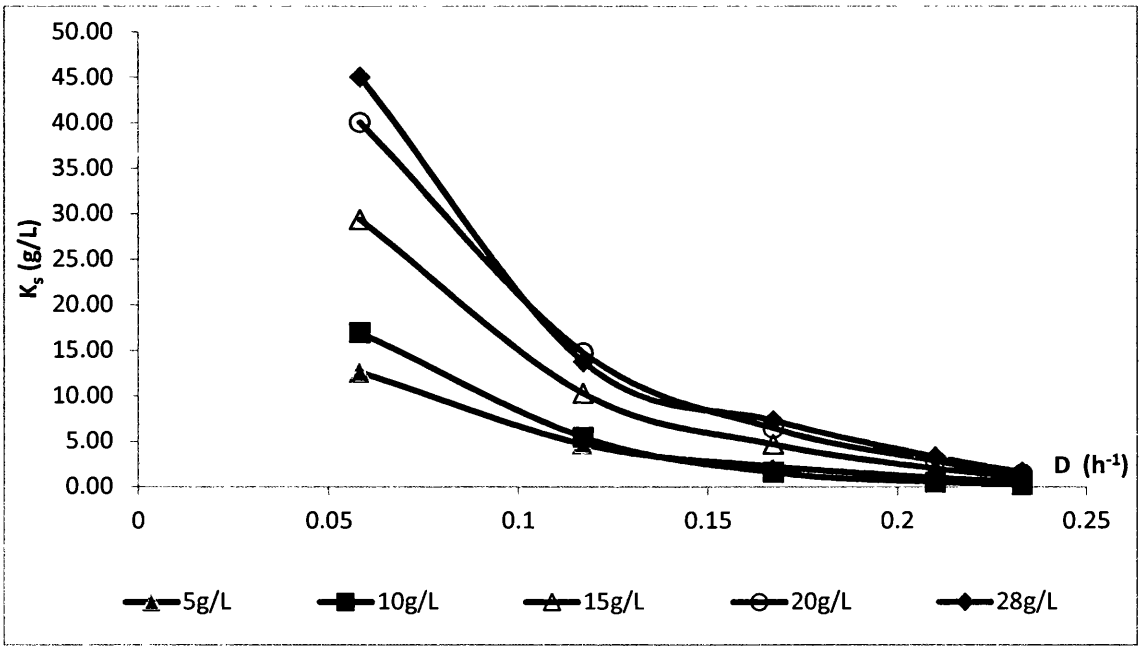

Figure 5.4: Concentration of K_s versus dilution rate.

There are potentially several explanations for such data. The first, observed in figure 5.4, is that at high glucose levels, the K_s increases and as such the glucose substrate uptake is inhibited. Another explanation may be that there is more than one glucose uptake process. i.e., that a passive system is present and active high affinity system is induced at high growth rates. Thus a passive process avoids energy consumption for glucose uptake so enhancing growth yields on glucose. It is also worth noting the maintenance coefficients are low at low growth rates but increase substantially above 10 g/l. Another explanation may be that the yeast extract in the medium at low growth rate represses the sugar uptake systems. Also there were high acetate:butyrate ratios (Figure 4.8 and table 5.4) at low sugar concentrations. This indicates that, electron transport processes to the reduced electron acceptor in the yeast extract may be operating. These observations require further experimental investigations to look more carefully at this problem.

5.5 Product formation

The samples taken from the continuous cultures were then analyzed for fatty acid content so that product formation kinetics may be investigated.

5.5.1 Relationship between dilution and acetic acid production

Figure 5.5 shows the production of acetic acid for a range of glucose concentrations and dilution rates.

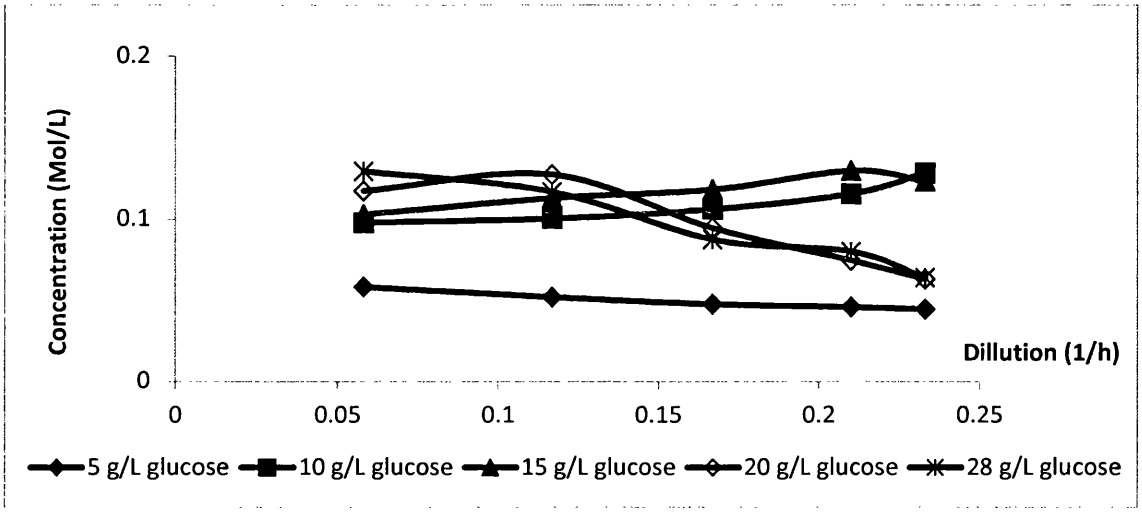

Figure 5.5: The effect of dilution rate and glucose concentration on acetic acid formation in continuous cultures of *C. butyricum.*

The highest production of acetic acid was using 15g/L glucose concentration as a feed. From $0.058h^{-1}$ to 0.21h the level of acetic acid increases in the culture and then drops at $0.233h^{-1}$. At a dilution level of $0.058h^{-1}$ the production of acetic acid is 0.103 mol/L, increasing to 0.129 mol/L at $0.21h^{-1}$ of dilution and then decreasing to 0.123 mol/L at $0.233h^{-1}$.

The 10g/L concentration of glucose gives the second highest level of acetic acid production. However, the results at 10g/L concentrations of glucose are slightly different to those at 15g/L of glucose in that acetic acid production increases from 0.098 mol/L at $0.058h^{-1}$ to, to 0.128 mol/L at $0.233h^{-1}$

At 20g/L glucose, acetate increases from $0.058h^{-1}$ to $0.167h^{-1}$, and then decreases thereafter. Even though the glucose concentration is higher, the production of acetic is less than that for 10 and 15g/L of glucose. Probably another product has been produced, from glucose. It is not uncommon for addition products such as solvents to be formed, i.e. ethanol, butanol or vicinal diketones, glycerol or 1.3 propandiol (Mendes et al., 2001 and Hui et al., 2012). This would be consistent with the observations associated with alkali consumption and the poor carbon recoveries observed at high sugar concentrations. The amount of acetic acid was 0.117 mol/L at $0.058h^{-1}$ and 0.127 mol/L at $0.167h^{-1}$. However at $0.233h^{-1}$ acetic acid production decreased to 0.063 mol/L.

At 28g/L of glucose, a similar but more exaggerated pattern to that at 20 g/l glucose was observed. Therefore the pattern of acetic acid production dramatically decreases from 0.129 mol/L acetate at $0.058h^{-1}$ to 0.064 mol/L acetate at $0.233h^{-1}$, suggesting a further shift into solvent production.

Finally, the lowest level of acetic acid production was at the 5g/L glucose concentration and the dilution rate had little or no effect on acetate production with a slight decrease detected, from 0.058 mol/L at $0.058h^{-1}$ to 0.044 mol/L at $0.233h^{-1}$.

5.5.2 Relationship between dilution and butyric acid production

As with acetate, samples of the cultures were assayed for the butyrate content. Figure 5.6 shows the effect of glucose concentration and dilution rate on the pattern of butyric acid production.

The patterns exhibit some similarities with acetate at high glucose concentration (15 to 28 g/L), with an initial increase at low dilution rates followed by dramatic decreases as the dilution rate is increased. The highest levels of butyrate production in these system was over 0.06 mol/L at 28g/L. With 10g/L there is a

different pattern with no decrease butyrate concentrations at high dilution rates and butyrate increases from 0.023 mol/L at 0.058h^{-1} to 0.040 mol/L at 0.233 h^{-1}.

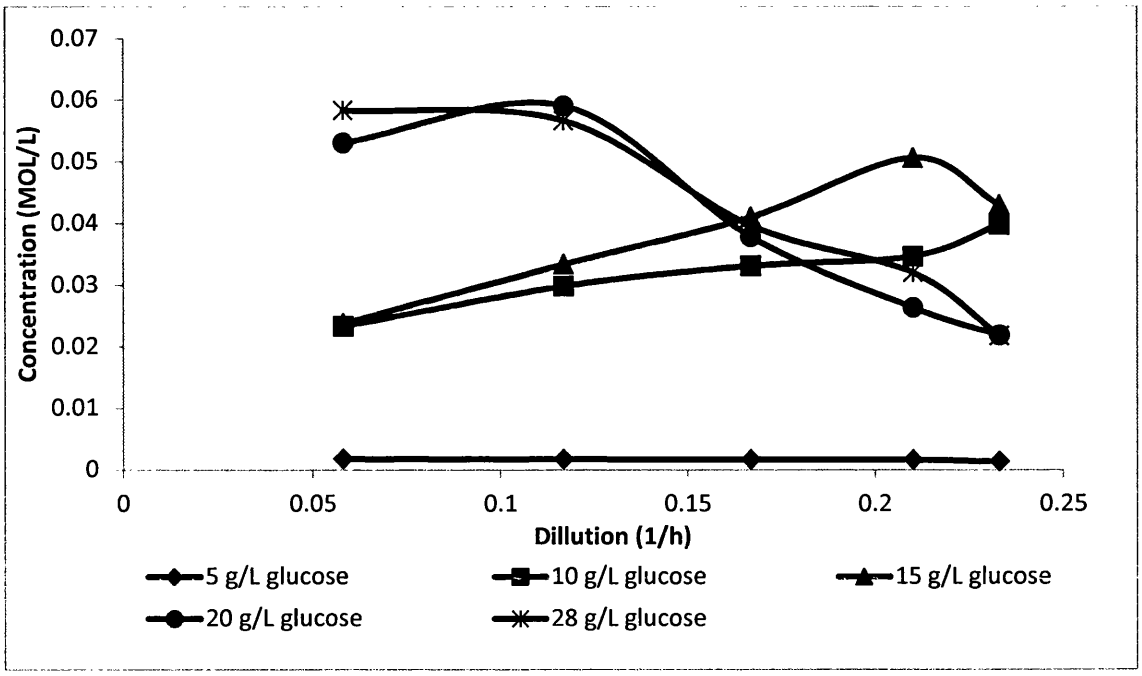

Figure 5.6: Relationship between butyric acid production and dilution rate for a range of glucose concentrations.

In contrast to the other sugar concentrations, the 5g/L level shows very low concentrations and slight decreasing butyrate production pattern over the whole range of dilution rates (figure 5.6). As observed with acetate, fatty acid production at these low glucose fermentations were markedly different showing a marked shift towards acetate production over butyrate. Glucose uptake kinetics are poor and the subsequently rates of carbon and electron flow pathways from glucose to butyrate are low. At 0.058h^{-1} the amount of butyric acid is 0.0017 mol/L and decreases slowly over the range of dilution rates until at 0.233h^{-1} where only 0.0013 mol/L of butyric acid was detected.

5.5.3 Relationship between dilution rate and carbon balance

The results of the analysis above prompted further analysis by carrying out carbon balance calculations for each dilution rate and sugar concentration at steady state. To carry this out, a number of assumptions were made.

1. Cell mass was 50% C by weight. This is a typical value for bacterial cells (Atkinson and Mavituna, 1991).
2. Yeast extract was also 50% C by weight (Bridson, 1998).
3. For each mole of acetate produced 1 mole CO_2 was produced (see biochemical pathways. This assumption can be illustrated in figure 2.1 which shows the metabolic pathway for the fermentation of glucose in this organism).
4. For each mole of butyrate produced 2 moles of CO_2 were produced (see also figure 2.1).

Figure 5.7 shows the percentages of carbon recovery through acetic and butyric acid formation. This figure was developed by considering calculations in table 5.3.

Figure 5.7 shows, for 10 and 15g/L glucose there was high percentages of carbon, suggesting that most of the carbon from yeast and glucose in this concentration was converted to acetic acid, butyric acid and carbon dioxide. Low growth rates gave the poorest recovery while high growth rate gave the best results for carbon recovery. However, for 5, 20 and 28 g/L glucose feeds, there was poor carbon recovery.

For 5g/L, cell concentrations were at their lowest. In these conditions there was only poor carbon uptake with only low levels of butyrate detected. Also it was assumed that all the carbon present in the yeast extract was consumed and maybe this provided an over estimate of the carbon utilised. Clearly, considering the glucose consumed, a good proportion of the carbon in the fatty acids came from the yeast extract.

Table 5.3: Table of carbon balances for variety of glucose concentration.

Initial glucose (g/L)	D (h-1)	Glucose (mM)	Yeast (mM)	Total input C (mM)	Cell(mM)	Acetate $+CO_2$ (mM)	Butyrate+ $2CO_2$ (mM)	Total output C (Mm)	% C recovery
	0.058	46.63	410.00	456.63	13.75	174.00	12.00	199.75	43.74
	0.117	39.96	410.00	449.96	12.50	156.00	12.00	180.50	40.11
5	0.167	36.63	410.00	446.63	11.67	141.00	12.00	164.67	36.87
	0.21	33.30	410.00	443.30	9.58	135.00	12.00	156.58	35.32
	0.23	26.64	410.00	436.64	9.17	132.00	6.00	147.17	33.70
	0.058	169.85	410.00	579.85	15.42	292.50	139.50	447.42	77.16
	0.117	185.83	410.00	595.83	18.75	300.00	178.50	497.25	83.45
10	0.167	239.45	410.00	649.45	26.67	317.20	198.16	542.03	83.46
	0.21	261.77	410.00	671.77	30.00	345.60	207.36	582.96	86.78
	0.23	269.76	410.00	679.76	36.67	384.00	238.08	658.75	96.91
	0.058	280.08	410.00	690.08	20.42	307.20	142.08	469.70	68.06
	0.117	298.40	410.00	708.40	32.08	337.92	199.68	569.68	80.42
15	0.167	314.72	410.00	724.72	43.33	353.28	245.76	642.37	88.64
	0.21	339.70	410.00	749.70	57.50	387.84	303.36	748.70	99.87
	0.23	333.04	410.00	743.04	44.17	368.64	257.28	670.09	90.18
	0.058	449.60	410.00	859.60	22.08	351.00	318.00	691.08	80.40
	0.117	459.26	410.00	869.26	40.00	411.00	354.00	805.00	92.61
20	0.167	467.58	410.00	877.58	63.75	282.24	226.56	572.55	65.24
	0.21	455.60	410.00	865.60	59.58	222.72	157.44	439.74	50.80
	0.23	443.61	410.00	853.61	52.08	188.16	130.56	370.80	43.44
	0.058	547.85	410.00	957.85	31.25	387.00	348.00	766.25	80.00
	0.117	559.17	410.00	969.17	68.33	348.00	342.00	758.33	78.25
28	0.167	501.22	410.00	911.22	63.33	261.00	240.00	564.33	61.93
	0.21	482.57	410.00	892.57	59.58	240.00	192.00	491.58	55.07
	0.23	497.56	410.00	907.56	51.67	192.00	132.00	375.67	41.39

Note: Yeast extract at 10 g/L contains approximately 50% C

For 20 and 28g/L poor carbon recoveries were also observed, and this was more pronounced at high growth rates. The factor which contributed to this situation is that other products such as solvents or other types of fatty acid were formed. Then, this would affect the calculation for carbon recovery because the calculation in table 5.3 only considered acetate, butyrate and carbon dioxide formation. Previous results from the study of batch cultures (table 4.2, chapter 4) also suggest the poor carbon

recovery could be explained by fact that undetected end products are not taken into account. These are most probably neutral non-volatile solvent materials as the amount of neutralisation by alkali is also low in these conditions (figure 4.13, Chapter 4)

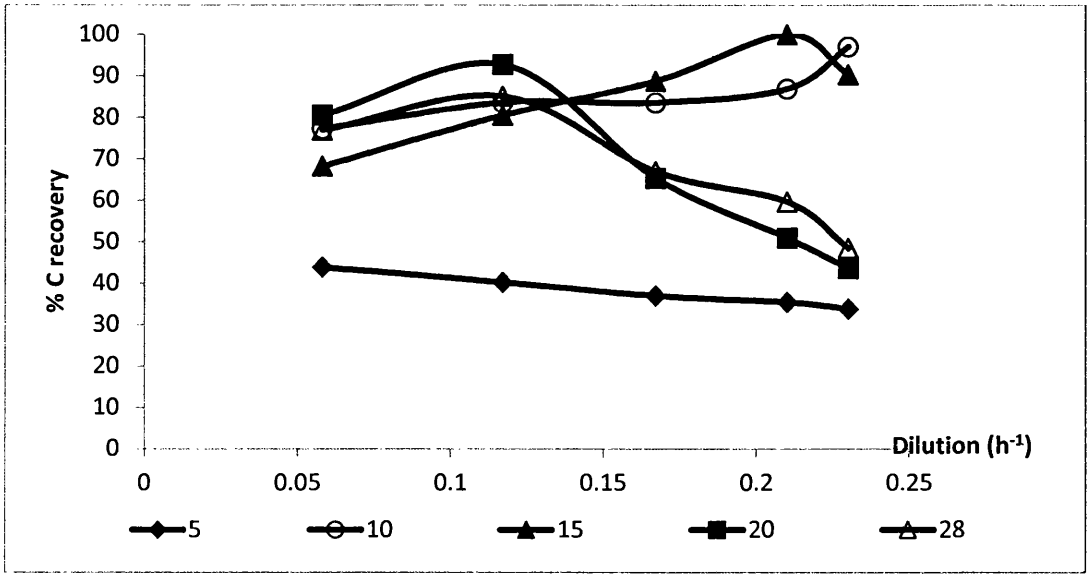

Figure 5.7: Percentages of carbon recovery for variety of glucose concentration and dilution rates by acetic and butyric formation.

5.5.4 Relationship between dilution rate and total acid production

Figure 5.8 shows the amount of total acid (butyric and acetic acid) production for a range of dilutions and glucose concentrations.

The highest production of total acid at $D=0.058h^{-1}$ was 0.19mol/L in 28g/L glucose concentration. This was followed by the concentration at 20g/L being 0.17 mol/L, then at 15 g/l and 10 g/L and with 0.13 and 0.12 mol/L total acid productions respectively. Generally, concentration of product formation was directly related to the glucose consumed, especially at 20 and 28 g/L glucose feeds.

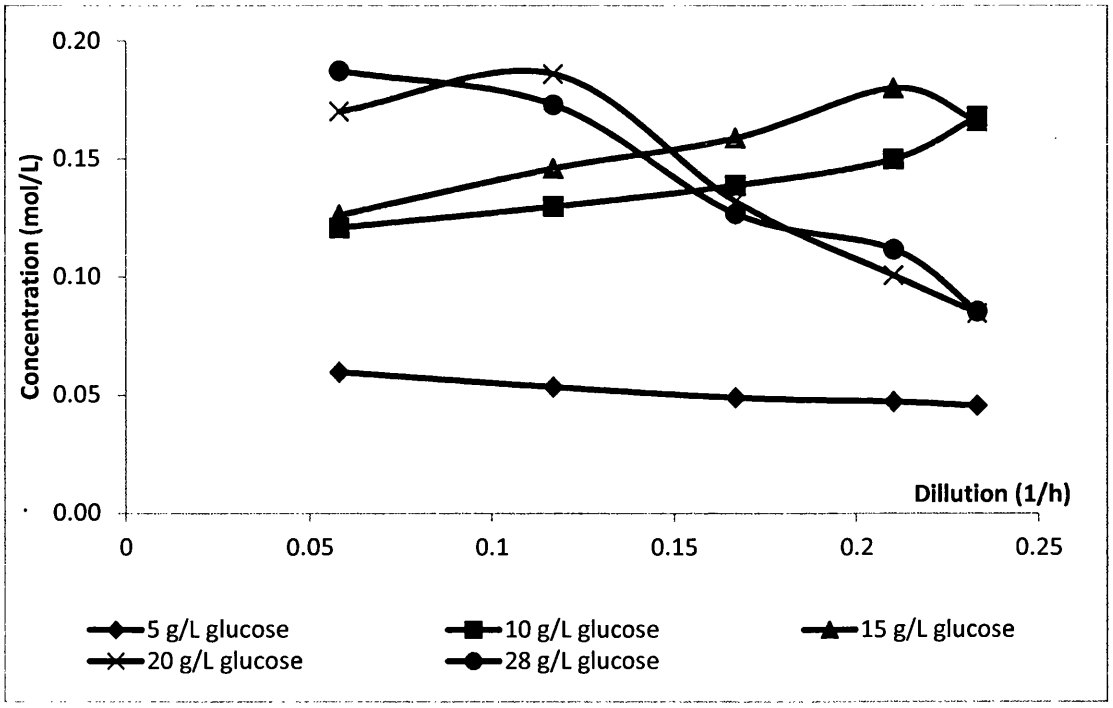

Figure 5.8: Relationship between total acid production and dilution rate for a range of glucose concentrations

5.5.5 Ratio between acetic and butyric acid

From the results in the section 5.5.1 and 5.5.2, a comparison of the ratios of acetate to butyrate was made. Table 5.4 shows the ratio between acetic acid to butyric acid for a range of dilution rates and glucose concentrations

Table 5.4: The ratio between production of acetic and butyric acid for a range of dilution rates and glucose concentrations

Dillution rate (h^{-1})	0.058		0.1167		0.1667		0.21		0.233	
	q_s	Ratio	q_s	Ratio	q_s	Ratio	q_s	Ratio	q_s	Ratio
5 g/L	0.059	34.16	0.134	31.29	0.161	29.71	0.121	29.75	0.155	35.30
10 g/L	0.802	4.19	1.433	3.36	1.416	3.20	2.081	3.32	3.33	3.23
15 g/L	0.980	4.32	1.341	3.38	1.487	2.88	1.525	2.56	2.559	2.87
20 g/L	1.450	2.21	1.639	2.15	1.509	2.49	1.989	2.83	2.435	2.88
28 g/L	1.260	2.21	1.014	2.05	1.405	2.20	1.800	2.49	2.378	2.93

The ratios were calculated by dividing the concentration of acetic acid (mol/L) with concentration of butyric acid (mol/L).

The highest ratio between acetic to butyric acid was at a 5g/L glucose concentration, as previous measurements had suggested, and was over 30 mol acetic to 1 mol butyric. For 10 and 15 g/L initial of glucose, the ratio between acetic and butyric acid was almost similar but much lower than at 5 g/l culture at approximately 4 to 3 mol acetic to 1 mol butyric.

The acetate butyrate ratios, for 20g/L and 28g/L of initial glucose concentrations were around 2 to 3 mol acetic to 1 mol butyric acid. Interestingly the ratios remain stable over the range of dilution rates but were altered by the amount of glucose present in the fermentation.

5.6 Conclusions

In this chapter, continuous culture studies have establish the kinetics parameters for growth. The effect of dilution on the growth of *C. butyricum* allowing the evaluation of growth kinetics and product formation was investigated. In addition, the effect of glucose concentration on growth performance was also analysed.

Five rates of dilution representing a range growth from slow and rapid growth were investigated for high and low glucose concentrations (5, 10, 15, 20 and 28g/L). The kinetics in these systems were then determined and in most cases were typical of an anaerobic organism. However, most notable was the relative sugar uptake of systems which showed that the affinity for glucose uptake was related to glucose concentration with the affinity increasing with increasing sugar concentrations.

The effect of dilution rate and glucose concentration on the growth and product formation of *C. butyricum*, were also investigated. The studies with 10g/L glucose concentration gave a typical response for the five rates of dilution as the dilution increases the biomass concentration increases and fatty acid productivity also increased. These systems also gave good carbon recovery in the products of the fermentation. The kinetic data obtained provides a good understanding of the culture in these conditions. However, this cannot be said of cultures with high

glucose concentration, for example Table 5.3 shows the proportion concentration between glucose, acetic and butyric acid. Here at high glucose concentration and high dilution rates there are distinct shifts in the fermentation towards butyrate with poor carbon recovery. The explanation for the shifts indicate the rate of glucose feed has a significant affect on the fermentation balance, suggesting that the carbon and electron flow pathways within the cells are altered allowing new product formation and a shift of metabolism toward more reduced end product in butyrate. This suggests especially that the capacity of electron flow to hydrogen becomes saturated or altered so that additional reducing electron equivalent are passed to butyrate so reducing the proportion of the oxidise product, acetate. Further study would be required to evaluate and confirm these interesting possibilities, for example the measurement of hydrogen and the determination of other potential end products that are also most certainly formed in cultures that contain high sugar concentration (i.e. >20 g/L).

The yield coefficients obtained from the cultures show some interesting findings. At 5g/L glucose the yield was quite different compared to the yield coefficient (refer to table 5.2) at other glucose concentrations. The amount of yield coefficient for 5g/L was relatively high, around 0.24-0.28 g cell/g sugar; while at other concentrations just around 0.07-0.20 g cell/g sugar (table 5.2). This is because, for 5g/L glucose a considerable amount of growth is based upon consumption of the yeast extract present in the medium with only limited amount of glucose consumption. Therefore, the growing cells have used other nutrients in yeast extract, consequently the growth yields will be higher as the amino acids and nucleotides in the yeast extract do not have to be synthesised within the cell.

The value of the maintenance coefficient (m_s) is dependent on experimental conditions, clearly the m_s increases with increased glucose concentration (figure 5.3). Therefore, the amount of m_s for a range of glucose concentrations differed between 0.02 to 1.1 g/substrate/g/cell/h. This is most probably due to high end-product fatty

acid concentrations know to inhibit the growth causing a diversion of resources (energy) to maintain cell homeostasis in aggressive environments. Further work on this aspect would have been useful especially the effects of pH, temperature and end product concentrations as this parameter can be used to control the concentration of biomass formed (i.e. cell yield).

Chapter 6: Membrane bioreactor

6.1 Introduction

This chapter reports the results of an investigation of the growth and performance of *C. butyricum* in a membrane bioreactor. The performance of the reactor was assessed using a range of glucose concentrations in the feed. This is a very intensive form of growth and product formation as the cells are retained within the system whilst the spent medium is removed as a permeate through the membrane. As a result the rate of feed can be very high, well above that observed in continuous culture and as such allows an intensified fatty acid production process. Another benefit of this approach is that the toxic end-products, fatty acids, are removed and this should also allow an enhancement of kinetic performance of the cells. In addition the products stream is cell free and therefore is more amenable to cell processing.

This chapter also reports and demonstrates the main areas of technical novelty of this thesis in that there are no prior reports of the growth *C. butyricum* with a membrane bioreactor.

Membrane bioreactors can be classified by their combination of bioreactor type and membrane. In this study membrane filtration has been used in the separation process. As membrane technology has developed, the application of membrane separation technology to biological process systems has attracted many researchers as a method of process intensification (Brindle et al., 1996; Chiemchaisri et al., 1993). The MBR system has allowed an increase in cell reactors, thereby encouraging the process to operate faster with an increase in productivity (Buisson et al., 1998; Cote et al., 1997). Previous researchers have reported that MBR have been applied in high concentration cell cultivation to produce organic products

(Tanugch et al., 1987; Borch et al.,1991; Kurosawa et al., 1991; Kai et al,. 1992; Chang et al., 1994).

6.2 Membrane bioreactor theory

Darcy's law has been applied and subdivided to investigate membrane resistance. The permeate flux (equation 6.1) has been used (Choo et al., 1996; Boyaval et al., 1996).

$$J_V = \frac{dV}{dt} x \frac{1}{A} \qquad (6.1)$$

where;

J_v= Permeate flux (L h^{-1} m^{-2})

dv/dt= Volume consumption with time (L/h)

A = Total membrane surface area(m^2)

Also the flow is a function of the changing volume of the reactor then

$$F = \frac{dV}{dt} \qquad (6.2)$$

where;

F= Flow rate (L/h)

dv/dt= Volume consumption with time (L/h)

Therefore, flux is described as

$$J_V = \frac{F}{A} \qquad (6.3)$$

When a cake builds up on the membarne surface then the total resistance to flow, R_T, is made of two components, the membrane resistance, R_m, and the cake resistance R_c,

$$R_T = R_M + R_C \quad (6.4)$$

The total resistance may be related to flow:

$$R_T = \Delta P/\mu J_V \quad (6.5)$$

Then, reorganising equation 6.4 and 6.5 gives;

$$R_C = \frac{\Delta P}{\mu J_v} - R_M \quad (6.6)$$

where;

μ = permeate viscosity in (kg $m^{-1}s^{-2}$)

These defined parameters can be used to predict the membrane performance.

6.3 The permeate flux and transmembrane pressure

An investigation of the MBR was conducted using distilled water to determine relation between flow and pressure and also to benchmark the flow rate for cleaning the membrane bioreactor system after each operation or experiment. The investigation was conducted with a cleaned membrane. The performance was measured with water and compared to initial flow rates or fluxes calculated. From this, after a run, when the performance is significantly reduced, then, that the reactor needs further cleaning. These methods are described in section 3.7.2.

The MBR system in this investigation was operated at 37°C and at 6 different levels of transmembrane pressure (TMP), starting with 3.6psi and increasing stepwise to 10.9psi. For every TMP, the flow rate was determined by measuring the time for 100mL of pure water to pass through the membrane. From these measurements the permeate flux (J_v) could be determined as a function of TMP.

Figure 6.1 shows the variations of transmembrane pressure on permeate flux for distilled water. The illustration shows that increasing TMP increases the flow rate

of fluid through the membrane and was linear and thus directly related to TMP. From this, the equation for the relationship between flux and pressure is J_v =0.0401TMP, where Jv is the flux (L/h/m^{-2}) and TMP is trans membrane pressure (psi).

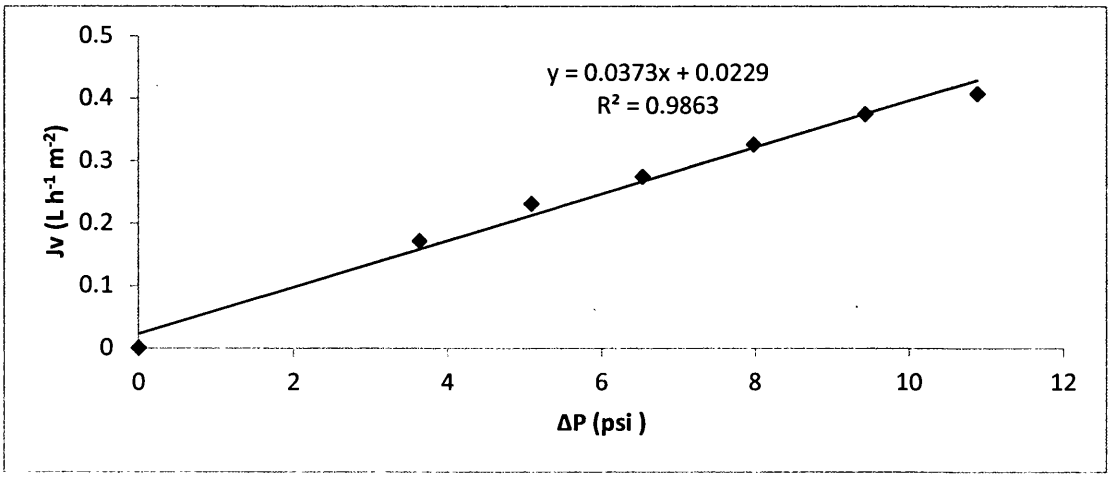

Figure 6.1: The effect of pressure on the flux obtained during the operation of MBR using distilled water.

6.3.1 Effect of media on membrane performance

A further experiment on membrane flux was carried out using the growth medium using the same techniques as described in section 6.3 i.e the flux rate of the medium as a function of pressure. This investigation was carried out at 37°C and pH 6.5 using the standard medium as described in table 3.5. Figure 6.2 shows the effect of media on membrane performance as a function of TMP. The flux increases with increase in TMP. The relationship between J_v and pressure (ΔP) is linear. However, the flux for the growth medium was less than that for distilled water, showing that medium causes some fouling, although the flux is still governed by TMP. The fouling was thought to affect the virtual pore size of the membrane by binding or interaction within the pores of the membrane rather than the formation of an actual cake on the membrane surface.

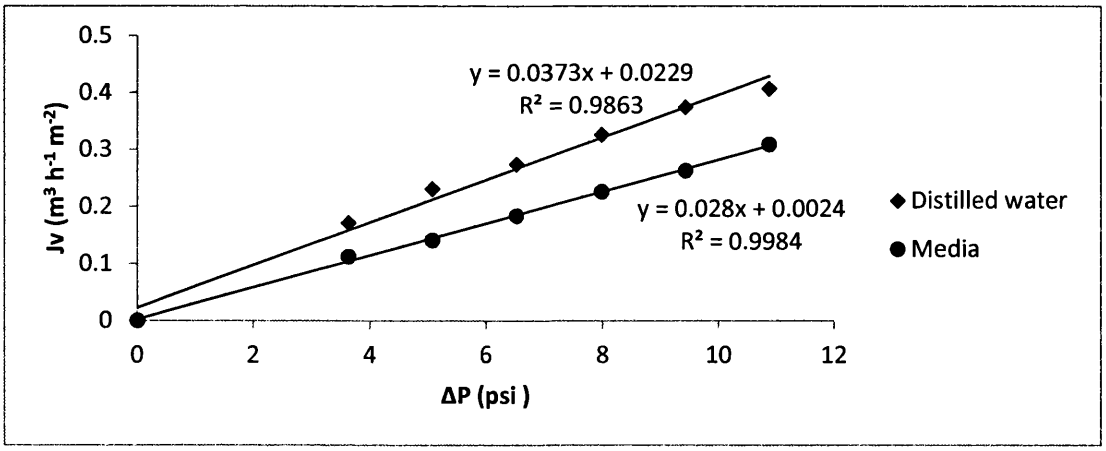

Figure 6.2: A comparison of fluxes for distilled water and growth media.

Cake resistance was calculated by using equation 6.6. Figure 6.3 show the estimation of cake resistance as a function of TMP, ΔP. Cake formation units are psi m^2 h / m^3 and this is around 10-11 (psi m^2 h / m^3) during the operation with pressure around 3-11psi.

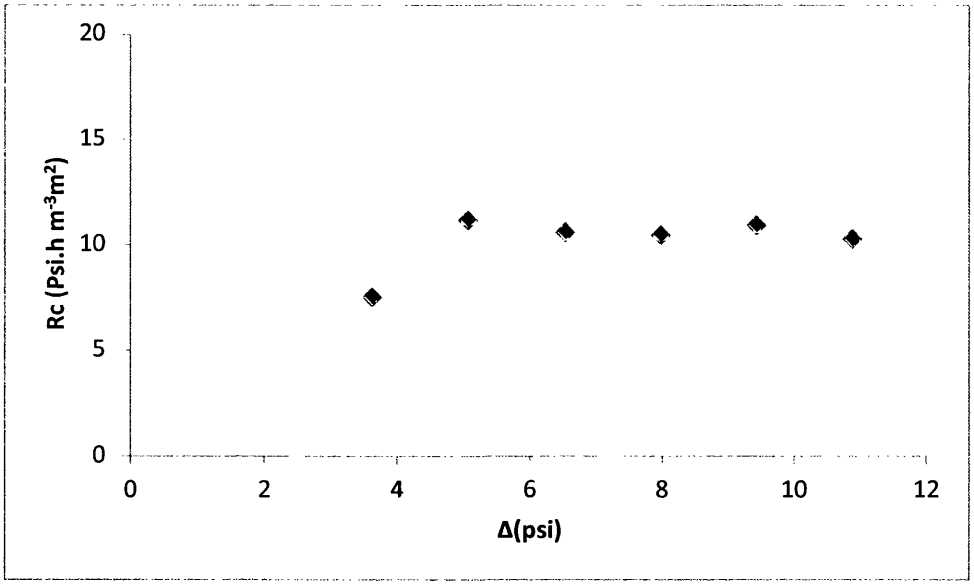

Figure 6.3: Little effect of ΔP on cake resistance at 37°C and pH 6.5.

6.4 Membrane bioreactor culture

The construction and operation of the membrane bioreactor for culturing bacteria was discussed in section 3.7.4. The MBR was set up according to the methods outlined in section 3.7. A 20 litre inoculum of *C. butyricum* was prepared and pumped into the MBR where it was filtered until all 20L were added. The pH and temperature were set at pH 6.5 and 37°C. The medium pump then started and the feeding strategy was then applied. This relied on the fact that as the cells are retained and the cell concentration increases as the organisms grow, then the feed rate was increased in proportion with the aim of maintaining the medium in excess. The feed rate was initialy set at 4 litre per hour then this increased to 8 L/ hour after 1 doubling time, and then 16 L/h after a further doubling time and finally 32 L/h after another doubling period. During these experiments samples were taken periodically for analysis of cell concentration, substrates and products.

6.4.1 Cell growth and glucose consumption

Figure 6.4 shows the relationship between biomass and glucose consumption in the membrane bioreactor for three feed glucose concentrations (Figure 6.4 I, II and III) and four permeation rates (Zones A-D) at each feed concentration. For all the concentrations; the consumption of glucose increased whilst, at the same time, the concentration of biomass increased.

Figure 6.4(I) shows the results for a 5g/L feed glucose concentration. In zone A, with a permeation rate of 4L/h, and from 0 to 10 hours, the amount of biomass almost doubles from 0.367g/L to 0.739 g/L, for glucose it decreased from 4.87g/L to 2.63g/L. Next, in zone B, with a permeation rate of 8L/h, the concentration of biomass increases to 3.068g/L, approximately four times, while the concentration of glucose dropped to 0.84 g/L. In zone C with a permeation rate of 16L/h, the biomass concentration gradually increased to 4.232g/L while the glucose concentration decreased to 0.289 g/L. Subsequently, zone D, with a permeation rate of 32L/h

shows the biomass concentration increased slowly to 5.676 g/L and glucose concentration was 0.524 g/L.

Figure 6.4 (II) shows that for an initial 10g/L of initial glucose. In the feed, in zone A, biomass concentration increases slowly from 0.377g/L to 0.826g/L and glucose consumption decreased from 9.76g/L to 4.51g/L. Then, in zone B, biomass increases rapidly to 2.989g/L, approximately a three and half times increase. At this point, the glucose concentration was 0.64g/L. In zone C, biomass still increased to 4.819g/L with a glucose concentration of 0.152g/L. Next, in zone D, the biomass slowly increased to 5.861g/L with glucose concentration of 0.193g/L.

Figure 6.4 (III) shows results for the 15g/L feed glucose. In zone A the biomass started with a concentration 0.380 g/L, increasing to 1.55 g/L with consumption of glucose, while reducing the glucose concentration from 13.5g/L to 4.4g/L. Then, in zone B, the biomass concentration increased substantially to 3.26g/L with glucose concentration being reduced to 0.39g/L. In zone C, biomass increased gradually to 4.78g/L and the concentration of glucose dropped to 0.096g/L. Then, in zone D the biomass slowly increased to 5.88g/L and with the concentration of glucose at 0.028g/L.

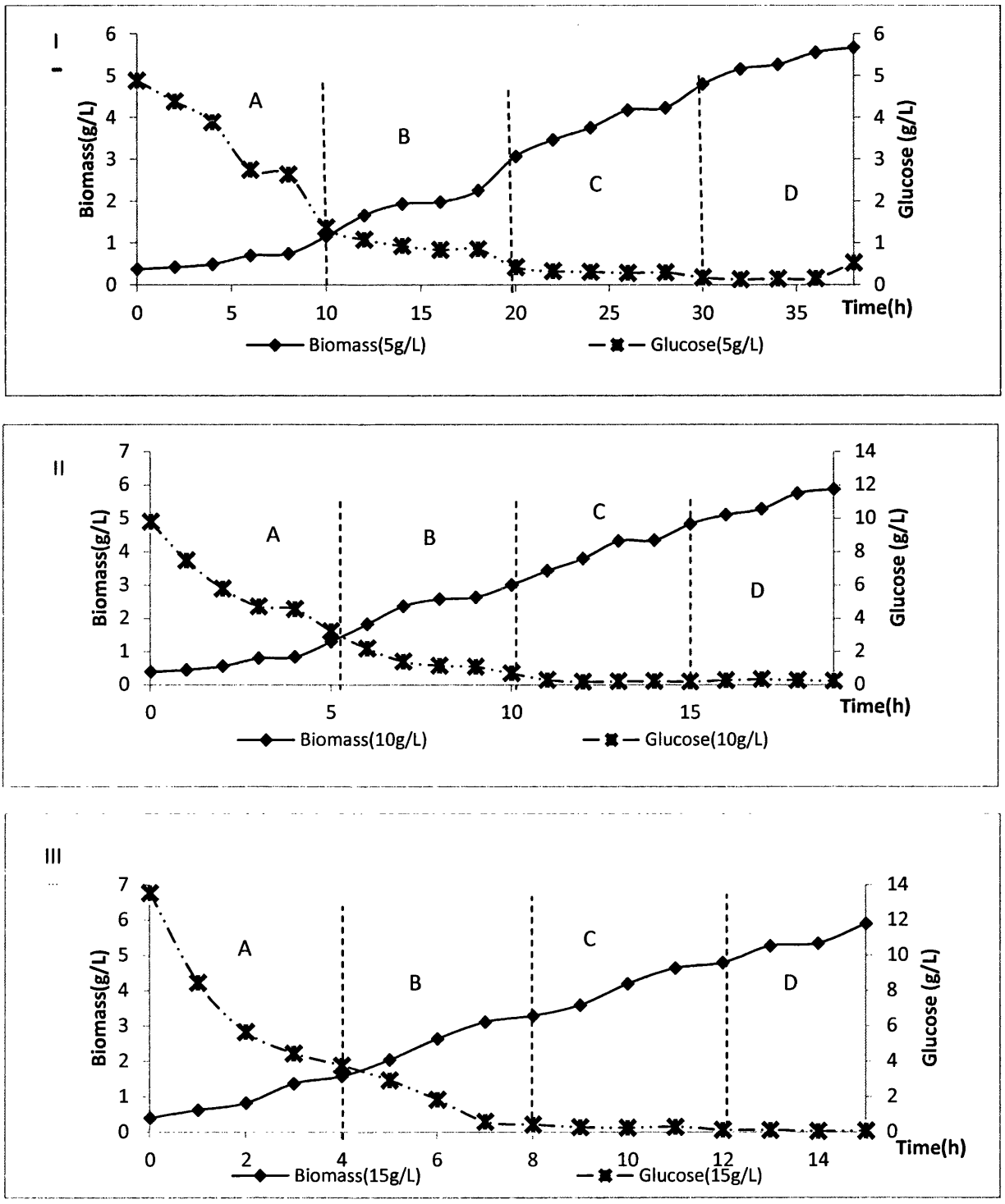

Figure 6.4: The growth of *C. butyricum* on glucose media at three ceontrations of glucose. (I) for 5g/L feed,(II) 10g/L feed and (III) 15 g/L, at permeation rates: A: 4L/h, B: 8L/h, C:16L/h, D:32L/h

6.4.2 Relationship between permeation rates with growth rate (μ)

Using the data gathered previously, the growth rates within the MBR system were investigated. The specific growth rate, μ was calculated using equations 3.12, 3.13, 3.14 and 3.15, from section 3.16. This was evaluted by plotting log graph between biomass against time.

Figure 6.5 shows the log graphs of biomass concentrations against time for MBR system with feeds containing 5, 10 and 15g/L glucose over a range of permeate rates (4, 8, 16, 32 L/h). Thus the slope for each permeate rate and feed glucose concentration were determined, and hence the growth in these conditions as shown in figure 6.5 and table 6.1.

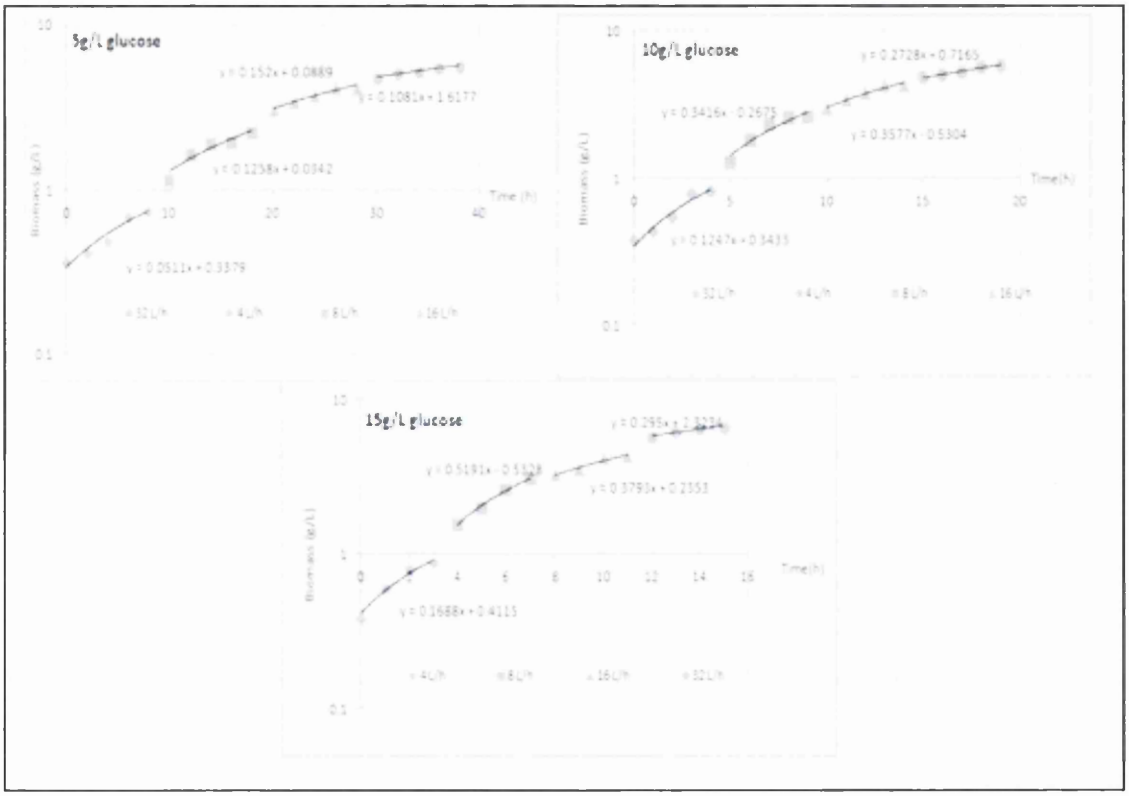

Figure 6.5: The measurement of growth rate within the MBR. The log of biomass is plotted against time and the slopes obtained are used to calculate values of μ for a range glucose concentrations and feed rates.

Table 6.1 shows, the analysis data from figure 6.5. K in this table is the slope the graph in figure 6.5. Then, equation 3.15 was applied to determine value of growth rate (μ) and subsequently doubling time.

Table 6.1: The values of specific growth rate derived from figure 6.5 for a range of glucose feed concentrations and permeate rates.

5g/L glucose

Flowrate(L/h)	4 L/h	8 L/h	16 L/h	32 L/h
K (Slope of graph)	0.051	0.126	0.152	0.108
μ (growth rate)	0.074	0.182	0.219	0.156
t_D (doubling time)	13.562	5.509	4.559	6.417

10g/L glucose

Flowrate(L/h)	4 L/h	8 L/h	16 L/h	32 L/h
K (Slope of graph)	0.125	0.342	0.358	0.273
μ (growth rate)	0.180	0.493	0.516	0.394
t_D (doubling time)	5.557	2.029	1.937	2.540

15g/L glucose

Flowrate(L/h)	4 L/h	8 L/h	16 L/h	32 L/h
K (Slope of graph)	0.169	0.519	0.379	0.295
μ (growth rate)	0.244	0.749	0.547	0.426
t_D (doubling time)	4.105	1.335	1.828	2.349

Table 6.1 and figure 6.6, show the trends of growth rate at different glucose concentrations used in these experiments.

The highest μ was achieved from a 15g/L glucose concentration. It started with a μ of 0.244h^{-1} at a 4L/h permeation rate. Then, it increased dramatically to 0.749 h^{-1} at 8L/h. After this, the pattern of μ decreased gradually at 16L/h with 0.547 h^{-1}. At a 32L/h flow rate, the trend of μ continuously decreased to 0.426 h^{-1}.

With 10g/L of glucose, at 4L/h a μ value of 0.180 h^{-1} was observed. At 8L/h the μ value increased to 0.493 h^{-1}. Above this, μ slowly increased to 0.516 h^{-1} at 16L/h, followed by a slight decrease to 0.394 h^{-1}at 32L/h.

At 5g/L a μ of 0.074 h^{-1}at 4L/h was observed, this then increased to 0.182 h^{-1} at 8L/h, 0.219 h^{-1} 16L/h and 0.156 h^{-1} at 32 L/h.

There is a common trend in all experiments and that is that the specific growth increases initially and then falls at the higher rate feed rates. This is thought to be related mainly to two factors. The first being substrate limitation (see figure 6.4, zone C and D) and the second is the relatively high biomass concentrations observed towards then end of the fermentations. At high biomass concentrations, the maintenance component of glucose consumption which is dependent on the biomass concentration (see equation 5.14), means that the proportion of carbon utilised for maintenance increases. The implications of this are that growth rates will decline and so will cell yields on glucose.

If these values of specific growth are compared with those obtained in batch culture (table 4.1) then the growth rates are higher than those observed in equivalent batch cultures. The main reasons for this are the lower end product concentrations and substrate concentrations observed in the MBR system, meaning that inhibition caused by these factors are not present.

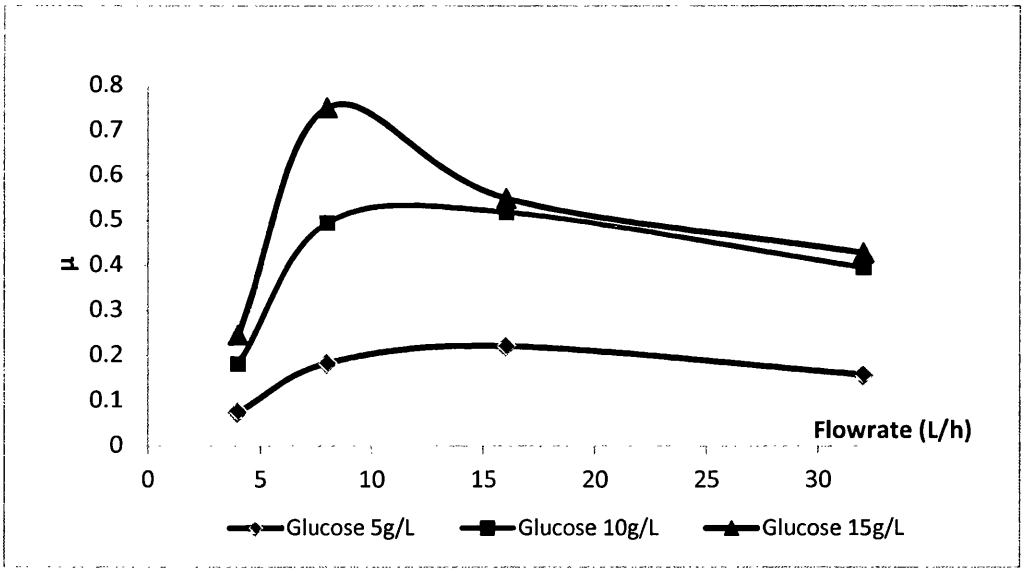

Figure 6.6 : The effect of flow rate on specific growth rate of *C. butyricum* in the MBR rates.

6.5 Acetate and butyrate production rate

Acetic and butyric acid in the membrane bioreactor concentrations were also monitored in the MBR fermentations where three feed concentrations of glucose, at 4 feed rates were investigated as described above. In the MBR, the fatty acids produced are removed in the permeate, however, there are still considerable amounts of fatty acids present in the reactor during the operation.

Figure 6.7 shows the production of acetic and butyric acid at different permeation rates using 5g/L glucose feed. Figure 6.7 shows, that for every increase of permeation rate that the production of acetic and butyric acids increased.

In zone A, acetic acid production, with a permeation rate of 4L/h, started at 0.14g/L at 0h and increased to 2.27g/L. The permeate flow rate was then increased to 8L/h (zone B). Here, there was an immediate, and dramatic, increase in the concentration of acetic acid in the bioreactor from 3.71g/L at 8h gradually increasing to 4.39g/L at 18h. Next, when the permeation rate was increased to 16L/h as shown in zone C, there was small incremental increase from 4.79g/L at 20h to 4.93g/L at 28h. Then in the zone D, the rate of increment decreases, and at 30h the amount of acetic acid production was 5.067g/L, which only marginally increased to 5.08g/L at 36h.

For butyric acid, the changes in butyrate concentrations did not change much over the range of permeation rates. For this concentration, the amount of butyric acid was measured at levels of 0.11g/L to 0.15g/L over the period of 0h to 32h.

The explanation for this behaviour suggests that the conditions within the MBR favour acetate production over butyrate production.

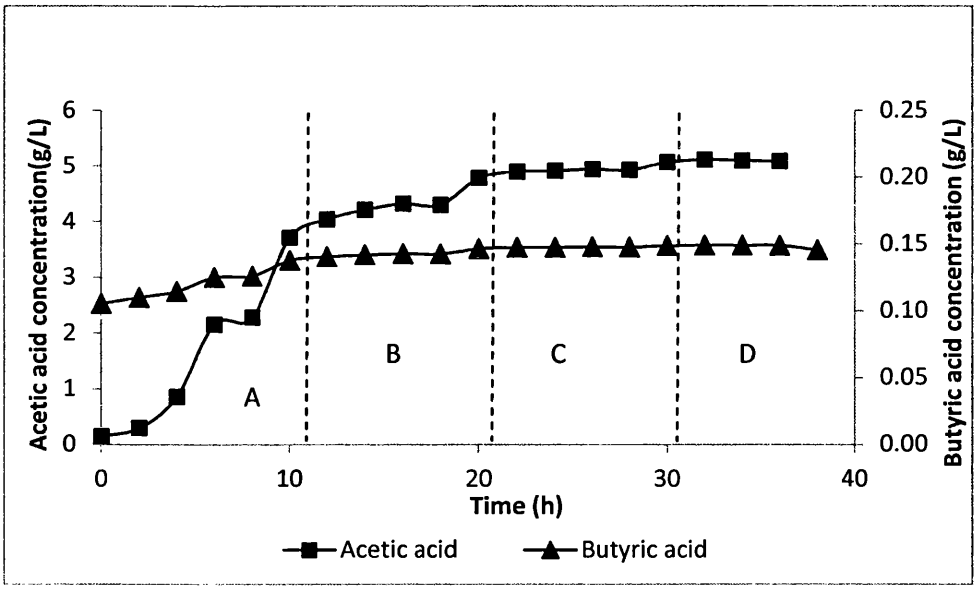

Figure 6.7: The production of acetic and butyric acid by *C. butyricum* grown on 5g/L glucose, at different permeation rates. *A: 4L/h, B: 8L/h, C:16L/h, D:32L/h*

Figure 6.8 shows the production of acetic and butyric acid for a 10g/L glucose substrate at the different permeation rates. Clearly, 10g/L glucose produces higher concentrations of acetic and butyric acid compared to the 5g/L substrate. The process to produce acetic and butyric acid was also faster than at 5g/L.

The acetic acid concentration started at 1.5g/L, at 0h, and increased to 4.91g/L at 4h with a 4L/h permeation rate (zone A figure 6.8). In zone B with permeation rates of 8L/h, the concentration of acetic was 5.76g/L and gradually increases to 7.16g/L at 9h. Then, for zone C (where the permeation rate is 16L/h) acetic acid increases from 7.42g/L at 10h and to 7.72g/L at 14h. At a permeation rate of 32L/h, there is little further increase in acetic acid production. It starts with 7.64g/L at 15h and only slowly increased to 7.68g/L at 19h.

Figure 6.8 also shows the trend for butyric acid production at 10g/L glucose for a variety of permeation rates. In zone A, with a permeation rate 4L/h, the concentration of butyric acid was 0.03g/L at 0h and increased to 1.26g/L at 4h. Then, the permeation rate was changed to 8L/h zone B in figure 6.8, where the butyric acid concentration changes from 1.601g/L at 5h to 2.16g/L at 9h. Next, in zone C the rate

of increase slows, starting at 10h with a concentration of butyric acid 2.26g/L and increasing to 2.38g/L at 14h. Lastly, in the zone D, the butyric acid concentration levels were maintained at 2.38 g/L from 15h to 19h.

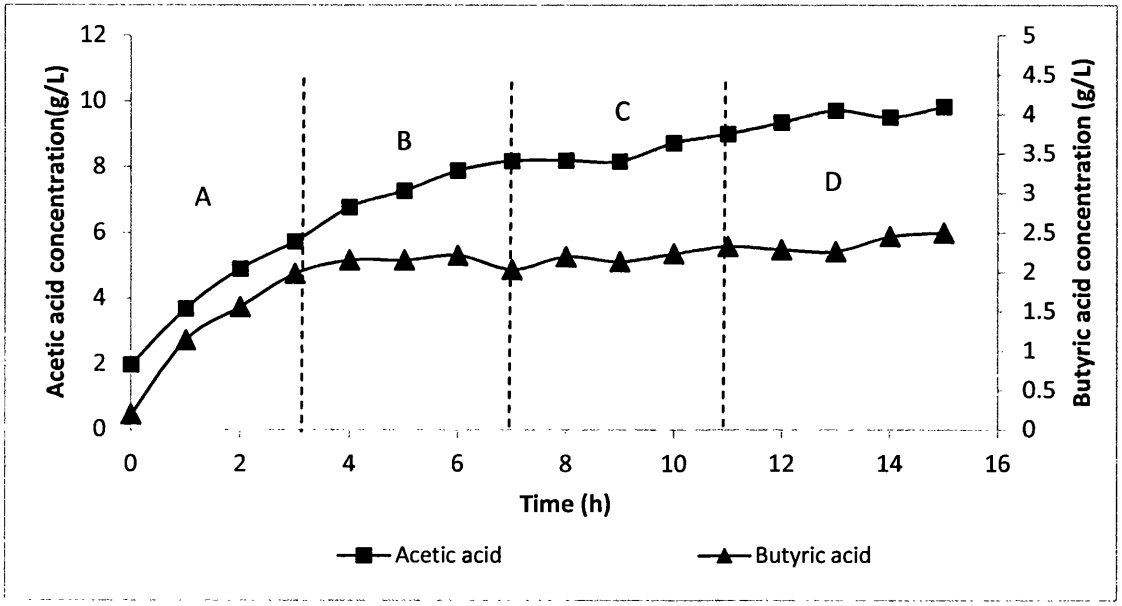

Figure 6.8: The production of acetic and butyric acid using *C. butyricum* grown on 10g/L, at different permeation rates. *A: 4L/h, B: 8L/h, C:16L/h, D:32L/h*

Figure 6.9 shows the trend of acetic and butyric acid production for 15g/L of butyric acid in the 15g/L glucose substrate for a variety of permeation rates. The production of acetic and butyric acid for this glucose concentration was faster than for the 5 and 10g/L glucose substrates.

In zone A, with a permeation rate of 4L/h, the concentration of acetic acid started at 1.96g/L and increased to 5.72g/L at 3h. Then in the zone B, the acetic acid concentration increase climbs from 8.17g/L at 7h. Next in zone C (16L/h permeation rate) and zone D (32L/h) the acetate concentrations was stable and the concentration was 8.17g/L at 12h and 9.82g/L 15h.

For butyric acid, the trend is almost the same as that for acetic acid. In zone A with a permeation rate of 4L/h, it started with 0.187g/L of butyric acid and increased

to 1.976 g/L at 3h. Then, the concentration increases further in zone B, (permeation rate at 8L/h) the concentration of butyric acid increased to 2.03g/L at 7h. After this (in zone C and D) the amount of butyric acid started at 2.320 g/L only increased slightly to 2.494 g/L at 15h.

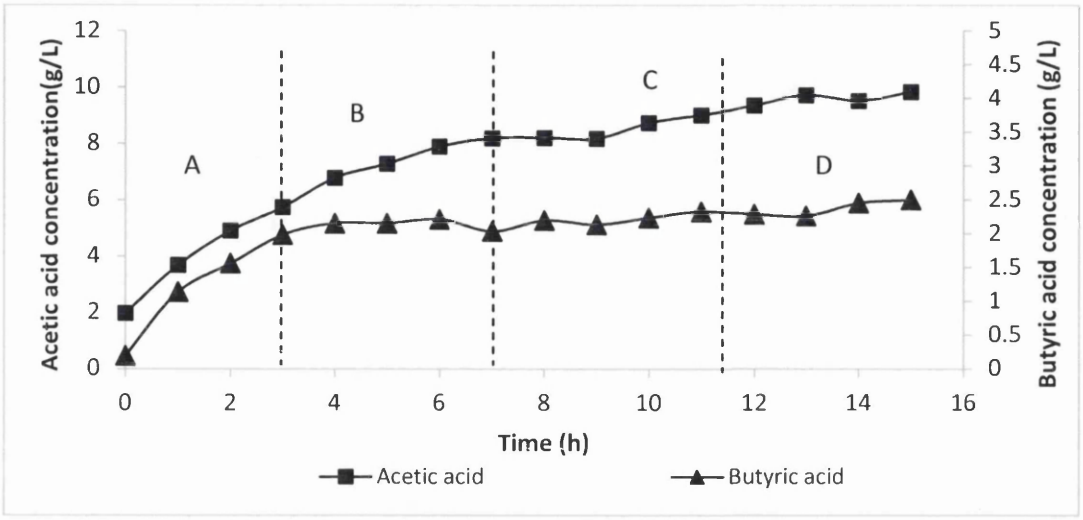

Figure 6.9 : The production of acetic and butyric acid using *C. butyricum* grown on 15g/L, at different permeation rates. *A: 4L/h, B: 8L/h, C:16L/h, D:32L/h.*

6.5.1 Ratio between acetic and butyric acid

Figure 6.10 shows the ratio between acetic and butyric acid for the 5, 10 and 15g/L glucose concentrations at 4, 8, 16 and 32 L/h permeation rates. The amounts have been calculated by dividing the amount of acetic acid by the amount of butyric acid.

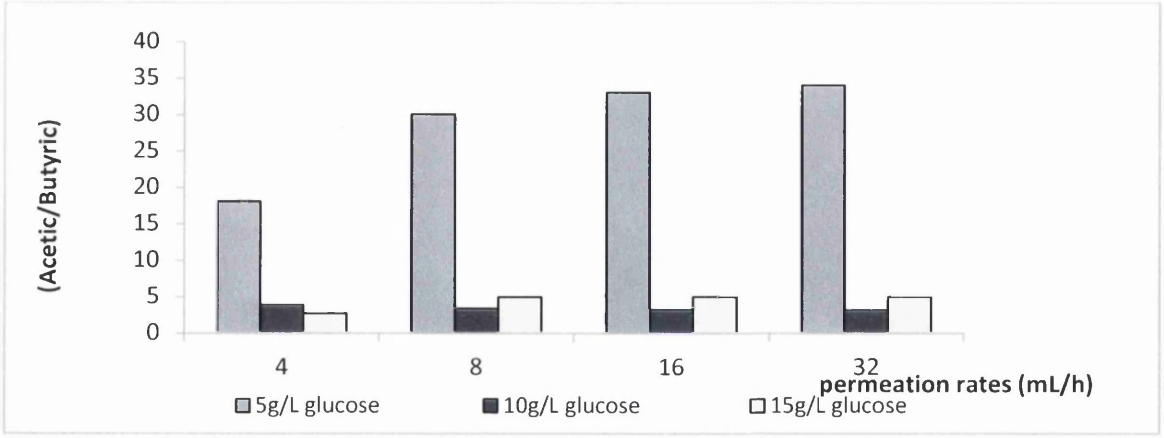

Figure 6.10: The effect of glucose concentration on the ratio between acetic to butyric acid produced by *C. butyricum.*

At a permeation rate of 4L/h, the highest ratio between acetic and butyric acid is achieved with a substrate of 5g/L glucose. The ratio was approximately 18 mol acetic per mol butyric acid. For a substrate with 10g/L glucose the ratio was reduced to 3.8:1, while at 15g/L a ratio of 2.64:1 was observed.

Subsequently, at a permeation rate of 8L/h, the highest ratio of 30:1 was observed for the 5g/L glucose substrate. At higher glucose concentrations ratios were 4.91:1 at 10g/L and 3.3:1 at 15g/L.

At 16L/h, the ratio at 5g/L glucose substrate was 33:1, with the ratio dropping to 3.1:1 and 4.93:1 at 10g/L and 15 g/L glucose concentration respectively.

Finally, for 32L/h, the ratio of acetate to butyrate for 5g/L glucose was 34:1 dropping 3:1 at 10g/L glucose and 4.95 at 15g/L glucose.

In summary, at 5g/L glucose the ratio between acetic to butyric acid is always high and increases with increasing permeation rate. This is because cell concentration increases as the permeation rate increases, but the source of carbohydrate was limited. Following this the carbohydrate had a large capacity to transform glucose to acetic acid with just a little of the carbohydrate remaining for transformation to butyric acid. At about 10g/L the ratio of acetic acid to butyric acid decreased as the permeation rates increased. Probably the source of carbohydrate was adequate for butyric acid production, with the reason for this being that while the permeation rates had increased, the production of butyric also increased. Then at 15g/L glucose the ratio increased in the 4L/h permeation rate, thereafter becoming similar to that at other permeation rates.

6.5.2 Relationship between cumulative of NaOH to cumulative of total acid production.

Figure 6.11 shows the relationship between cumulative NaOH and total acid production for 5, 10 and 15g/L glucose substrates. All of the concentrations show very strong correlation with a regression coefficient of more than 0.96.

10g/L glucose shows a higher correlation with a regression coefficient of 0.9967. This is then followed by the 15g/L substrate with a coefficient of approximately 0.9936 and finally 5g/L with a regression coefficient of 0.9684.

Investigation of the correlation is very important to understand the requirement for sodium hydroxide. The illustration shows that the sodium hydroxide is required to keep the pH in the reactor at pH 6.5 due to the pH value being affected by total acid production. The correlation between consumption of sodium hydroxide and total acid production is important to industrial scale for early prediction total production will be produced.

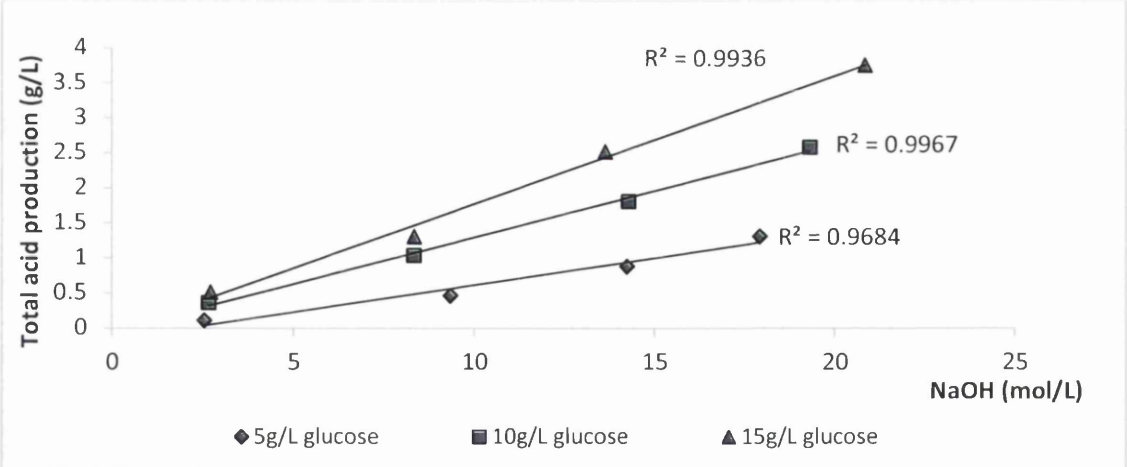

Figure 6.11: The relationship between NaOH required with total acid production for three glucose concentration.

6.6 Productivity

Productivity was calculated by feed flow rate (permeate rates) and by product concentration thus productivity of cells or acetate or butyrate could be determined. It is very important to understand the influence of feed (permeation rates) productivity on cell, acetic or butyric acid production with respect to time.

6.6.1 Cell productivity

Table 6.2 shows the cell productivity in the MBR system for the three different glucose concentrations. In the MBR system, cells were recycled and retained by membrane system. Therefore, the calculation of cell productivity is identical to that of the batch system as shown in chapter 4, i.e. cell productivity is calculated by dividing total cell production with the time taken.

Table 6.2 shows that the cell productivity increased as the initial glucose concentration was increased. The reason for this situation is due to the content of carbon in the glucose which supplies energy and carbon to the cell.

The high cell productivity also enhances the fermentation process, becoming faster, increasing the reaction between cell and substrate consumption (refer to figure 6.4).

Table 6.2: The effect of glucose concentrations on the cell productivity of the MBR.

Concentration (g/L)	Total cell (g/L)	Time (h)	Cell productivity (g/L/h)
5	5.68	38	0.15
10	5.76	19	0.30
15	5.88	15	0.39

6.6.2 Acetic acid productivity

Figure 6.12 shows the acetic acid productivity versus time. For the three glucose concentrations presented in figures 6.7, 6.8 and 6.9.

Figure 6.12 (I) shows the data for a 5g/L glucose concentration with the different permeate rates. In the zone A, obviously, productivity of acetic was increased from approximately 0.1 to 1.65 g/L/h. Then in the zones B and C, the trend looks to level out. The amount of acetic productivity in zones B and C were 5.39-6.27 and 13.92 -14.33 g/L/h respectively. Figure 6.12(I), shows the acetate productivity is flat in zones B and C. Probably, in these conditions, some of the carbon flow is switched into butyric acid production. Then in the zone D acetic acid productivity was 29.25-29.68 g/L/h.

In figure 6.12 (II), all of zones (A, B, C and D) followed a similar trend and fatty acid concentrations dramatically increased. The 10g/L glucose concentrations provide sources of carbohydrate that were well balanced for cell, acetic and butyric acid productivity. In zones A, B, C and D the amount of acetic acid productivities were 1.09-3.57, 8.4-10.4, 15.58-18.68 and 35.04-37.88 g/L/h respectively.

In the figure 6.12 (III) in zones A and B, a dramatic increase in acetic acid productivity was observed. Then in the zone's C and D increases in acetic productivity level out. In the zone's A, B, C and D acetic productivities were 1.4-4.1, 12.75-25.14, 28.29-48.55 and 50.67-57.16g/L/h respectively.

From figure 6.12, the conclusion for acetate production is that productivity increased for every increment of permeate rate and with higher glucose concentation. There is still scope to increase the final productivity of these systems.

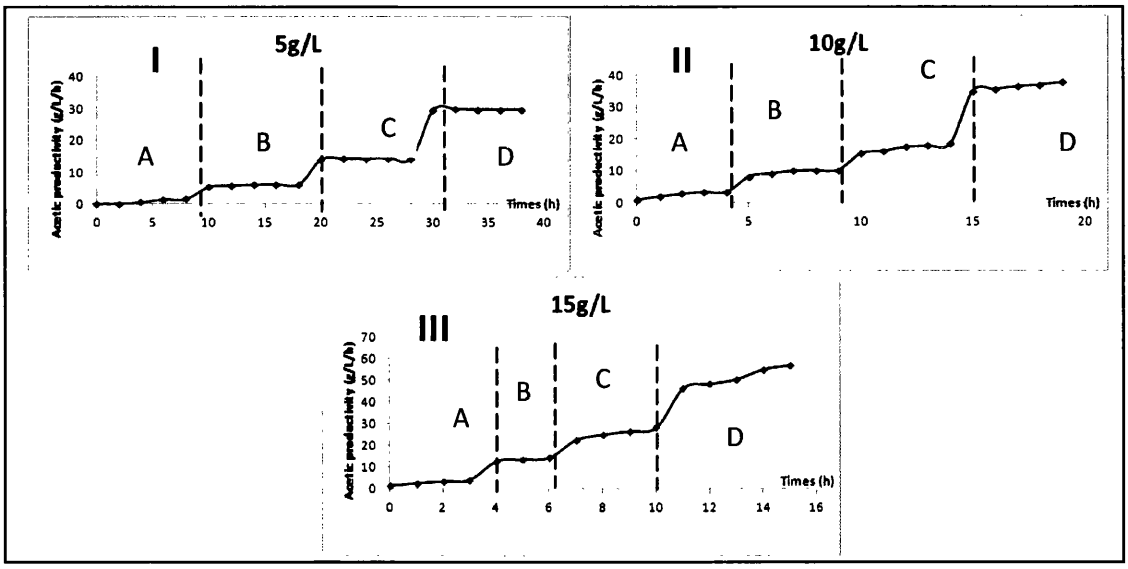

Figure 6.12: Acetic acid productivity versus time in 5, 10 and 15 g/L glucose concentrations for various permeate rates. *A=4L/h, B=8L/h, C=16L/h and D=32L/h*

6.6.3 Butyric acid productivity

Figure 6.13 shows butyric acid productivity for 5, 10 and 15g/L glucose concentration with various permeate rates.

Figure 6.13(I) shows the butyrate productivity for 5g/L. In zone's A and B, obviously the butyric productivity increased from 0.077-0.09 to 0.18-0.29 g/L/h respectively. In zone's C and D the trend of graph increases slightly where the productivity of butyrate was 0.625-0.63 and 0.845-0.86 g/L/h respectively.

Figure 6.13 (II) shows the 10g/L glucose fermentations. The butyric acid productivities in zones A, B, C and D were 0.002-0.9, 2.3-3.1, 6.5-6.9 and 13.6-13.8 g/L/h respectively.

Figure 6.13(III) shows the butyric acid productivity for 15g/L glucose. The butyric acid productivities for zones A, B, C and D were 0.13-1.6, 3.21-3.41, 5.91-6.49 and 9.43-14.51 g/L/h respectively.

The conclusion form figure 6.13, is that butyric acid productivity had been related to glucose concentration and the permeate rates. There is still scope for further improvements in productivity.

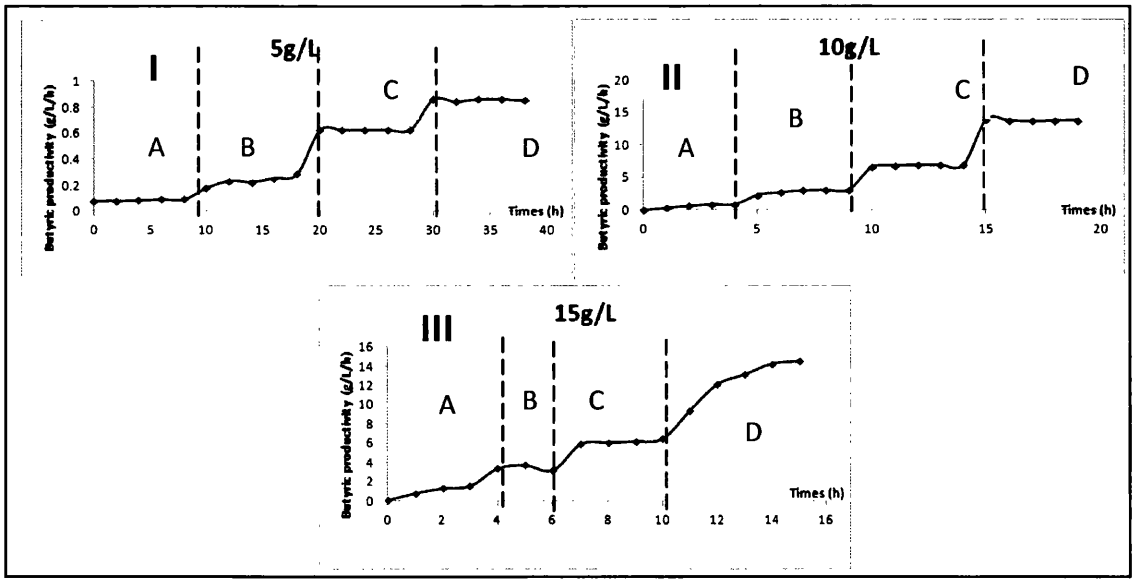

Figure 6.13: Butyric acid productivity versus time in 5,10 and 15 g/L glucose concentrations for various permeate rates. *A=4L/h, B=8L/h, C=16L/h and D=32L/h.*

6.6.4 Relationship between permeate rates and specific productivity of acetate and butyrate.

Table 6.3 shows the specific productivity for acetate and butyrate in the MBR system for a variety of permeate rates and initial glucose concentrations. The identification of specific productivity for acetate and butyrate was calculated by dividing acetate or butyrate productivity by cell concentration.

From table 6.3, specific acetate and butyrate productivity was plotted versus permeate rates such as in figures 6.12 and 6.13.

Table 6.3: Specific acetate and butyrate productivity of *C. butyricum* in the MBR

Concentration (g/L)	Flow rate (L/h)	Cell concentration (g/L)	Acetate productivity (g/L/h)	Butyrate productivity (g/L/h)	Specific acetate productivity (g/g/cell/h)	Specific butyrate productivity (g/g/cell/h)
5	4	0.74	1.65	0.09	2.23	0.12
	8	2.25	6.26	0.29	2.78	0.13
	16	4.23	14.33	0.63	3.39	0.15
	32	5.68	29.68	0.85	5.23	0.15
10	4	0.83	3.56	0.92	4.29	1.11
	8	2.62	10.42	3.14	3.98	1.20
	16	4.33	18.68	6.92	4.31	1.60
	32	5.76	37.88	14.34	6.46	2.45
15	4	1.35	4.16	1.58	4.48	1.17
	8	2.62	14.37	3.21	5.48	1.23
	16	4.17	28.29	6.49	6.78	1.56
	32	5.88	57.19	14.51	9.73	2.47

Figure 6.14 shows, the specific acetate productivity versus permeate rates for 5, 10 and 15 g/L of initial glucose concentration. The trend of all the plots was that acetate productivity increased as the permeate rate increased.

Specific acetate productivity, the rate of acetate productivity per unit cell, for example, at 32 L/h of permeate in the 10g/L initial glucose substrate was 6.46 g substrate/g cell/h. The lowest specific acetate productivity is 5g/L and followed with the 10g/L glucose substrate.

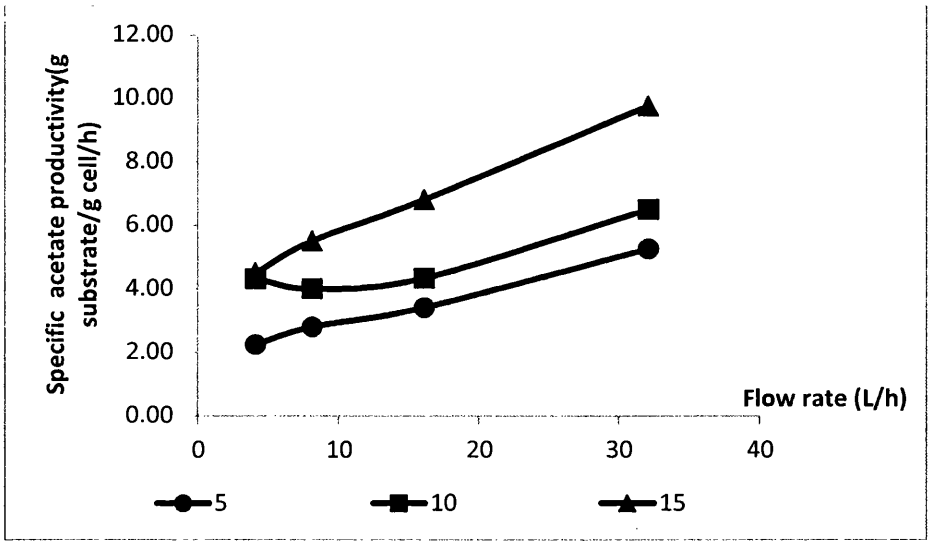

Figure 6.14: The effect of permeate flow rate and glucose concentration on specific acetate productivity of *C. butyricum* in the MBR at 37°C and pH 6.5.

Figure 6.15 shows the specific butyrate productivity. Obviously for 10g/L and 15g/L the butyrate productivity increased as the permeate rate increased. However, for the 15g/L just a small increase was observed when the permeate rate increased. This trend shows that butyrate productivity was directly related to the glucose concentration in the feed.

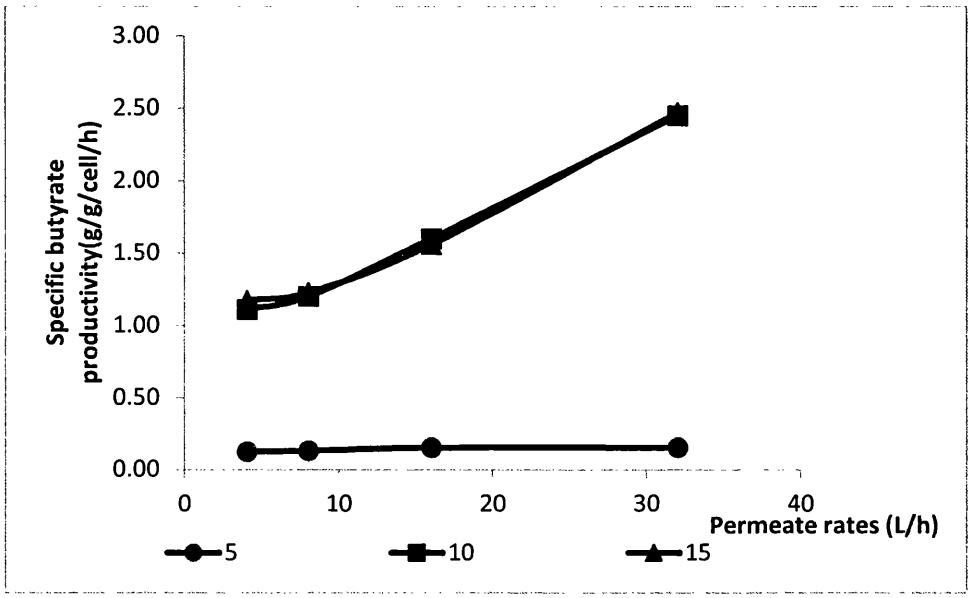

Figure 6.15: The effect of flow rate and glucose concentration on the specific productivity of butyrate using *C. butyricum* at 37°C and pH 6.5.

6.6.5 Relationship between permeate rates and carbon balance

In the MBR experiments, carbon balance was determined by using 4 assumptions as described in section 5.5.3 for the continuous experiments. Table 6.4 shows the carbon balance for 5, 10 and 15 g/L of glucose concentration in the MBR system.

In the input carbon side, two parameters contain carbon, the glucose consumed (Δ S) and the yeast extract (assumed to be completely consumed). Then on the output carbon side 3 parameters were used i.e., cell concentration, acetate concentration + carbon dioxide and butyrate concentration+ 2 moles carbon dioxide. Then, percentages of carbon recovery were determined as shown in table 6.4.

The percentage of carbon recovery was plotted in the figure 6.14 for 5, 10 and 15g/L to investigate the trends carbon recovery and the effects of permeate rates.

Table 6.4: The effect of glucose concentration and permeation rates on fermentation carbon balances of C butyricum fermentation in the MBR system.

Initial glucose (g/L)		Input carbon		Total input C (mM)	Output carbon			Total output C (mM)	% C recovery
	Permeate rate (L/h)	Glucose (mM)	Yeast (mM)		Cell (mM)	Acetate +CO_2 (mM)	Butyrate +2CO_2 (mM)		
	4	78.88	410	488.88	30.83	113.55	8.57	152.95	31.29
	8	138.53	410	548.53	93.75	214.94	9.69	318.38	58.04
5	16	146.89	410	556.89	176.25	246.13	10.04	432.42	77.65
	32	158.68	410	568.68	236.67	232.87	9.89	479.43	84.31
	4	182.54	410	592.54	34.58	245.1	85.85	365.53	61.69
	8	298.17	410	708.17	92.50	357.74	146.83	597.07	84.31
10	16	326.67	410	736.67	180.42	320.71	162.07	663.20	90.03
	32	327.07	410	737.07	240.00	325.26	167.83	733.09	99.46
	4	352.73	410	762.73	56.25	286.01	147.61	489.87	64.23
	8	481.20	410	891.20	109.17	493.69	150.47	753.33	84.53
15	16	490.84	410	900.84	173.75	485.89	152.09	811.73	90.11
	32	498.64	410	908.64	245.02	491.07	169.88	905.97	99.71

Figure 6.16 shows the trends in carbon recovery for 5, 10 and 15 g/L initial glucose and a variety of permeate rates in the MBR system. Obviously, all of the graphs show the incremental increases as the permeate rates were increased. For 5 g/L initial glucose, only 31.29% was recovered at a 4L/h permeate rate and this increased to 84.31% at a 32 L/h permeate rate. For 10g/L initial glucose, 61.69% was recovered at a 4L/h and then increased to 99.46 % at a 32L/h rate. Using 15g/L initial glucose, 64.23% of the carbon was recovered at 4L/h and this increased to 99.71% carbon recovery at 32L/h.

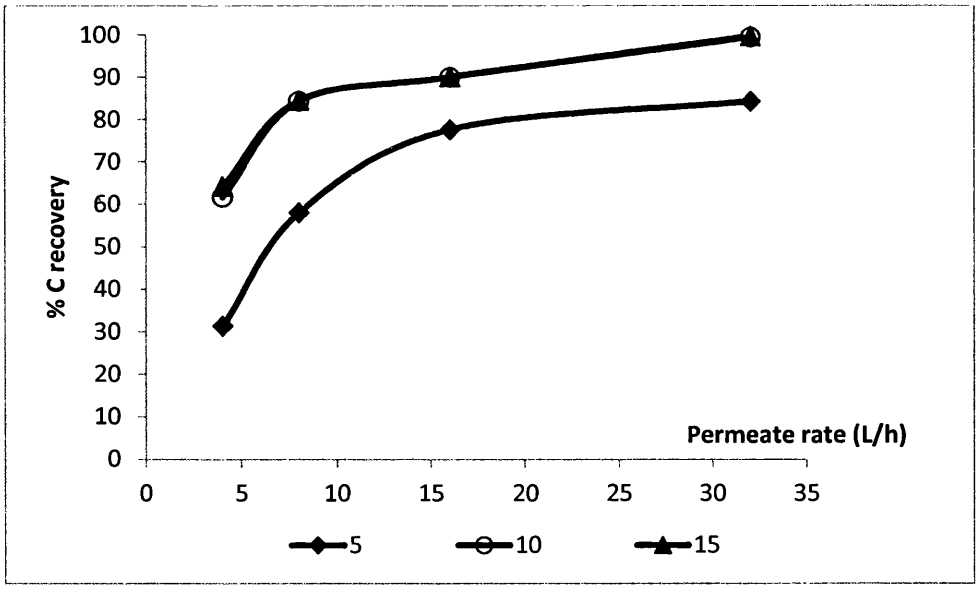

Figure 6.16: The effect of permeate flow rate and glucose concentration on the carbon recovered from the fermentation of glucose by *C butyricum* in the MBR.

6.7 Conclusion

The MBR culturing method was successfully applied to the growth of *C. butyricum*. Membrane filtration technology was successful in producing acetic and butyric acid with high productivities, and in some conditions, with good yields.

The observations associated with carbon recovery and end product shifts were similar to the previous studies in continuous and batch cultures in that at low or high glucose concentrations the balances were incomplete.

The studies do show that the rate of permeation and the concentrations of glucose affect the product yield and product spectrum. Clearly, the most productive conditions were with 10 or 15 g/l glucose and at high permeation rates. However, the permeate rate influences the production and growth rate of *C. butyricum* and the end products of metabolism.

In terms of growth rate (μ) for MBR systems good growth rates were archived comparable with the batch cultures and in some cases better. The growth in MBR system at 15 g/L initial glucose was around 0.244-0.547 h^{-1} (table 6.1), meanwhile for the batch system was just 0.263h^{-1} (table 4.1) or 0.233 h^{-1} in the continuous cultures.

Chapter 7: Conclusion

7.1 Introduction

The production of fatty acids and hydrogen from wastes can be considered an alternative technology to the anaerobic fermentation of wastes for production of methane. The additional benefit of these systems is that the carbon materials, if recovered, represent a capture of carbon for chemical synthesis rather than conversion to carbon dioxide with the release of energy..

This thesis set out to study these problems by the investigation of the growth and fatty acid production of *Clostridium butyricum* as a model system. These investigations established a basic growth medium for the study and then the organism was grown in batch, continuous and MBR cultures at controlled pH and temperature. As the main products of growth were fatty acids, pH control was essential to produce high concentrations of fatty acids. A substantial amount of fatty acids were produced and in the MBR system were produced rapidly.

Generally, these studies have shown that *C. butyricum* could be grown successfully in batch, continuous and MBR cultures and that pH control was necessary to give good growth and performances. To control pH in the reactor, an alkali feed system was employed. These data showed that alkali addition would be a good indicator for fatty acid production and it is possible to consider alkali consumption as the key control parameter for permeate flux, for example.

This chapter of conclusions is therefore set out to discuss the most notable discoveries and other significant discoveries related to the aims and objectives of the thesis.

The nutrient studies established a suitable growth medium (chapter 3) in which carry out the experiments. Each method of production was then studied in

detail in chapters 4,5, and 6. The most novel aspect of the research was application of MBR for *C.butyricum* and fermentation of glucose in the continuous and MBR system. Currently, there are no reports of work in this area.

7.2 The growth kinetics of *C.butyricum*

7.2.1Test tube and batch reactor (STR)

A study of medium optimisation was carried out, this showed that a relatively simple medium could be formulated that would support good growth and acid production, particularly with pH control. The organism could grow at a specific growth rate of 0.263h^{-1} in batch culture and would also be the basis of media for continuous and MBR cultures.

Table 7.1: The effect of glucose concentration and other carbohydrates on the growth rate and the maximum cell concentration in the pH controlled batch system.

Material	Concentration (g/L)	Time (h)	Growth rate μ (h^{-1})	Maximum of cell concentration (g/L)	Doubling time (td) (h)	Concentration (g/L)		Productivity (g/L/h)	
						A	B	A	B
Glucose	0	5	0.094	0.099	7.38	0.120	0.001	0.024	0.000
	5	8	0.111	0.187	6.21	4.730	0.300	0.591	0.03
	10	11	0.200	0.243	3.438	10.43	1.600	0.948	0.145
	15	11	0.263	0.291	2.62	12.94	3.84	1.176	0.349
	20	11	0.150	0.366	4.588	6.270	3.79	0.5700	0.345
	28	31	0.119	0.374	6.319	6.270	4.1	0.202	0.133
Starch	15	11	0.192	0.178	3.594	2.280	1.95	0.207	0.178
Xylose	15	10	0.123	0.151	5.61	4.530	1.30	0.452	0.1301

*A = Acetic acid; B=Butyric acid

Table 7.1 shows the result for growth rate and OD maximum for variety of glucose concentrations and other carbohydrates studied. The results show that *C.butyricum* required yeast extract (figure 4.2) but only poor growth was obtained with yeast extract alone. (the growth rate was less than 0.1h^{-1}). Increasing glucose concentration increased the yield with successive additions of glucose it also showed that glucose is the best source of carbohydrate in term of growth compared to starch

and xylose. Obviously, in term of growth rate (μ) in the glucose concentration, 15g/L was shown to be the fastest. Comparison between 15g/L glucose, starch and xylose showed that glucose was the best carbohydrate to grow the *C. butyricum* both in terms of growth rate and maximum cell concentration obtained.

The production, concentrations of acetic and butyric acid increased as the glucose increased. Even though, the productivity of acetic acid and butyric acid did not increase for feeds above 15g/L glucose. This was thought to be because of either by shifting of the fermentation away from acid production to solvents or by failing to metabolise the sugar added above these levels.

In comparison the growth rate in 15g/L glucose with 15g/L starch and xylose, showed that the growth rate of *C. butyricum* in glucose is much better than in the other substrates. The reason for was explained in the chapter 4.

Table 7.1 shows; the higher acetic and butyric acid productivity for glucose concentration occurs at a substrate concentration of 15g/L. This was the optimum due a fast growth rate in 15g/L and the lack of inhibition from end products or the fact that carbon is not being converted fatty acids but to other non-detected products..

The effect of glucose concentration in the batch reactor showed that as glucose concentration increased then the product formation was mainly acetic acid with significant amounts of butyric acid with an average acetate:butyrate ratio of 3:1. The exception to this was when low concentrations of glucose were used where acetate butyrate ratios were over 30:1. The explanation for almost pure acetic acid fermentations was thought to be due to the fact that much of the growth could be derived from the yeast extract rather than from glucose. In this case the oxidised constituents can act as electron acceptors so allowing the oxidation of glucose and the formation of acetate. An alternate explanation is the redox balance must be maintained on glucose, so that end-products must be produced to allow acetate to be

formed, and thus large quantities of hydrogen are formed. Similar results were observed in the continuous cultures

7.2.2 Continuous Culture (CSTR).

Apart from the ability to produce fatty acids more productively than the batch culture, the studies of the continuous cultures revealed the main kinetics of growth. These studies enabled the basic kinetic constants to be determined and how they varied as a function of growth rate, substrate concentration and substrate feed rates. It was thought the poor performance at high glucose feed rates was due to end product inhibition and the subsequent physiological response to produce other end-products that were not measured in this study.

In the continuous study, kinetic constant parameters were determined including yield coefficient, maintenance coefficient, true yield, and substrate coefficient and sugar uptake (refer to section 5.4). Table 7.2 shows the kinetic constants and other parameters determined from the continuous experiments.

Table 7.2: The kinetic data of obtained during the investigation of the glucose fermentation in continuous cultures of *C butyricum*

Con. initial S (g/L)	D (h^{-1})	ΔS (g/L)	X (g/L)	Con. (g/L)		Productivity (g/L/h)		Specific Productivity (g/g/cell/h)		$Y_{x/s}$	$Y_{x/s}^{max}$	m_s
				A	B	A	B	A	B			
5	**0.058**	1.4	0.34	3.49	0.18	0.2	0.01	0.59	0.03	0.24	0.26	0.02
	0.117	1.2	0.3	3.13	0.18	0.37	0.02	1.23	0.07	0.25		
	0.167	1.1	0.29	2.83	0.18	0.47	0.03	1.62	0.10	0.26		
	0.21	1	0.23	2.71	0.18	0.57	0.04	2.48	0.17	0.23		
	0.23	0.8	0.22	2.65	0.09	0.61	0.02	2.77	0.09	0.28		
10	**0.058**	5.1	0.48	5.87	2.05	0.34	0.12	0.71	0.25	0.09	0.2	0.03
	0.117	5.58	0.56	6.02	2.62	0.7	0.31	1.25	0.55	0.1		
	0.167	7.19	0.9	6.36	2.91	1.06	0.49	1.18	0.54	0.13		
	0.21	7.86	1.08	6.93	3.05	1.46	0.64	1.35	0.59	0.14		
	0.23	8.1	1.25	7.7	3.5	1.77	0.81	1.42	0.65	0.15		
15	**0.058**	8.41	0.5	6.16	2.09	0.36	0.12	0.72	0.24	0.06	0.18	0.64
	0.117	8.96	0.78	6.78	2.93	0.79	0.34	1.01	0.44	0.09		
	0.167	9.45	1.06	7.08	3.61	1.18	0.6	1.11	0.57	0.11		
	0.21	10.2	1.4	7.78	4.46	1.63	0.94	1.16	0.67	0.14		
	0.23	10	1.05	7.39	3.78	1.7	0.87	1.62	0.83	0.11		
20	**0.058**	13.5	0.54	7.04	4.67	0.41	0.27	0.76	0.50	0.04	0.21	1.05
	0.117	13.79	0.98	8.24	5.2	0.96	0.61	0.98	0.62	0.07		
	0.167	14.04	1.56	5.66	3.33	0.95	0.56	0.61	0.36	0.11		
	0.21	13.68	1.45	4.47	2.31	0.94	0.49	0.65	0.34	0.11		
	0.23	13.32	1.27	3.77	1.92	0.87	0.44	0.69	0.35	0.1		
28	**0.058**	17.65	0.77	7.76	5.11	0.45	0.3	0.58	0.39	0.04	0.22	0.83
	0.117	14.49	1.67	6.98	5.03	0.82	0.59	0.49	0.35	0.12		
	0.167	13.05	1.55	5.23	3.53	0.87	0.59	0.56	0.38	0.12		
	0.21	12.49	1.46	4.81	2.82	1.01	0.59	0.69	0.40	0.12		
	0.23	11.03	1.27	3.85	1.94	0.89	0.45	0.70	0.35	0.12		

*Note: A= Acetic acid; B= Butyric acid; X= cell concentration; S=initial glucose; ΔS= glucose consumption; Con= concentration

7.2.3 Culture in the membrane bioreactor (MBR).

In the membrane bioreactor, the concentrations of cells are higher than other systems and as a consequence these systems with increased catalytic power were also more productive. The fact that products are also removed may stimulate the

productivity as the end product inhibition may be avoided. Compared to continuous culture where substantial glucose concentrations remain unconsumed, the substrates are almost completely consumed in the MBR.

Table 7.3: The effect of sugar concentration and permeates rates, on the specific productivity of fatty acids of *C. buyricum* in the MBR.

Concentration (g/L)	Flow rate (L/h)	Specific acetate productivity (g/g cell/h)	Specific butyrate productivity (g/g cell/h)	Glucose consumption (g/L)
5	4	2.23	0.12	2.26
	8	2.78	0.13	4.17
	16	3.39	0.15	4.72
	32	5.23	0.15	4.85
10	4	4.29	1.11	5.32
	8	3.98	1.2	8.88
	16	4.31	1.6	9.35
	32	6.46	2.45	9.81
15	4	4.48	1.17	10.59
	8	5.48	1.23	14.45
	16	6.78	1.56	14.74
	32	9.73	2.47	14.97

The productivity of the MBR system will be limited by the permeate rate of the fluid leaving the reactor. Ultimately, the membrane filtration rate will be limited by the cell concentration in the reactor and the consequent cake layer on the filter as described by equation 6.6. Thus, if all other conditions are constant, and the cell concentration increases, then the potential permeation rate will decline. There will therefore be an optimum set of conditions which can be calculated from the maintenance coefficient and the area of the membrane present in the systems.

A comparison of growth and productivity for Batch, continuous culure and MBR are summarised in table 7.4. The productivity of acetic and butyric acid has increased as the dilution rate increased for 5, 10 and 15g/L glucose. Then, productivity for acetic and butyric acid also increased as the initial glucose concentration increased.

7.3 Comparison of growth, productivity in the batch, continuous and MBR

Table 7.4 shows the productivity for acetic and butyric acid in g/L/h for the batch, continuous and MBR systems. Obviously, the MBR system gave higher acid productivity compared to continuous and batch system.

For the continuous reactor, the acetate productivity rate was 4.6 times and butyrate productivity rate was 7.7 times productive than batch system. Meanwhile the MBR system shows 40 times acetate productivity rate and 96 times butyrate productivity rate better than batch system. Obviously the MBR system is better than other system in terms of productivity.

Table 7.4: Comparison of productivity of acetate and butyrate in the batch, continuous and MBR systems

Type of reactor	Initial glucose (g/L)	Productivity of acetate (g/L/h)	Productivity of butyrate (g/L/h)	Ratio of acetate productivity base on batch reactor	Ratio of butyrate productivity base on batch reactor
Batch	10	0.95	0.15	1.0	1.0
Continous	10	4.41	1.27	4.6	8.7
MBR	10	37.88	14.44	39.9	96.0

In terms of carbon balance, 10g/L initial glucose substrate are the best for carbon recovery compared to other concentrations. Table 7.5 shows, the percentages of carbon recovery for three systems are almost gave same results. However, the MBR shows higher glucose consumption.

Table 7.5: Carbon balance in batch, continuous and MBR reactors for 10g/L glucose.

Reactor	S (g/L)	Δ S (g/L)	Yeast Extract (Mm)	Total C input	Cell	Acetate + CO_2	Butyrate + $2CO_2$	Total C output	% C recovery
Batch	10	255.66	410.00	665.66	10.11	487.50	129.32	626.94	94.18
Continuous	10	269.76	410.00	679.76	36.67	384.00	238.08	658.75	96.91
MBR	10	327.07	410.00	737.07	240.2	325.26	167.83	733.31	99.49

*Note: S= initial glucose.

7.4: Comparison with previous studies

Table 7.6 shows the results from previous researchers investigating the fermentation by *C.butyricum*. All use different nutrients or substrates compared to this research for the fermentation process. As with this research, investigations of glucose concentration concerning the carbohydrate used have been investigated. The different concentrations of glucose have caused a change in the ratio between acetic to butyric acid. The ratio and shifts of products (acetic and butyric acids) is not as great as that observed here, while the medium ingredients were different to those studied in this work. According to Solomon et al, (1995), in the continuous study with various dilution rates was produced good component of acetic and butyric acids. However, due to the different media formulation, then the production was affected especially the ratio of acetic to butyric acid and the cell concentration. In this study, the proportion of acetic acid was always higher compared to butyric acid, in contrast, Solomon's work shows butyric acid was higher than acetic acid.

Wang & Jin (2009) also reported that butyric acid concentration was higher than acetic acid in the high concentration (20-120g/L) of molasses in batch culture fermentation (table 7.6). From table 7.6, Wang & Jin (2009) have shown the percentages of butyric acid (80-70%) and (10-20%) acetic acid for variable molasses concentration. This fact is supported by Vandak et al, (1997), who found that using 30g/L of glucose as as carbon source in the batch fermentation mode, produced high concentrations of butyric acid. Vandak et al, (1997) stated that 11.75g/L butyric acid was produced compared to 4.0 g/L acetic acid.

However, in this study (figure 4.8), acetic acid production was higher than butyric acid. The explanation for this is that if hydrogen production is higher in the current study then the proportion of acetate can increased at the expense of butyrate. A comparison of the two strains on the same medium and growth conditions would be most informative. Further is there are still differences in the proportions of

fermentation products it would suggest a slight but significant difference in the biochemistry of the fermentations. i.e. the enzymes involved in the integration of carbon and electron flow pathways may be different.

Table 7.6 Summary of results from previous researcher

Researcher	Ingredient	Mode	Dilution (h^{-1})	Cell concentration (g/L)	Production (g/L)		Productivity (g/L/h)		Strain
					Acetic	Butyric	Acetic	Butyric	
Solomon et al, 1995	Glycerol, K_2HPO_4 KH_2PO4 $(NH_4)_2 SO_4$ $Mg SO_4.7H_2O$ $CaCl_2.2H_2O$ FeSO4 . 7H2O CaCO3 Yeast extract	Continous (20g/L glycerol)	0.052	0.69	0	31.7	0	1.648	*C.butyricum* strain DSM 5431 (=sh1;European patent applied for, No. EP 0361082A2)
			0.098	0.92	1.4	29.1	0.137	2.852	
			0.198	1.16	4.2	27.1	0.832	5.366	
			0.286	1.19	17.3	19.9	4.948	5.691	
			Molasses concentration (g/L)	**Maximum cell concentration (g/L)**	**Production (g/L)**				
					Acetic	**Butyric**			
Wang & Jin,2009	Tryptone soya broth Molasses $(NH_4)NO_3$	Batch	20	1.3	3	5	0.25	0.42	*C.butyricum* W5 (GenBank accesion number is DO831124) isolated from hydrogen-producing sludge
			40	2.4	0.5	9	0.04	0.75	
			60	3	4.5	13	0.25	0.72	
			80	4	3	13.5	0.12	0.71	
			100	5	4	16	0.21	0.84	
			120	5.5	11	17	0.35	0.55	
Vandak et al, 1997	Glucose (NH4)SO4 KH2PO4 MgSO4 FeSO4 MnSO4	Batch	**Glucose (g/L)**	**Maximum cell concentration (g/L)**	**Production (g/L)**				
					Acetic	**Butyric**			
			30	3.1	4	11.75	0.083	0.24	*C.butyricum* S21 was isolated from soil

7.4.1 Productivity

In terms of productivity, a comparison of the data table 7.6 and the table 7.4, shows that the MBR system productivity is far greater than other modes of fermentation and previous results. A comparison of productivity in the previous studies (table 7.6) with MBR fatty acid productivity (table 7.4) i.e acetate productivity or butyrate productivity shows that the MBR still the achieves the highest rates.

Table 7.6 shows that the best productivities for Solomon et al, 1985 were 4.948 g/L/h for acetate and 5.69 g/L/h for butyrate. Meanwhile 0.35 g/L/h for acetate and 0.55 g/L/h for butyrate were reported by Wang & Jin, (2009), Vandak et al, (1997) recorded productivities 0.083g/L/h for acetate and 0.24g/L/h for butyrate. However, in the MBR system used in this study, 44.89 g/L/h and 13.63 g/L/h production rates were found for acetate and butyrate acid respectively. Therefore, the comparison shows, the MBR used in this study was more productive and is over 50 times greater for butyrate and over 500 times greater for acetate when compared to previous studies. Further comparisons need to be made to confirm these improvements

7.5 Future work

Overall, this research has shown the potential for the conversion of fatty acids from carbohydrates and that the MBR as a production system are a good system to produced fatty acids. However, there are a number of areas in which this research can be improved and progressed in the context of waste treatment technology.

7.5.1 Investigation of carbohydrate mixtures and more extensive measurements of products.

In this research, all of the substrates have been studied as individual compounds at a few concentrations. For future investigations, mixtures of

carbohydrates could be investigated. Therefore, the content of carbohydrate in the substrate will be similar to that found in food wastes in the real environment. The work here, shows that other products yet to be identified are formed. A study of potential products needs to be extended to ensure the complete fermentation balances can be obtained and to better quantify the conversion of glucose. Similarly the measurement of hydrogen formation would be most informative as this may be manipulated by the substrate concentration and the feed rate.

7.5.2 Modification MBR system to concentrate products and recycle water

The results in this study have shown that the MBR system is most effective compared to other reactor systems although this is technically the most demanding of the three systems studied. Clearly further work can improve the systems in a number of areas. The use of a good control system to control the medium addition is needed. In this study this was carried out in a stepwise fashion, however, the feed rate should be altered continuously as the cell concentration is raised. This will bring about a more efficient use of the substrate. The best measure of growth and metabolic activity on the culture is the production of acids which can be measured as alkali is added. It would thus be a good idea to control fed rate by monitoring the alkali consumption by the culture. The work here has shown that there is a direct relationship between acid produced and the alkali consumed to neutralise it. This could be exploited to provide positive feedback control of the feed.

Another area of further development is the possibility of recycling water from the product stream. As the product stream is cell free the water could be separated from the volatile fatty acids (VFA) and returned to the fermentation. To achive this, nanofiltration or RO membranes could be installed after the MF permeate that separates water and VFA. Subsequently, water could be recycled into the reactor. The advantage for this system, is that the VFA level could be reduced so avoiding

end-product inhibition of *C.butyricum*. At the same time the fatty acid stream could be concentrated. Figure 7.1 shows such a scheme

The high concentration of VFA's produced will be of more value and could be further concentrated or used in other bioprocesses that ferment VFA to produce polyhydroxyalkanoates (PHAs) and poly-3-hydroxybutyrate (PHB), for example (Chen & Page, 1997).

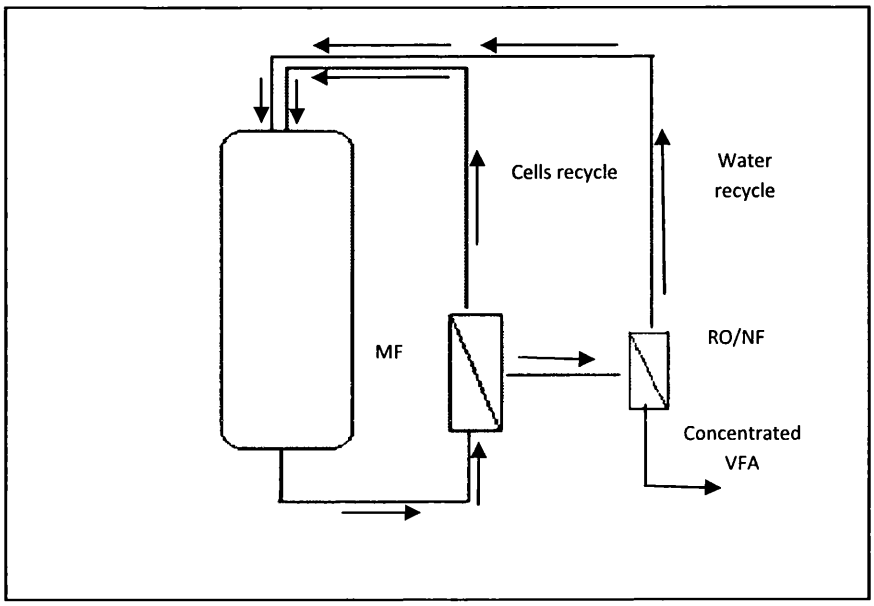

Figure 7.1: Improvement of the membrane bioreactor system by the incorporation of additional membrane separation such as RO and NF to recycle water and concentrate the fatty acids.

7.5.3 Optimization of MBR design

The design of MBR can futher explored, Depending on the objectives of fermentation the reactor could be optimized in a number of ways. For example, the relative size of the reactor and the membrane unit can be changed to allow the best cell concentration in relation to feed rates. Similarly, the reactor using the most economic configuration of vessel to membrane area, when used on large scale equipment, could be investigated.

This study was restricted to a ceramic filter of relatively low porosity. There are many types of commercially available membranes to investigate for the suitability for MBR application. In addition there are some fundamental relationships that need to be investigated to demonstrate and validate the theoretical limitations to growth in the MBR.

7.5.4 Development of mathematical modelling of the MBR with consideration of other substrates and products.

The development of mathematical modelling especially for MBR is requires further consideration with a more detailed understanding of the physiological responses of the organisms in complex situations such as those encountered in the MBR. This is particularly relevant to the description of end product inhibition and is influenced by the cells in the context of extractive fermentations. Thus, models are important tools in studies of simulation and optimisation of MBR for example. The models for fatty acid production on wastes that include hydrolysis of carbohydrate polymers or sugar mixtures should be considered especially in the context of waste conversion.

7.6 Epilogue.

The study of fatty acid as a conversion technology for waste has some interesting challengers to overcome before it becomes a reality. The work in this thesis shows that fatty acid production can be intensified and more work is needed on these systems to make them a viable alternative to conventional anaerobic digestion technology.

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Appendix 1

The peristaltic pump for continuous system was calibrated. The flow rate versus pressure was plotted.

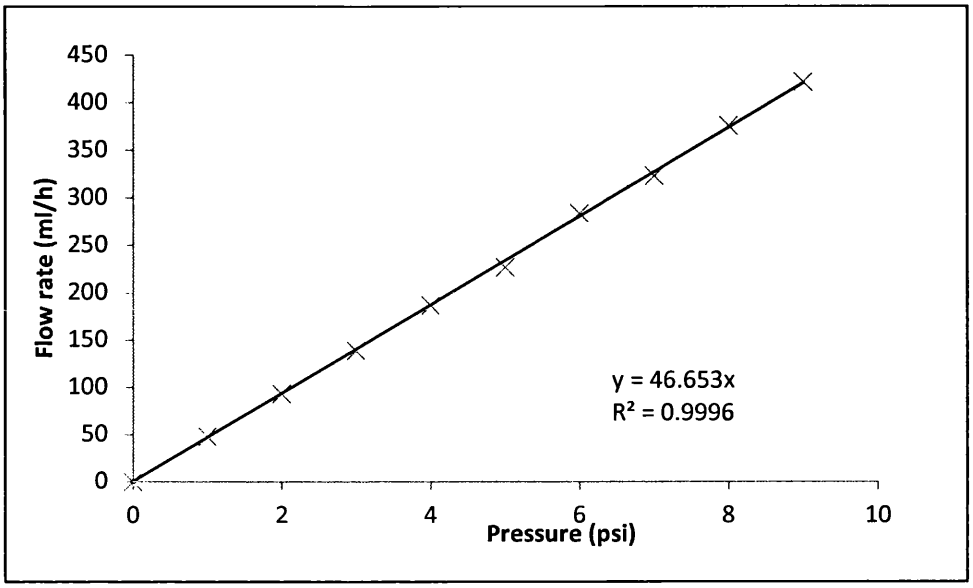

Figure 1a.1: Calibration graph for the pump in the continuous system.

Appendix 2

Figure 2a.1 and 2a.2 show calibration graphs to identify the concentrations of acetic and butyric acid. Figure 2a.1 and 2a.2 show the relationship between peak area and concentration of acetic and butyric acid.

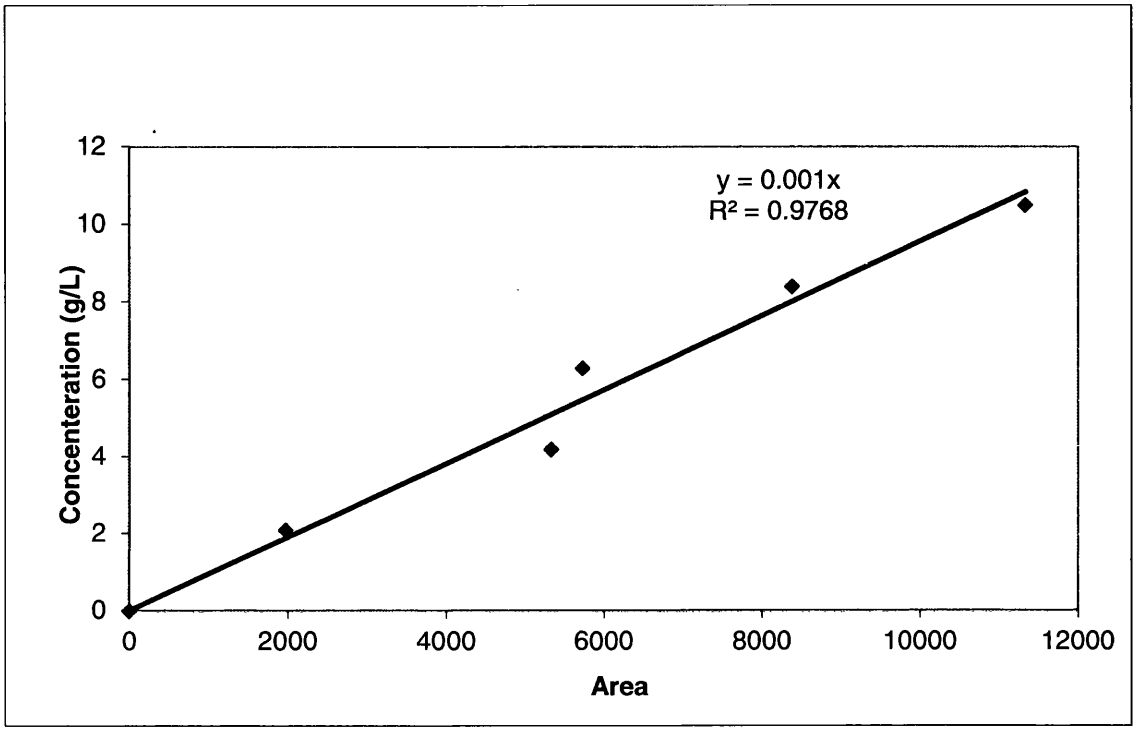

Figure 2a.1: Calibration graph for acetic acid.

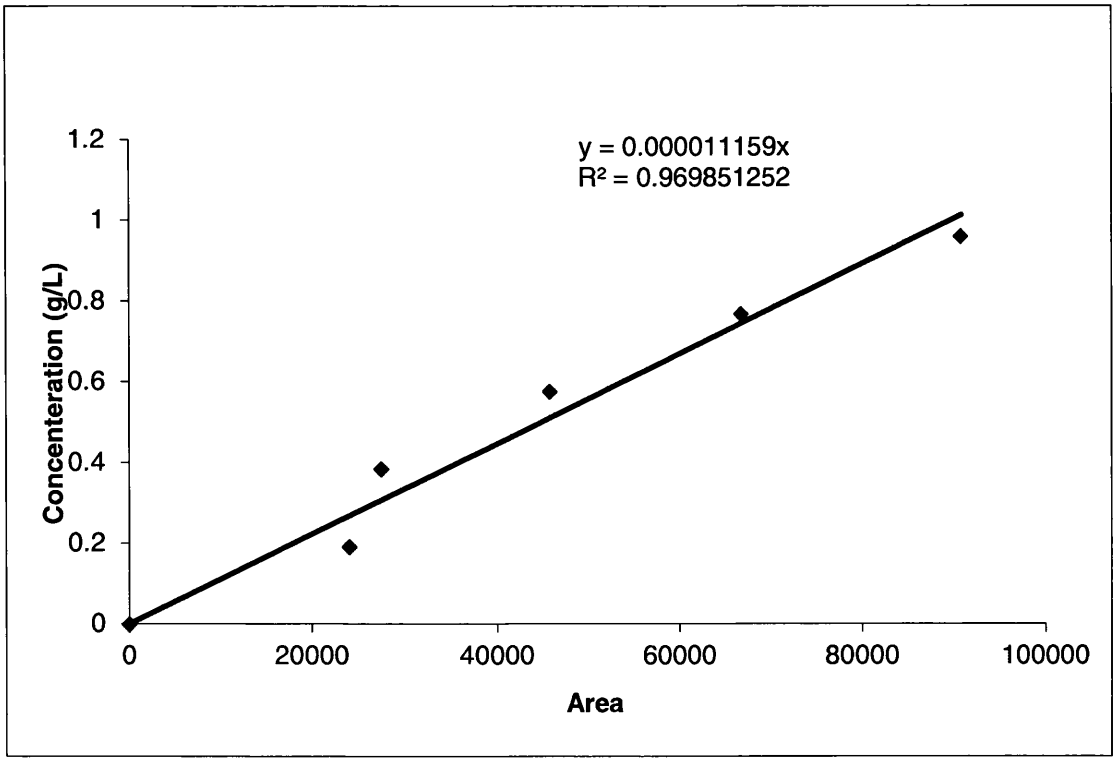

Figure 2a.2: Calibration graph for butyric acid.

Appendix 3

Figure 3a.1 shows calibrations of UV spectrometry. OD(actual) was plotted versus OD(measured). Figure 3a.1 shows the relationship between OD(measured) and OD(actual).

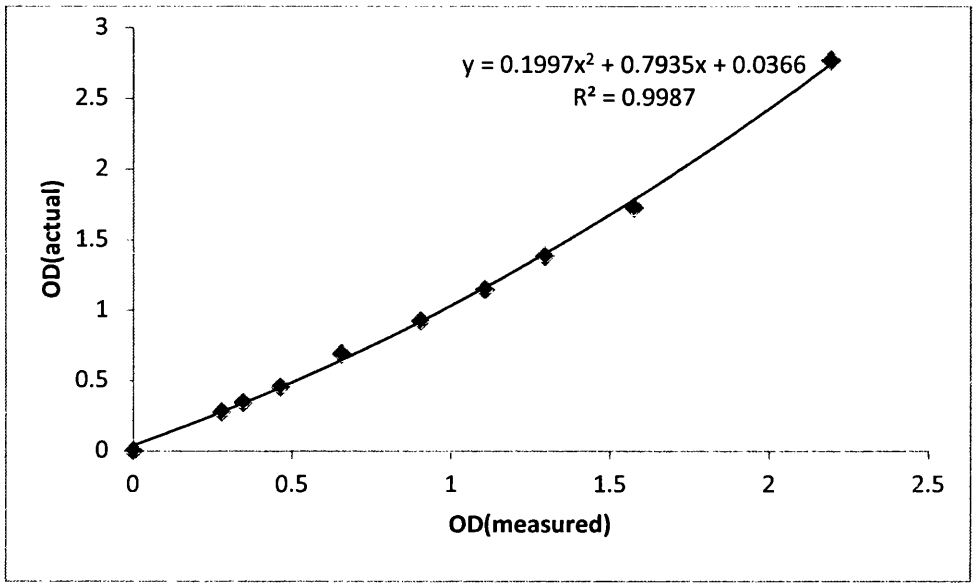

Figure 3a.1: Calibration of UV spectrometry.

Appendix 4

Figure 4a.1 shows the correlation that was used to identify the amount of dry weight or biomass in this study (refer to Section 3.9.1). This correlation was applied in batch, continuous and MBR systems to identify the biomass concentration.

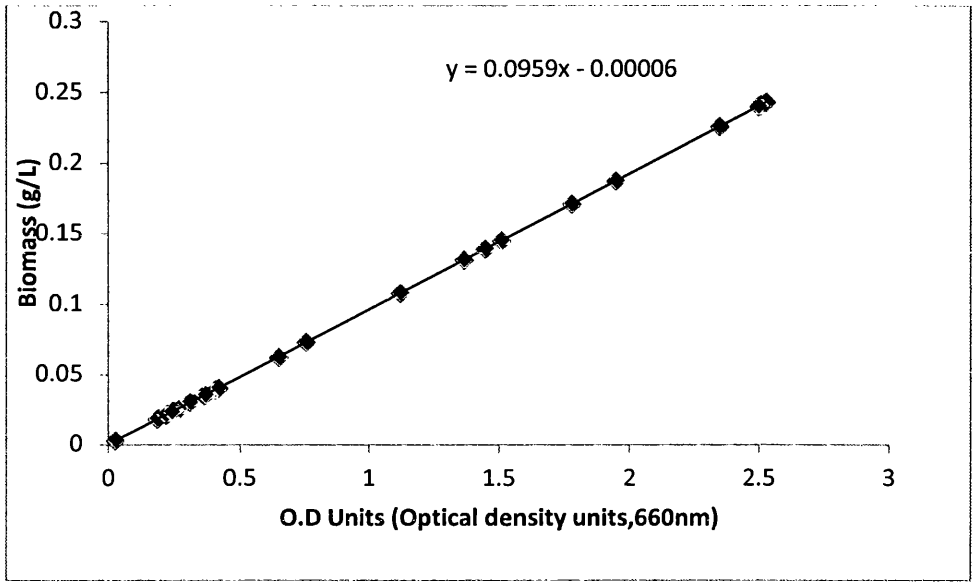

Figure 4a.1: Investigation of the relationship between OD and biomass.

Appendix 5

Figure 5a.1 shows the standard curve for glucose concentration versus absorbance using a 1.88 mm light path at 540 nm (refer to section 3.11).

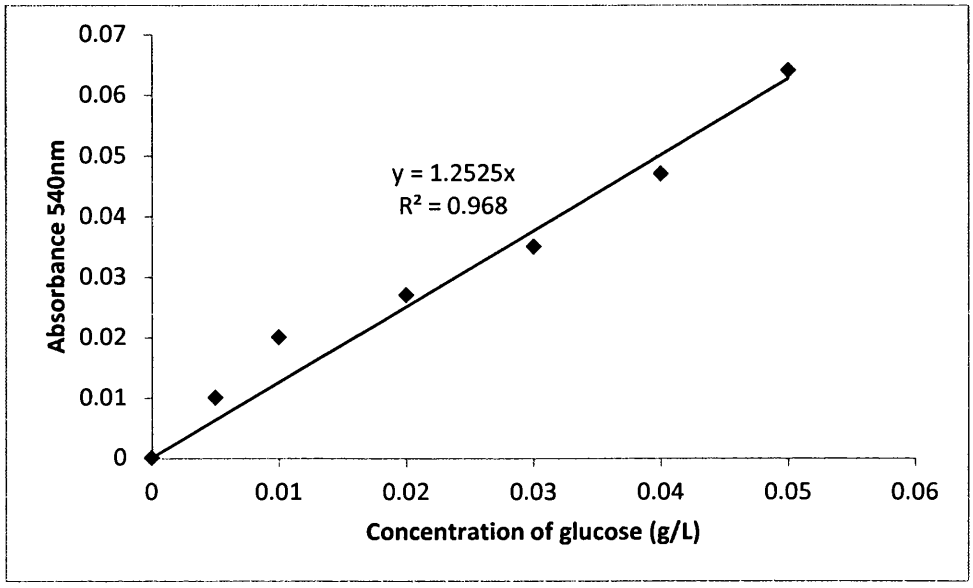

Figure 5a.1: Standard curve of glucose concentration from GOPOD assay.

Appendix 6

Figure 6a.1 shows the relationship between xylose concentrations with absorbance with a 1.88 mm light path at 540 nm (refer to section 3.12).

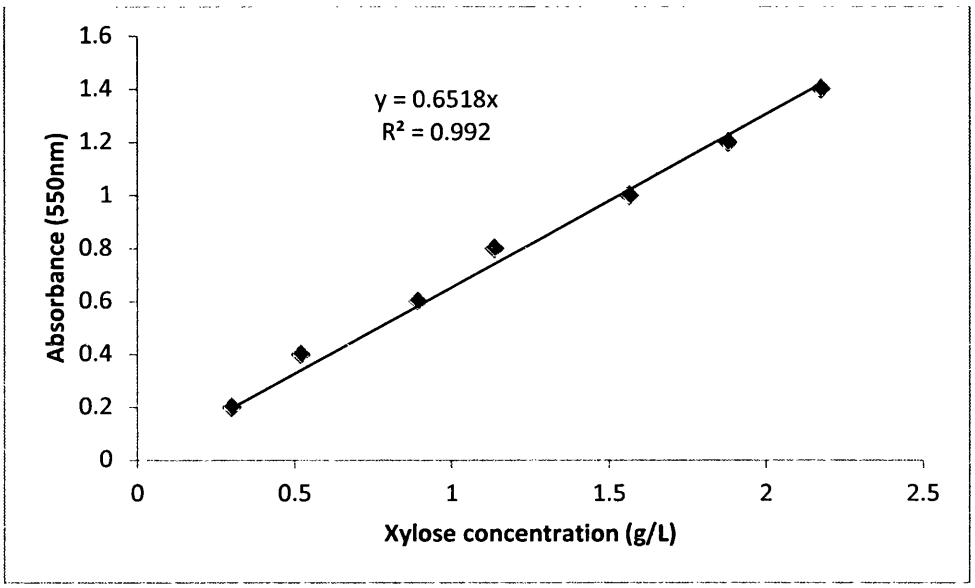

Figure 6a.1: Standard curve for Xylose concentration from DNS method

Appendix 7

7A.1 Data in the batch experiments

This section will describe the results in the batch experiments.

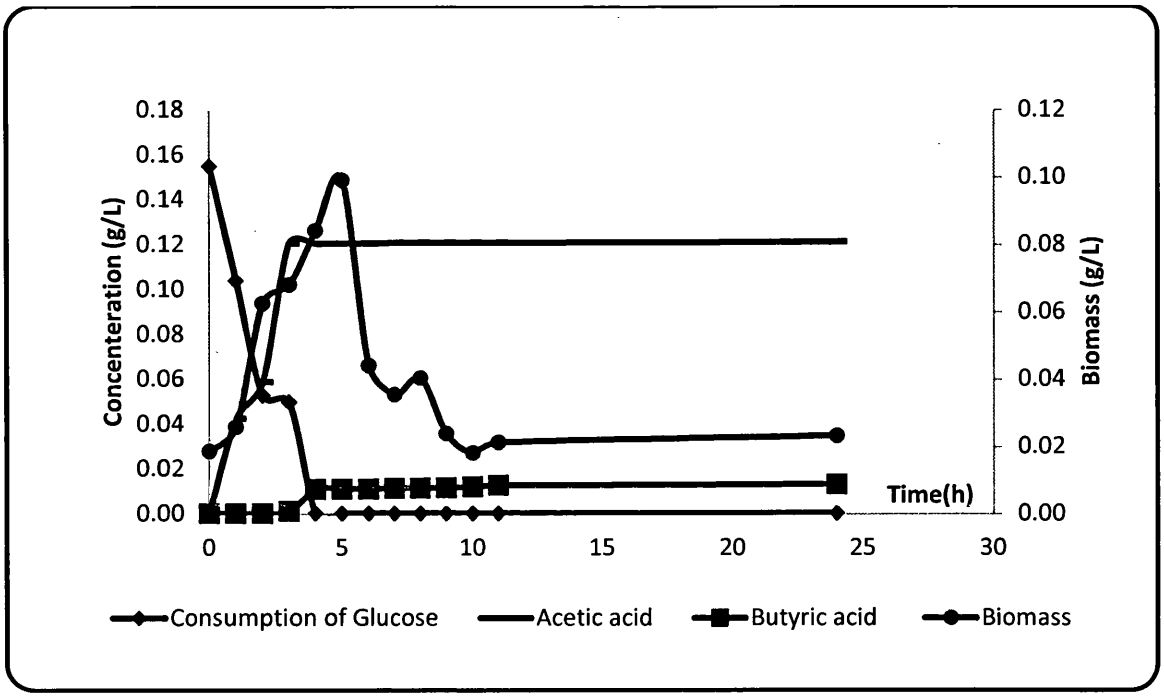

Figure 7a.1: The trend of the parameters in the 0g/L glucose concentration for batch culture.

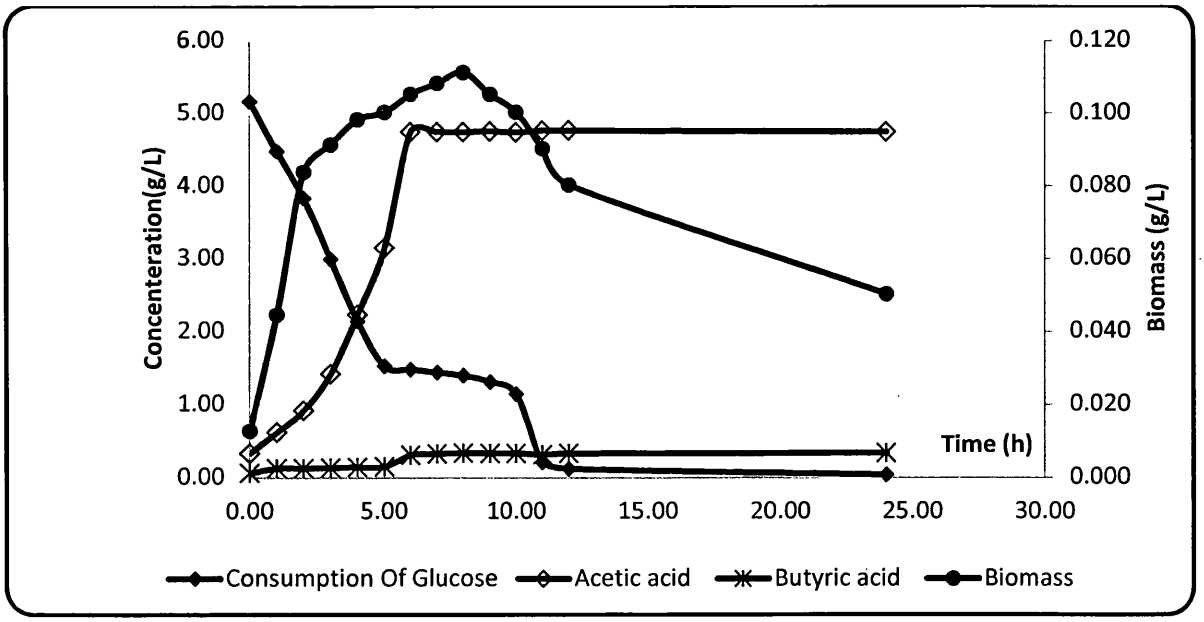

Figure 7a.2: The trend of the parameters in the 5g/L glucose concentration for batch culture.

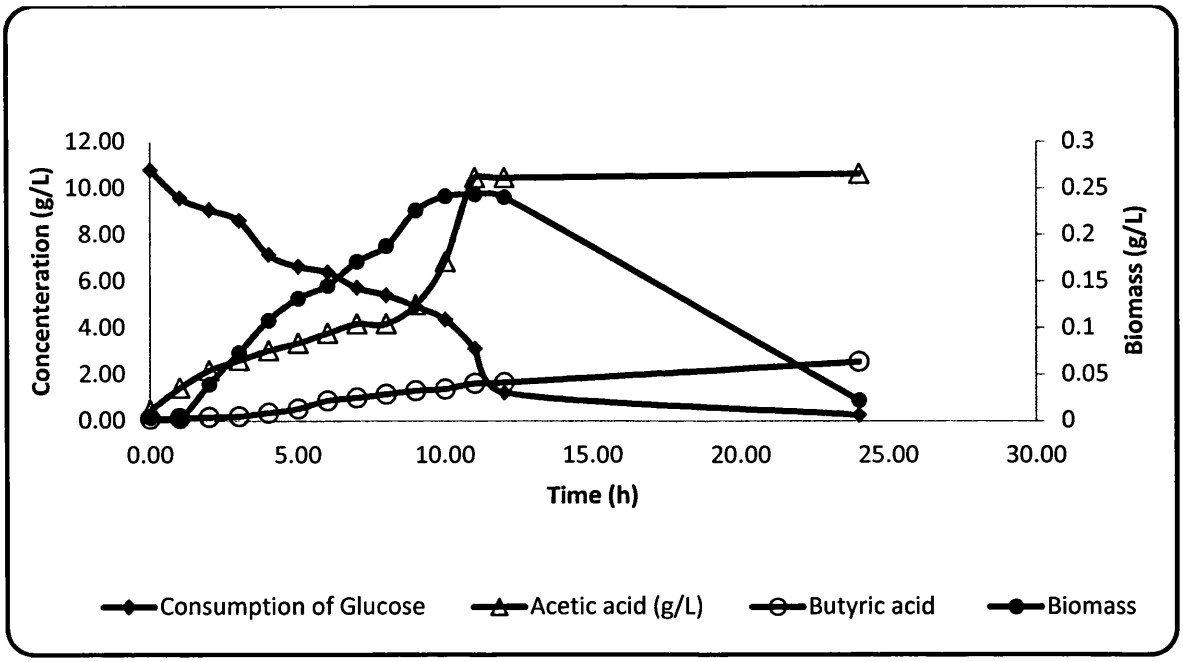

Figure 7a.3: The trend of the parameters in the 10g/L glucose concentration for batch culture.

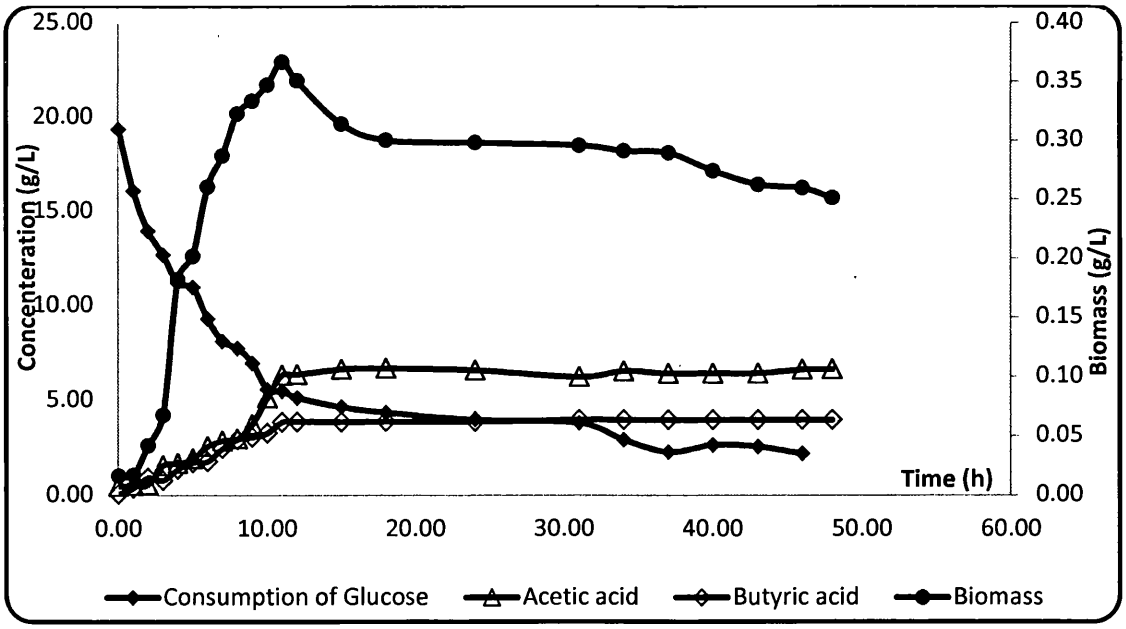

Figure 7a.4: The trend of the parameters in the 20g/L glucose concentration for batch culture.

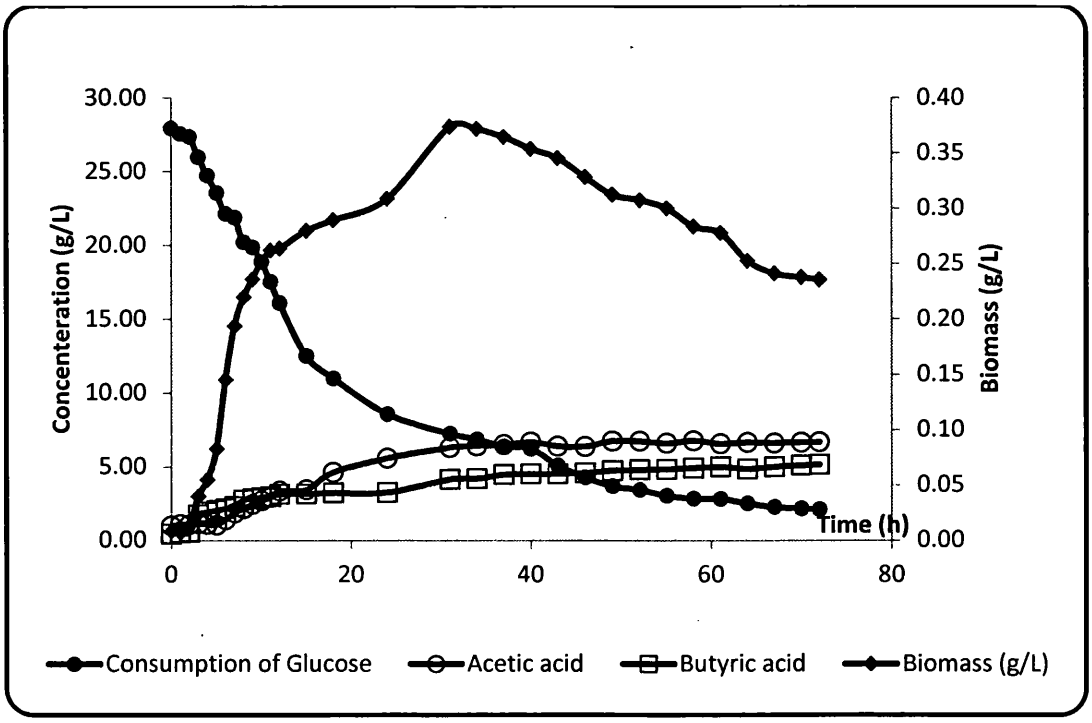

Figure 7a.5: The trend of the parameters in the 28 g/L glucose concentration for batch culture.

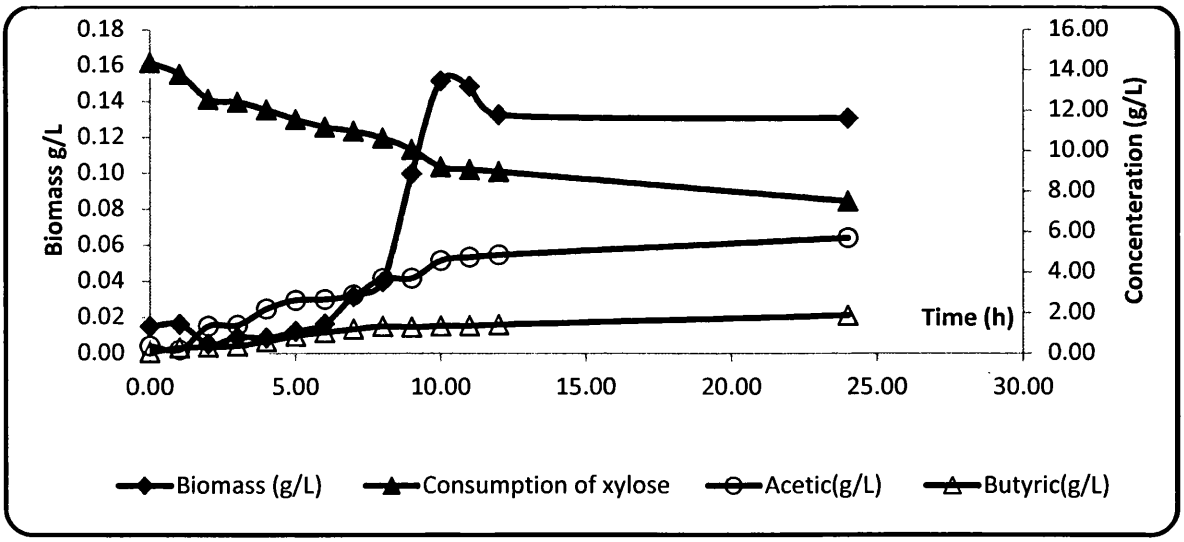

Figure 7a.6: The trend of the parameters in the 15g/L Xylose concentration for batch culture.

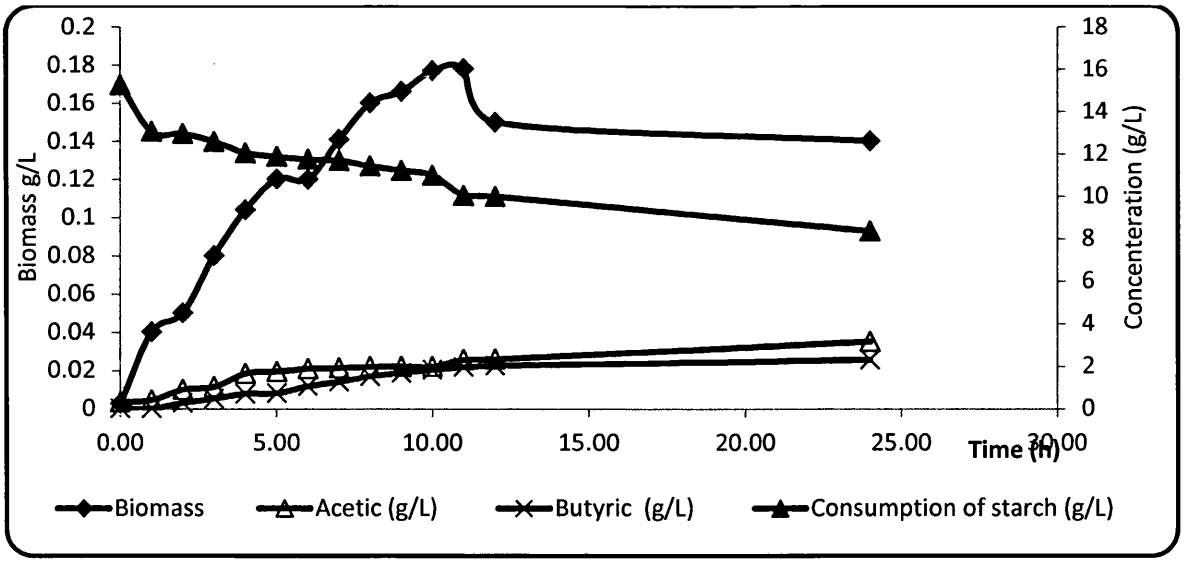

Figure 7a.7: The trend of the parameters in the 15g/L starch concentration for batch culture.

7A.2 Example calculations for analysis results in Chapter 4

1. Growth rate calculation

First results have been plotted in the normal graph. Then, the exponential phase had to be identified. This was then plotted on a log graph. Figure 7A.1 shows the exponential phase on the log graph. Next, the linear trend line had to be used to determine the slope for exponential phase.

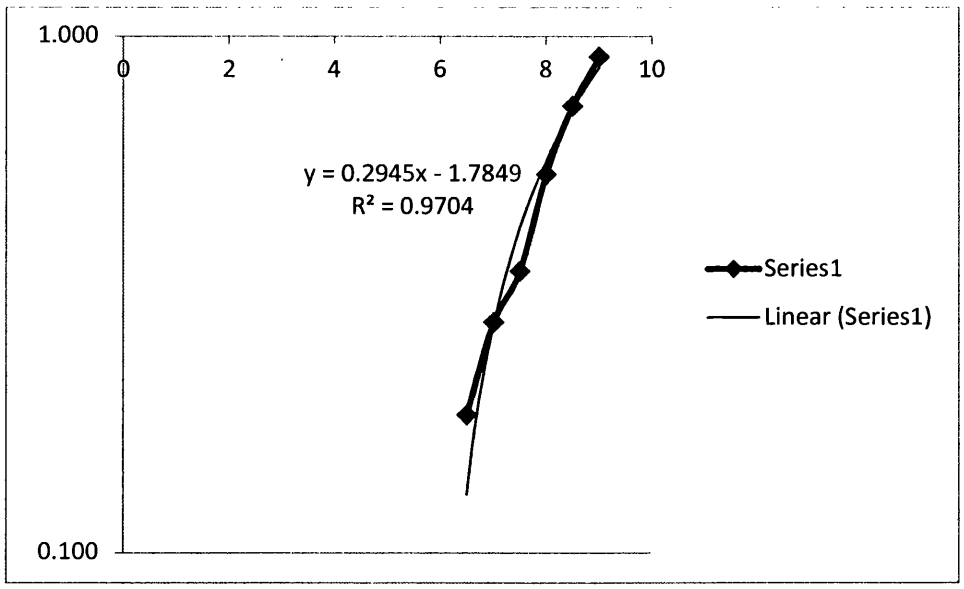

Figure 7a.8: Growth rate on the log graph.

Therefore;

Slope of graph = 0.2945 h^{-1} = K

g = 0.0693 / K = 0.42496 h

μ = 1/g = 0.42496 h^{-1}

2. Percentages of acetic and butyric acid production:

$$\%\ Acetic\ acid = Acetic\ acid(^{mol}/_{L}) /\ Total\ acid\ production\ (^{mol}/_{L})\ x\ 100$$

$$\%\ Butyric\ acid = Butyric\ acid(^{mol}/_{L}) /\ Total\ acid\ production\ (^{mol}/_{L})\ x\ 100$$

3. Percentage glucose converted to acetic and butyric acid

$$\%\ Glucose\ had\ been\ used = \frac{\Delta S}{S}\ x\ 100$$

Therefore; percentage glucose converted to acetic or butyric acid

$$Percentage\ glucose\ converted\ to\ acetic = \frac{\Delta S}{S}\ x\ \%\ acetic\ acid\ x\ 100$$

$$Percentage\ glucose\ converted\ to\ butyric = \frac{\Delta S}{S}\ x\ \%\ butyric\ acid\ x\ 100$$

4. Total acid production

$$Total\ acid\left(^{mol}/_{L}\right) = \frac{Concenteration\ of\ acetic\ acid(^{g}/_{L})}{Molecular\ weight\ acetic\ acid\ (\frac{g}{mol})} + \frac{Concenteration\ of\ butyric\ acid(^{g}/_{L})}{Molecular\ weight\ butyric\ acid\ (\frac{g}{mol})}$$

5. Ratio total acid production to glucose/xylose/starch

$$Ratio\ total\ acid\ production/Carbohydrate = \frac{Total\ acid\ production\ (mol/L)}{Initial\ Carbohydrate\ (mol/L)}$$

6. Ratio between acetic to butyric acid

$$Ratio\ (acetic/butyric) = \frac{Total\ acetic\ acid\ (mol/L)}{Total\ butyric\ acid\ (mol/L)}$$

Appendix 8

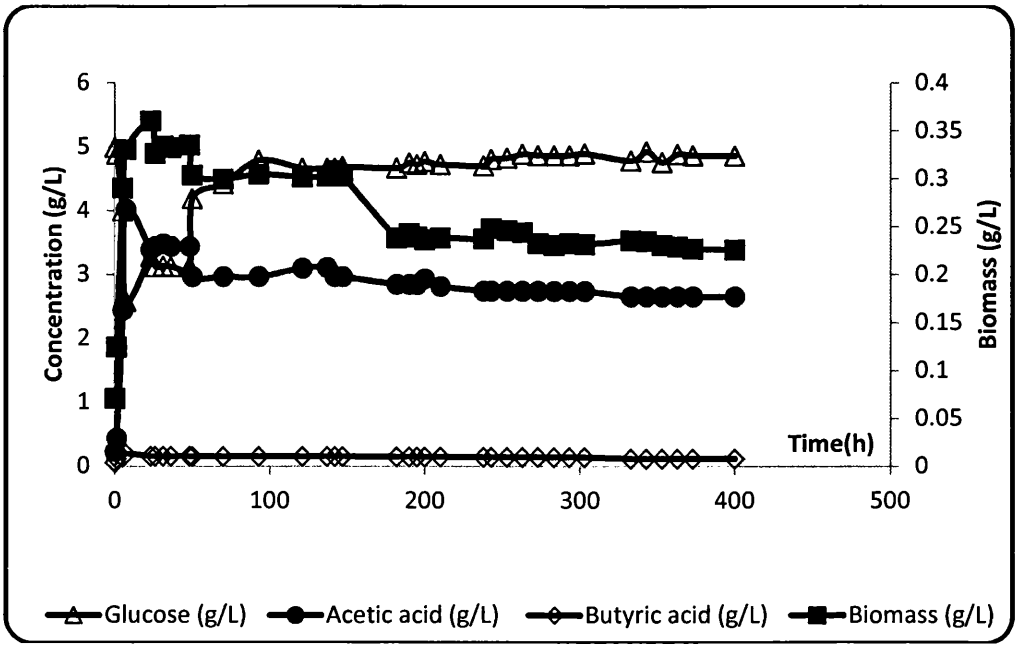

Figure 8a.1: The trend of the parameters in the 5g/L glucose concentration for continuous culture.

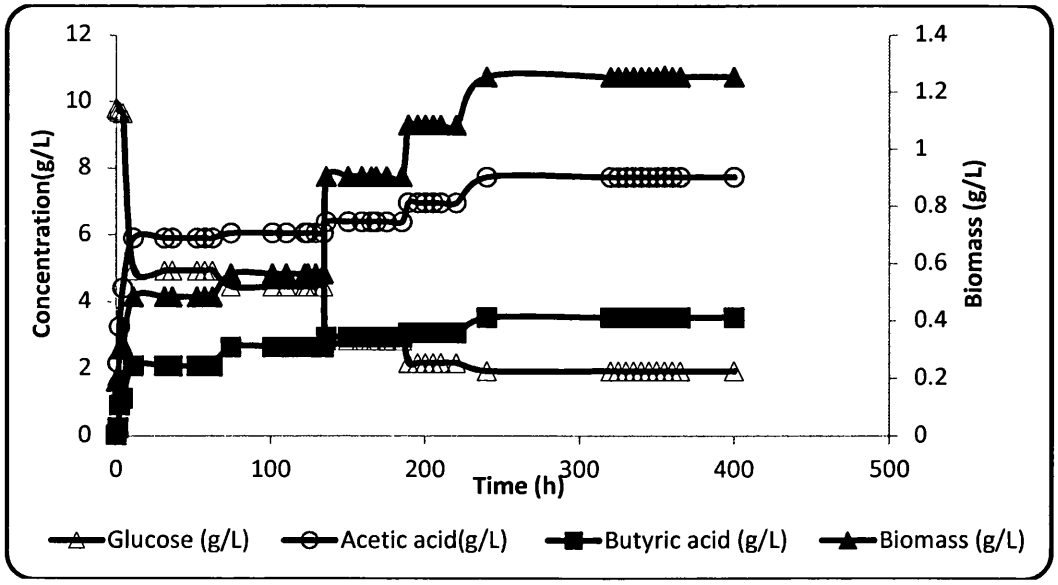

Figure 8a.2: The trend of the parameters in the 10g/L glucose concentration for continuous culture.

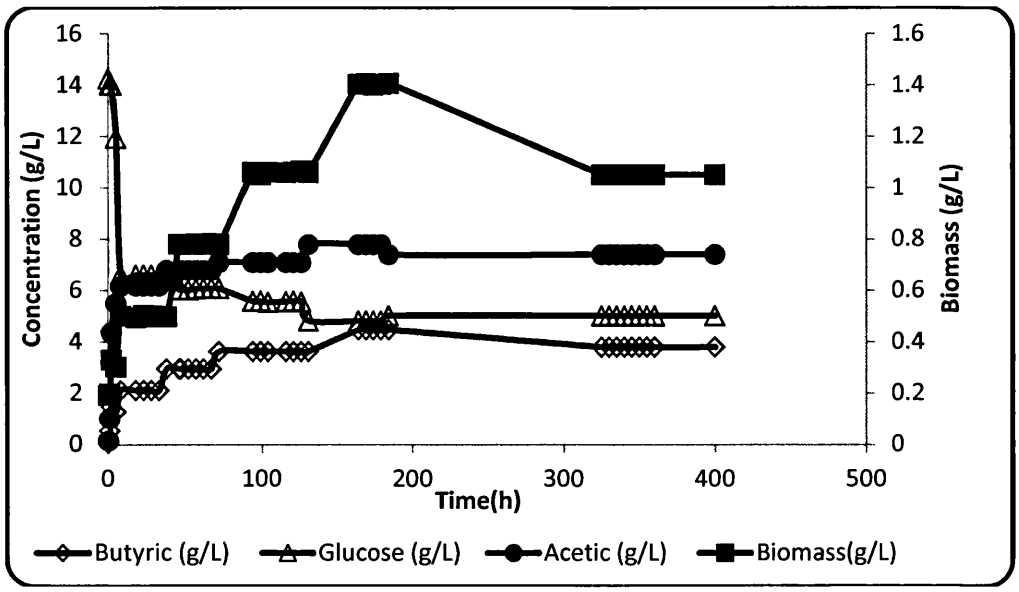

Figure 8a.3: The trend of the parameters in the 15g/L glucose concentration for batch culture.

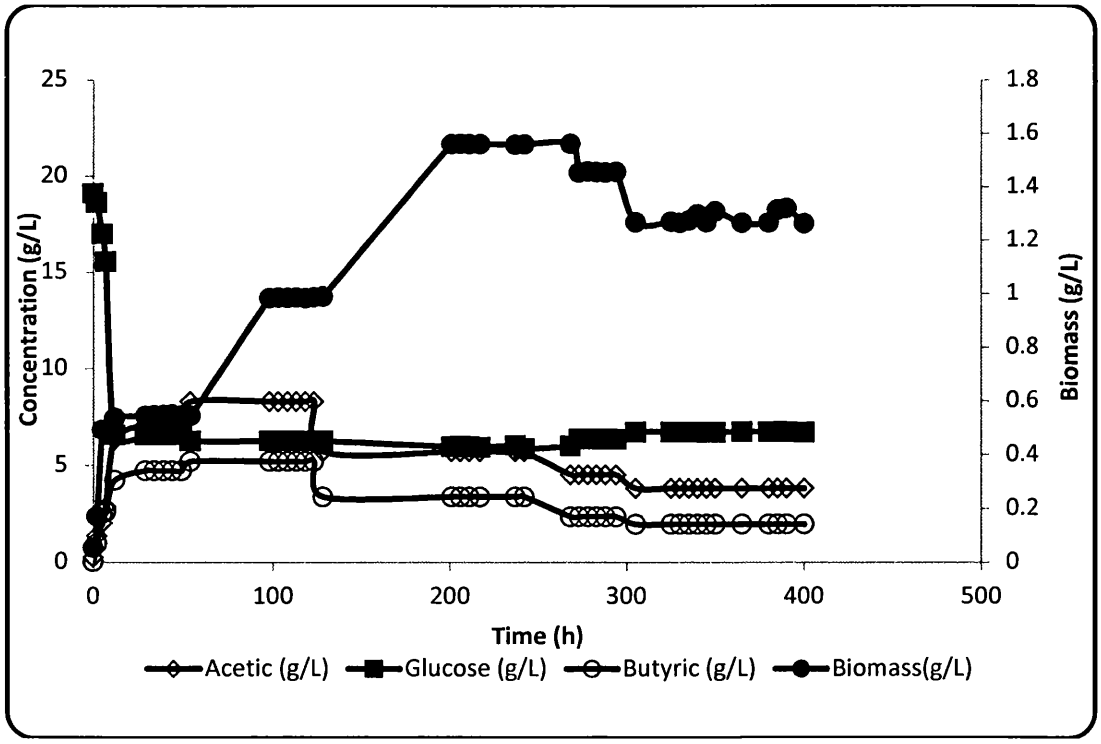

Figure 8a.4 : The trend of the parameters in the 20g/L glucose concentration for continuous culture.

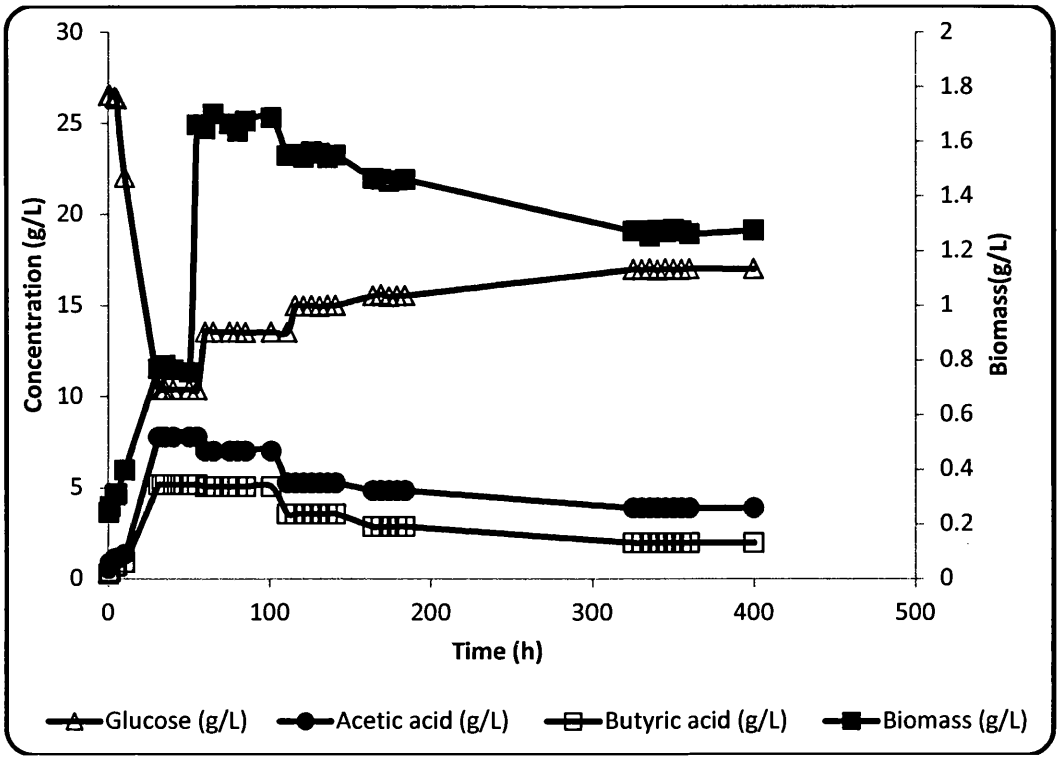

Figure 8a.5: The trend of the parameters in the 28g/L glucose concentration for continuous culture.

Appendix 9

Table 9a.1: Results for 5g/L glucose in MBR system with variety of dilution rates.

Hour (h)	Biomass (g/L)	Glucose (g/L)	Dilution (h^{-1})	Acetic acid (g/l)	Butyric acid (g/L)
0	0.37	4.877	0.727	0.14	0.11
2	0.42	4.381	0.727	0.29	0.11
4	0.49	3.885	0.727	0.85	0.11
6	0.70	2.742	0.727	2.15	0.12
8	0.74	2.632	0.727	2.27	0.13
10	1.15	1.364	1.455	3.71	0.12
12	1.66	1.075	1.455	4.04	0.16
14	1.93	0.923	1.455	4.21	0.16
16	1.97	0.827	1.455	4.32	0.18
18	2.25	0.840	1.455	4.30	0.20
20	3.07	0.413	2.909	4.79	0.22
22	3.46	0.317	2.909	4.90	0.22
24	3.75	0.303	2.909	4.91	0.22
26	4.17	0.276	2.909	4.94	0.22
28	4.23	0.289	2.909	4.93	0.22
30	4.79	0.165	5.818	5.07	0.15
32	5.16	0.124	5.818	5.11	0.15
34	5.27	0.138	5.818	5.09	0.15
36	5.56	0.152	5.818	5.09	0.15
38	5.68	0.524	5.818	5.10	0.15

Table 9a.2: Results for 10g/L glucose in MBR system with variety of dilution rates.

Hour (-)	Biomass (g/L)	Glucose (g/L)	Dilution (h^{-1})	Acetic acid (g/l)	Butyric acid (g/L)
0	0.38	9.764	0.727	1.50	0.00
1	0.43	7.440	0.727	3.01	0.51
2	0.55	5.745	0.727	4.11	0.94
3	0.78	4.684	0.727	4.80	1.22
4	0.83	4.519	0.727	4.91	1.26
5	1.28	3.196	1.455	5.76	1.60
6	1.81	2.149	1.455	6.44	1.87
7	2.35	1.364	1.455	6.95	2.07
8	2.56	1.116	1.455	7.12	2.14
9	2.62	1.047	1.455	7.16	2.16
10	2.99	0.648	2.909	5.36	2.26
11	3.41	0.248	2.909	5.62	2.36
12	3.77	0.138	2.909	6.03	2.39
13	4.31	0.165	2.909	6.16	2.38
14	4.33	0.179	2.909	6.42	2.38
15	4.82	0.152	5.818	6.02	2.39
16	5.09	0.234	5.818	6.14	2.37
17	5.27	0.289	5.818	6.28	2.35
18	5.73	0.234	5.818	6.37	2.37
19	5.76	0.193	5.818	6.51	2.38

Table 9a.3: Results for 15g/L glucose in MBR system with variety of dilution rates.

Hour (h)	Biomass (g/L)	Glucose (g/L)	Dilution (h^{-1})	Acetic acid (g/l)	Butyric acid (g/L)
0	0.38	13.502	0.727	1.96	0.19
1	0.60	8.404	0.727	3.68	1.13
2	0.80	5.621	0.727	4.89	1.85
3	1.35	4.409	0.727	5.72	2.17
4	1.56	3.720	1.455	8.77	2.35
5	2.02	2.893	1.455	9.27	2.56
6	2.62	1.791	1.455	9.88	2.21
7	3.09	0.551	1.455	7.78	2.03
8	3.26	0.386	2.909	8.59	2.09
9	3.57	0.234	2.909	9.17	2.13
10	4.17	0.207	2.909	9.73	2.23
11	4.62	0.262	2.909	8.00	1.62
12	4.78	0.096	5.818	8.34	2.08
13	5.25	0.083	5.818	8.71	2.26
14	5.33	0.014	5.818	9.50	2.45
15	5.88	0.028	5.818	9.82	2.49