Microbial Fuel and Chemical Production Using Sweet Potatoes

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A thesis submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy in Engineering.

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DECLARATION

I declare that this thesis is my own unaided work. It is being submitted to the Degree of Doctor of Philosophy to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination to any other University.

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ABSTRACT

Extensive use of fossil fuels over the years has resulted in increased petrol and electricity prices as well as negative impacts on the environment resulting from increased Green House Gas (GHG) emissions. There is therefore a need for a renewable and more environmental friendly source of energy. The aim of this study was to produce an alternative fuel source in a form of biofuels from sweet potato tuber using a laboratory-scale Fluidized Bed Bio-Reactor (FBBR). Sweet potato was a substrate of choice because of its high carbohydrate content. Although sweet potato is a food source, there is a huge surplus annually which is regarded as waste. Bacteria isolated from sweet potato tuber were identified based on their 16S rRNA sequence using colony PCR followed by sequencing. Strains identified belonged to species: *Klebsiella, Enterobacter, Rhodobacter, Bacillus, Citrobacter, Alcaligenes* and *Bordetella*. Industrial applications of each bacterial isolate were predicted from known bacterial species. Batch fermentation was operated using M9 minimal growth medium and GP medium and a consortium of the identified species. In these experiments, pH was measured but not controlled. Using M9 minimal growth medium, acetic acid (48.6 g/l), ethanol (29 g/l), propionic acid (29 g/l), butyric acid (22.9 g/l), methane (21.1 g/l), hydrogen (3.2 g/l) and carbon dioxide (6.2 g/l) were produced; however, lower concentrations were produced in GP medium (acetic acid, 13.1 g/l; ethanol, 7.3 g/l; propionic acid, 16.7 g/l, methane, 0.2 g/l; hydrogen, 1.7 g/l and carbon dioxide, 0.6 g/l) except for butyric acid, 23.6 g/l. Under fluctuating pH conditions, higher concentrations were obtained at a pH value of 6.0. Based on results, it appeared that M9 medium and pH 6.0 were preferred. However, liquid and gas products obtained at controlled pH 6.0 were lower than those obtained under uncontrolled pH conditions. This resulted in using M9 medium under uncontrolled pH conditions in a continuous FBBR. Three Hydraulic Retention

Times (HRTs) of 6 hours, 3 days and 12 days were investigated. FBBR was operated at each HRT over 42 days with a 14 day interval. HRT of 6 hours resulted in the highest productivity. Maximum concentrations of acetic acid (18.5 g/l), ethanol (5.2 g/l), propionic acid (16.9 g/l), butyric acid (16.9 g/l), hydrogen (16.3 g/l) and carbon dioxide (5.3 g/l) were obtained by day 42. Bacterial growth dynamics were monitored by plate counts while cell attachment on granular activated charcoal (GAC) was studied using scanning electron microscope (SEM). SEM micrographs showed attachment of bacterial cells as well as extracellular polymeric substance (EPS) indicating that mixed cultures used in this study were able to form biofilms.

DEDICATION

In memory of my parents

Mammako Ntoampe

&

Toka Letsie

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LIST OF SYMBOLS

μ	Specific growth rate
S	Substrate concentration
K_S	Saturation constant
μ_m	Maximum growth rate
X	Cell concentration,
X_0	Initial cell concentration
R_{batch}	Output of the culture in terms of biomass concentration
X_{max}	Maximum cell concentration achieved at stationary phase
t_i	Time during which the organism reached maximum growth
t_{ii}	Time during which the organism is not growing at maximum and includes the lag phase and the deceleration phase.
D	Dilution rate
F	Medium flow rate
V	Volume of the bio-reactor
R_{cont}	Culture output in terms of cell concentration
t	Time period prior steady-state establishment
T	Time period during which steady-state conditions prevail
CFU	Colony Forming Units

ABBREVIATIONS

FBBR	Fluidized Bed Bio-Reactor
PBR	Packed Bed Reactor
CSTR	Continuous Stirred Tank Reactor
TBR	Trickling Bed Reactor
ALR	Airlift Reactor
NAD	Nicotinamide Adenine Dinucleotide
NADHP	Nicotinamide Adenine Dinucleotide Phosphate
ATP	Adenosine Tri-Phosphate
LAB	Lactic Acid Bacteria
ABE	Acetone-Butanol-Ethanol
C	Control
CO_2	Carbon Dioxide
CO	Carbon Monoxide
H_2	Hydrogen
CH_4	Methane
PCR	Polymerase Chain Reaction
GAC	Granular Activated Carbon
VFA	Volatile Fatty acid
HRT	Hydraulic Retention Time
OD	Optical Density
NA	Nutrient Agar
NB	Nutrient Broth

SEM	Scanning Electron Microscopy
EPS	Extracellular Polymeric Substances
BP	Base Pairs
rRNA	Ribosomal Ribonucleic Acid

Chapter 1

Literature review

1. Introduction

Due to petrol and electricity price hikes, energy security has become one of the major concerns and as such there is a need for an alternative energy source (Hammond et al., 2008). Climate change due to carbon dioxide (CO_2) released from fossil fuel combustion is also a problem; however, the potential solution to these problems lies in the use of biofuels (Hammond et al., 2008). In a study performed by Brown et al. (1998), CO_2 emission reduction of up to 90% was reported when bioethanol was used as an automotive fuel relative to conventional petroleum fuels. The use of biofuels does not only reduce CO_2 emissions to the environment but is also a biodegradable and sustainable energy (Demirbas, 2008; Hammond et al., 2008).

1.1 Biofuels

The term biofuel refers to various liquid, biogas and solid fuels derived from biological materials (biomass) (Demirbas, 2008; Demirbas, 2009). Biofuels include products such as bioethanol, biomethanol, vegetable oils, biodiesel, biogas, bio-synthetic gas (bio-syngas), bio-oil, bio-char, Fischer-Tropsch liquids, and biohydrogen (Demirbas, 2008). Depending on the biomass used, biofuels can be classified in three categories, namely: First generation, second generation and third generation (Table 1.1).

1.1.1 First generation biofuels

First generation biofuels are fuels produced from agricultural crops such as grains, sugar crops and oil seed (Demirbas, 2009; Sims et al., 2010). Advantages of first generation biofuel include feasibility with current scientific methods, ease of production and use. Disadvantages include

land competition, negative effect on food prices and that not all crops can be used for biofuel production (Elshahed, 2010).

1.1.2 Second generation biofuels

Second generation biofuels are derived from non-food biomass i.e. lignocellulosic material such as forest residues, energy crops (vegetative grasses), stalks, husks and straws (Demirbas, 2009; Elshahed, 2010). For second generation biofuel production, a wide variety of materials can be used and with use of these biofuels, there is potential for improved energy, economic and environmental performance. However, these biofuels are not yet commercial because their conversion methods are still being researched intensively (Elshahed, 2010).

1.1.3 Third generation biofuels

Third generation biofuels are fuels produced from photosynthetic algae and are also known as oilgae (Demirbas, 2009). Compared to other sources of biofuels such as husks and grains, it has been stated that algae can produce up to 30 times more energy (Hartman, 2008).

There are contradicting reports on the effect of biofuels on food prices and food scarcity (Malik et al., 2009). Production of biofuels from maize and soybean contributes about 70% and 40% of the price increase in the USA respectively (Lipsky, 2008; Collins, 2008). On the other hand, some studies strongly argue that biofuels have no impact whatsoever on food market and prices since only about 1% of total agricultural land is used for biofuels (Malik et al., 2009).

Table 1.1: Biofuel categories depending on biomass used (Adapted from Demirbas, 2008)

Category	Feedstock	Examples
First generation	Sugar, starch, vegetable oils or animal fat	Bioalcohols, biodiesel, biosyngas, biogas bioalcohols, bio-oil, bio-DMF, biohydrogen
Second generation	Non-food crops, wheat s traw, corn straw, wood, solid waste, energy crops	Fischer-Tropsch diesel, wood diesel
Third generation	Algae	Vegetable oil, biodiesel

Use of biomass increases the market value of agricultural by-products (Annadana, 2007). Biomass is converted to biofuels through two main conversion technologies - thermochemical and biochemical routes which are distinguished by different decomposition methods and process specification (Table 1.2) (Demirbas, 2009; Sims et al., 2010). Thermochemical technology uses high temperatures (327 ºC - 977 ºC) to covert biomass to produce syn gas ($CO + H_2$), fuel gases and liquid fuels and includes processes such as pyrolysis, gasification and liquefaction (Fig 1.1).

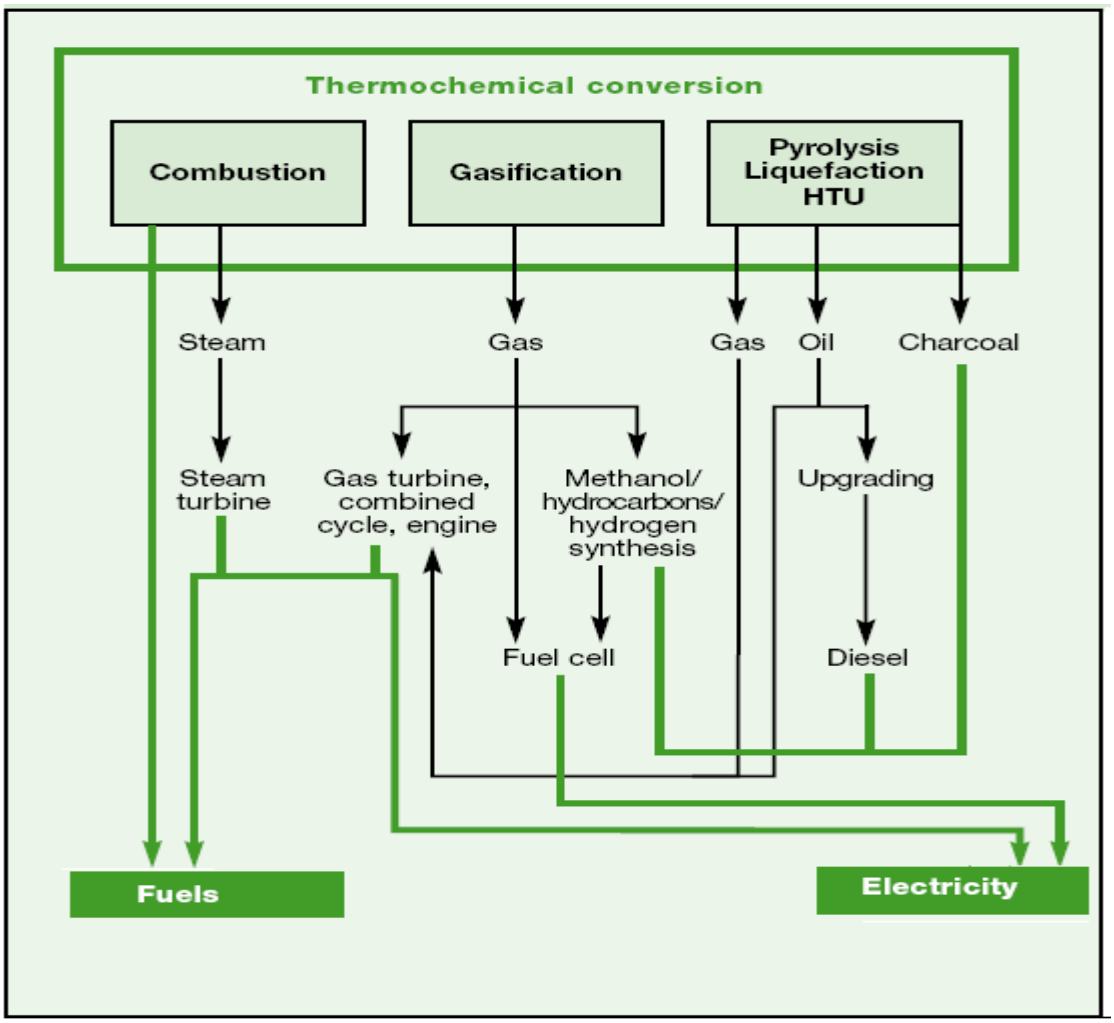


Figure 1.1: Summary of biomass thermochemical processes (Adapted from Faaij, 2006; Demirbas, 2009).

Biochemical conversion involves the use of biological agents such as enzymes and microorganisms to break down complex carbohydrate molecules into simple sugars for production of secondary energy carriers or useful energy (Sim et al., 2010). Biochemical conversion of the biomass leads to production of liquid and gas fuels such as ethanol and hydrogen (H_2) (Fig 1.2) (Faaij, 2006; Antonopoulou et al., 2009). In biochemical conversion, microrganisms are mostly used; as a result, fermentation process is the main technology in this category (Demirbas, 2008; Hammond et al., 2008).

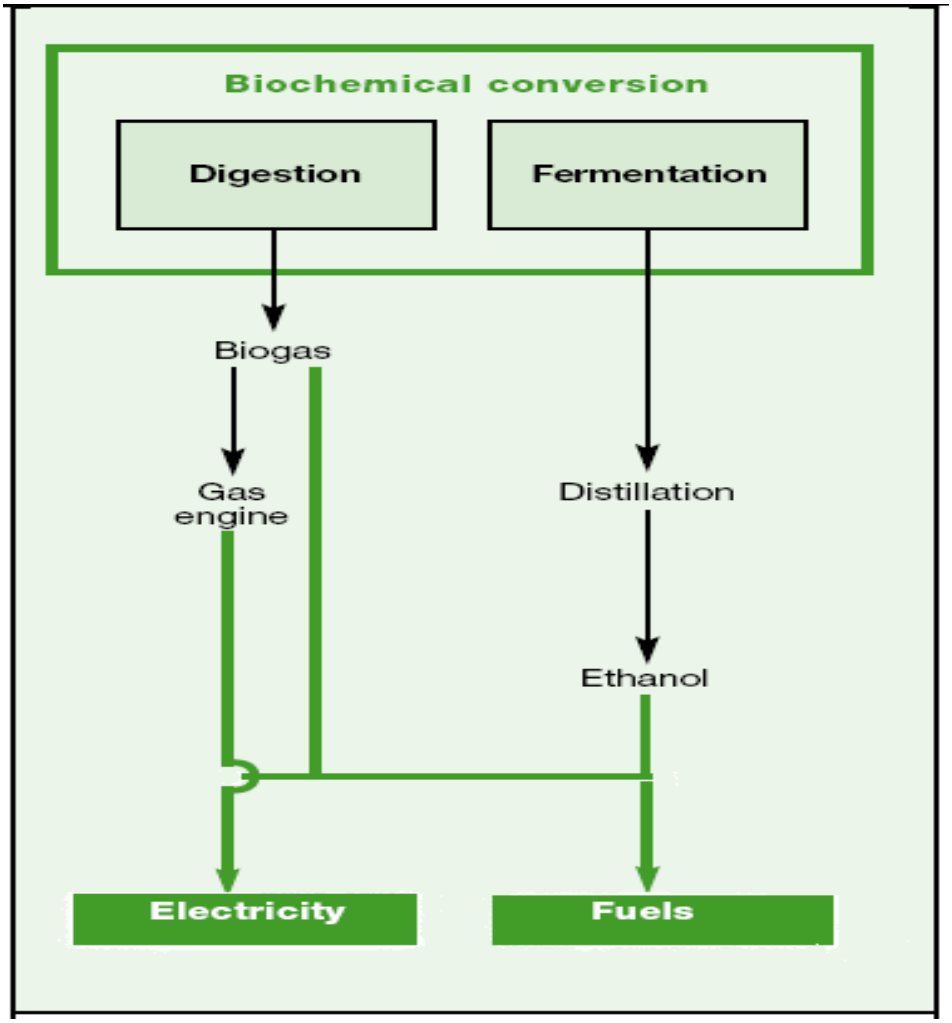


Figure 1.2: Biomass biochemical conversion to secondary energy carriers (Adapted from Faaij, 2006; Demirbas, 2009).

Table 1.2: Differences between thermochemical and biochemical conversion technologies (Demirbas, 2009)

Technology	Method of Decomposition	Process Specification
Thermochemical	Uses heat to break down biomass into intermediate products.	The two processes are mainly distinguished by either the presence or absence of oxygen.
	Include processes such as gasification and pyrolysis.	The presence of oxygen is gasification and the absence of oxygen is pyrolysis
Biochemical	Involves the use of enzymes and bacteria to break down biomass into products. It includes fermentation of lignocelluloses, starch and sugar, anaerobic digestion and aerobic digestion.	Processes are distinguished by the type of biomass used. E.g.starch and sugar biomass mean starch and sugar fermentation whilelignocellulosic biomass indicates lignocellulosic fermentation. Wet feedstocks (animal manure) result in anaerobic digestion and dry organic biomass mean aerobic digestion.

1.2 Fermentation

Fermentation is the conversion of carbohydrate into alcohols and short chain fatty acids by microorganisms' enzymes (Silva et al., 2008; Yuan et al., 2008). It is therefore a basis of many biological products which involves a process of chemical reactions with the use of microbes such as bacteria, yeast and filamentous fungi (Huang and Tang, 2007; Fortman et al., 2008). The success of fermentation is affected by physical and chemical factors such as culture used and process conditions (Huang and Tang, 2007). End-products of fermentation are determined by the way in which NADH (reduced form of Nicotinamide Adenine Dinucleotide (NAD)) is oxidized to NAD and how pyruvate is broken down (Fortman et al., 2008). Fermentation process can generally be classified as aerobic or anaerobic (Huang and Tang, 2007).

Aerobic fermentation requires a supply of oxygen as raw material (Ward, 1992). Oxygen has limitations, which is a major setback given that oxygen has low water solubility (Huang and Tang, 2007). Since oxygen availability affects microbial growth and activity, it is crucial to keep oxygen levels as high as possible in these systems. This is achieved by providing higher oxygen transfer rates with increased aeration or high agitation as practiced in stirred-tank reactors (Finn and Nowrey, 1958). Aerobic fermentation is popular in antibiotic and organic acids (i.e citric acid) production (Enzminger and Asenjo, 1986; Marcos et al., 2004).

Anaerobic fermentation on the other hand, occurs in the absence of oxygen and the process is much slower (Huang and Tang, 2007). Obligate anaerobic microbes are very sensitive to oxygen; therefore, specialized media and apparatus are needed to keep the environment as oxygen free as possible. In bio-reactors, anaerobic conditions are usually maintained by passing nitrogen gas (N_2) through the reactor. Anaerobic fermentation is mostly used for H_2 production where glucose is used as a substrate (Ren et al., 2006; Wu et al., 2010).

Anaerobic fermentation has advantages over aerobic processes such as minimized contamination, less energy requirement to keep cells alive as well as suitable for a wider range of substrates (Huang and Tang, 2007). However, the disadvantages are that microbial population changes with environmental conditions making them difficult to study as they also vary with nutrient availability.

Production of useful gases and chemicals via fermentation is more advantageous compared to traditional methods such as thermo-chemical means (Das and Veziroglu, 2001). Fermentation requires less energy and is environmental friendly (Wu et al., 2007).

1.2.1 Fermentation methods

Fermentation can be operated as batch, continuous or batch-fed systems (Stanbury and Whitaker, 1984; Olsson and Hahn-Hägerdal, 1996; Palmqvist and Hahn-Hägerdal, 2000). The choice of configuration depends on the process economics and property of microorganisms used (Olsson and Hahn-Hägerdal, 1996). Fermentation configuration requires that parameters such as yield and cost of equipment be considered and product yield should be high with minimum equipment cost (Palmqvist and Hahn-Hägerdal, 2000).

a) Batch fermentation

Batch fermentation involves the use of substrate, nutrients and separately grown cultures put together in a fermenter at the beginning of the fermentation process (Crueger, 1984; Ward, 1992). It is therefore defined by high initial substrate concentration and high final product concentration (Keim, 1983; Olsson and Hahn-Hägerdal, 1996). During this mode of operation, there is no input of substrates or output of products (Crueger, 1984). Reportedly, unstable conditions, such as exposure time that are associated with reactor operation can easily be controlled under batch operation resulting in high bacterial activity (Mohan et al., 2007). Batch cultures also result in constant circulation of the biomass leading to increased resistance to substrate shock (Kaballo et al., 1995). However, batch systems usually exhibit increased inhibitory effects on bacteria growth as a result of increasing product yields (Keim, 1983; Olsson and Hahn-Hägerdal, 1996; Bali and Sengul, 2002).

b) Fed-Batch fermentation

Fed-batch fermentation is a mode of operation that is between batch and continuous modes (Longobardi, 1994). It involves addition of substrate at a low rate; nonetheless, nothing is taken

out of the fermenter (Crueger, 1984; Palmqvist and Hahn-Hägerdal, 2000). Microorganisms are therefore exposed to low substrate concentrations (Olsson and Hahn-Hägerdal, 1996). This way substrate depletion is avoided and at the same time inhibition is minimized (Longobardi, 1994; Palmqvist and Hahn-Hägerdal, 2000). This also favours high cell densities hence improved productivity, unlike in continuous mode, there is no problem of cell wash-out (Palmqvist and Hahn-Hägerdal, 2000). However, productivity in these systems is hindered by feed rate and cell-mass concentration.

c) Continuous fermentation

Continuous systems are open systems which are described as steady-state systems where a constant supply of nutrient and a steady amount of biomass is maintained in the bio-reactor, i.e. there is an input and output flow (Crueger, 1984; Stanbury and Whaitaker, 1984). In continuous culture systems, the rate of dilution controls the rate of microbial growth and hence productivity (Palmqvist and Hahn-Hägerdal, 2000; Hoskisson and Hobbs, 2005).

The principal advantage of continuous system fermenters is reduced investment cost (Palmqvist and Hahn-Hägerdal, 2000). Continuous systems are also easy to control and less labour intensive (Olsson and Hahn-Hägerdal, 1996). However, continuous systems are associated with contamination (Stanbury and Whitaker, 1984). Continuous operations have also been reported to have limited activity or productivity due to uneven biomass distribution (Kaballo et al., 1995; Palmqvist and Hahn-Hägerdal, 2000; Mohan et al., 2007).

1.3 Bio-reactors

Bio-reactors can be defined as the class of fermenting vessels in which bacteria act as the biocatalyst (Saravanan and Sreekrishnan, 2006). Bio-reactors are grouped according to the way

they are assembled and operated and as such there are various kinds of bio-reactors. Bio-reactors range from Continuous Stirred Tank Reactor (CSTR - Agitating Continuous Reactors, and Rotary Continuous Reactors), Packed Bed Reactor (PBR), Trickling Bed Reactor (TBR), Airlift Reactor (ALR) and Fluidized Bed Bio-Reactor (FBBR) (Fig 1.3) (Nicolella et al., 1999; Qureshi et al., 2005; Singh et al., 2006).

CSTR is a stirred tank reactor with at least a single or multiple impeller/s to mechanically stir the reactor (Qureshi et al., 2005). In this reactor, reactants are fed at the same rate at which products are removed (Qureshi et al., 2005). It is therefore characterized by a continuous flow of reactants and products and a homogenous mixture (Nelson and Sidhu, 2002). In agitating continuous reactors, cell layers are clipped off dues to excessive growth, on the other hand rotating CSTRs are rated horizontally along the axis (Qureshi et al., 2005).

CSTRs have been used in fermentation for the production of gases such as H_2 (Wu et al., 2003). It has also been used for butanol production from corn using *Clostridium acetobutylicum* (Huang et al., 2007). However, low dilution rates have been reported to restrict their performance.

Packed bed reactors (PBRs) are packed with support material such as granular activated charcoal (GAC) prior to inoculation (Qureshi et al., 2005). They are fed with a nutrient rich medium which is supplied at the bottom of the reator while the product is collected at the top. They have been used for H_2 fermentation with reaction rate increase of up to 45 times, nonetheless; this reactor may result in inefficient mass transfer due to ecxcessive cell growth that is likely to result in blocakage (Wu et al., 2003).

Ulike in PBRs, in TBRs the feed is supplied at the top of the bioreactor while products are collected at the bottom (Qureshi et al., 2005). This set-up could result in insufficient nutrient

supply with great negative affects on productivity. The efficiency of the reactor may also be affected in cases where gases are produced as gases formed may take a lot space in the reactor and form inactive pockets. These reactors have been used successfully in large scale production of acetic acid (Qureshi et al., 2005).

Airlift reactors have two tubes – an inner tube called riser and an outer tube called a downcomer. Circulting air at the bottom of the reactor during operation brings about the required mixing in these reactors (Qureshi et al., 2005).

In FBBR, cell growth takes place around the support material and the particles are fluidized by the liquid flow (Singh et al., 2006). To achieve the fluidization, the fluid velocity through the bed should be sufficient (10-20 meters/hr) to allow for bed expansion (Nicolella et al., 1999). This reactor has been used for butanol production with no problem of blockage due to ecxcessive cell growth (Qureshi et al., 2005).

In addition to this, FBBR has been reported to habe minimal problems of diffusion and has evenly distributed liquid phase throughout. It also requires small volume and land for setting up and most importantly, it offers high specific surface area to microorganisms bringing about increased microbial activity and efficiency (Heijnen et al., 1989; Saravanane and Murthy, 2000; Bohlmann and Bohner, 2001; Ochieng et al., 2003). Furthermore, FBBRs are easy to scale-up; therefore, the FBBR technology is preferred and was used in this study (Wu et al., 2003).

Bio-reactors are very important in industry because their reaction rates and operation affects productivity and yield (Maddox, 1989; Qureshi et al., 2005). This implies that for high efficiency, the bio-reactor should maintain high cell density and reaction rate (Qureshi et al., 2005). Membrane reactors and immobilized cell reactors have been reported to have high

reaction rates since they are capable of maintaining high biomass densities (Mehaia and Cheryan, 1984; Maddox, 1989).

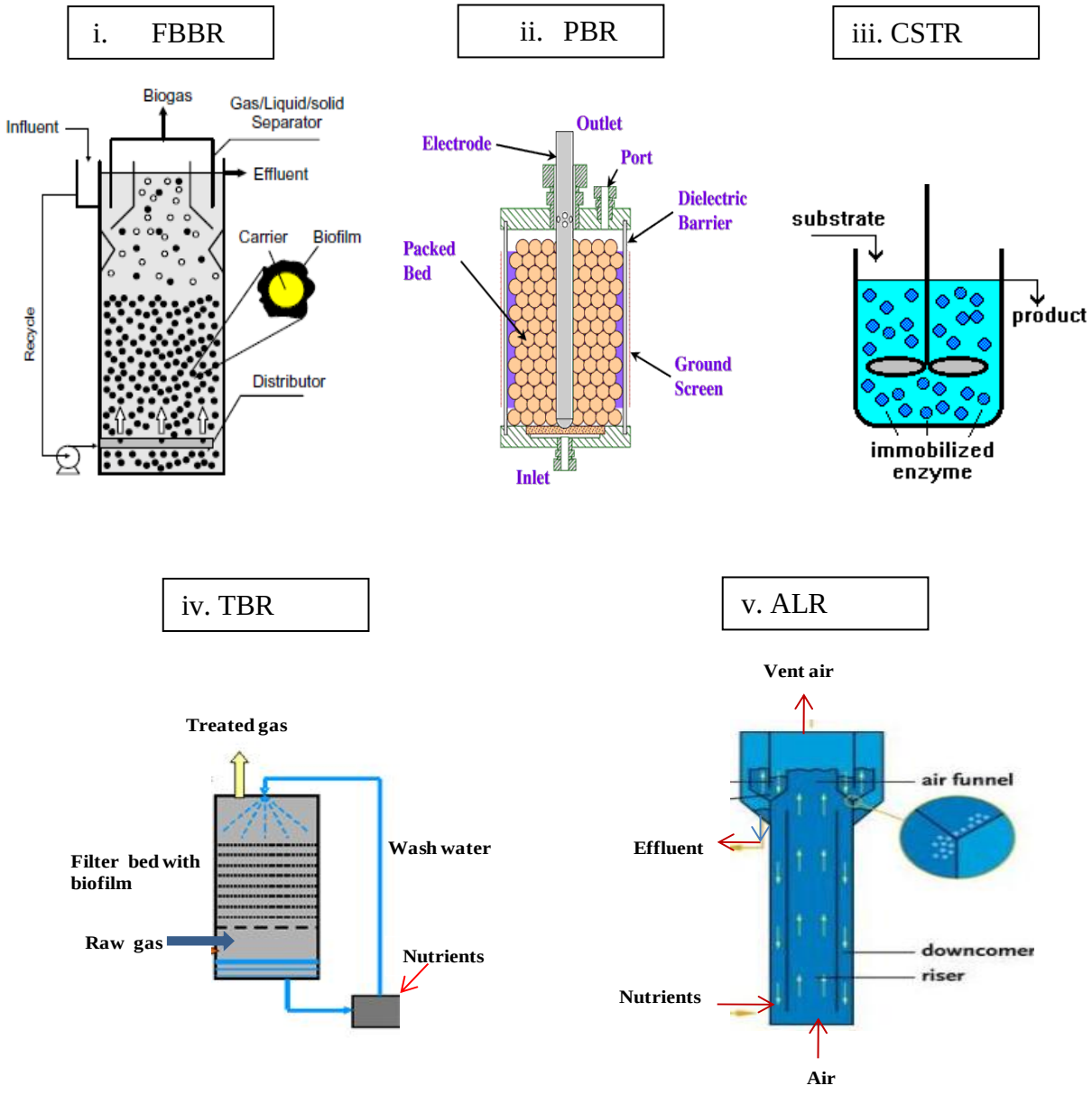


Figure 1.3: Different types of reactors i) Fluidized bed bio-reactor (FBBR), ii) packed bed reactor (PBR), iii) continuous stirred tank reactors (CSTR) iv) Trickling Bed Reactor (TBR) and v) airlift reactor (ALR) (Adapted from Qureshi et al., 2005).

1.4 Fermentation microorganisms

Microorganisms used in fermentation are the driving force that converts various substrates to precious products (such as industrial chemicals and fuels). The success of these microbes lies in their genetic nature. They possess specialized enzymes that are capable of metabolizing substrates. They break down a variety of substrates into different metabolites with concomitant production of heat and gases. Nowadays, bacteria and fungi are widely used in industries to produce useful chemicals and gases such as ethanol, acetic acid, methane and H_2 (Huang and Tang, 2007). Bacteria are used mostly because of their potential to be genetically engineered and their high growth rate (Wackett, 2008).

1.4.1 Fungi/Yeast

Fungi are microorganisms normally found in soils and on plants and are important in fermentation processes. They have been used industrially for the production of value-added products such as steroid hormones and a number of antibiotics. In addition to being used in production of chemicals such as amino acids and glycerol, fungi are also used for production of beer, bread and bio-ethanol (Table 1.3) (Huang and Tang, 2007).

Table 1.3: Industrial applications of fungi/yeast (Huang and Tang, 2007)

Applications	Examples
Baking and brewing	Bread, beer, wine, spirits
Bio-based fuels	Bio-ethanol from sucrose, glucose, and xylose
Bioremediation	Heavy metal removal, wastewater treatment
Chemicals	Glycerol, bio-surfactants, enzymes, organic acids
Health-care	Human therapeutic proteins, steroid hormones
Nutrition and animal feed	Biomass, polysaccharides, vitamins, single cell proteins

a) Aspergillus niger

Aspergillus niger, a black mold, is not a single organism. It includes a group of strains that have different biochemical and morphological characteristics. This group is usually used in fermentation for commecially production of organic acids, enzymes and antibiotics. It is normally found in damp places.

A. niger is normally used for commercial production of citric acid. It is usually an organism of choice for because is it easy to handle, it has the ability to ferment a variety of substrates and has high yields.

b) Saccharomyces cerevisiae

Saccharomyces cerevisiae, the budding yeast, usually exists as a diploid i.e. contains two full set of chromosomes one from each parent (Landry et al., 2006). Just like *Escherichia coli*, it has a complete sequenced genome (Huang and Tang, 2007). It is a unicellular organism that is found

in soils and exudates of fruits (Naumov et al., 2003). It has been used for baking, brewing and wine making (Landry et al., 2006; Huang and Tang, 2007).

S. cerevisiae is unable to use pentose sugars as its sole energy source (Gray et al., 2006; Katahira et al., 2008). However, this fungus has been used in industrial-scale production of ethanol (Huang and Tang, 2007). Even though it is known to have low tolerance to high temperatures (above 35 °C), species that ferment between 40 °C and 45 °C have been recently selected for using progressive cultures and selection of survivors after heat shock process (Rikhvanov et al., 2001; Edgardo et al., 2008). In ethanol fermentation, *S. cerevisiae* has been reported to be more tolerant to inhibition than most bacterial species (Edgardo et al., 2008).

S. cerevisiae mainly uses Embden-Meyerhoff-Parrnas (EMP) pathway to produce ethanol (Fig 1.4). In this pathway, one mole of glucose is broken down to produce two pyruvate molecules (Madigan et al., 2000; Bai et al., 2008; Wilkins, 2008). The pyruvate molecules are inturn reduced to ethanol with the production of two ATP (Adenosine Triphosphate) molecules (Wilkins, 2008). The ATP produced is used for yeast cell growth. This implies that in *S. cerevisiae*, ethanol production is coupled with cell growth therefore more cell growth would mean increased ATP consumption hence increased ethanol production (Bai et al., 2008).

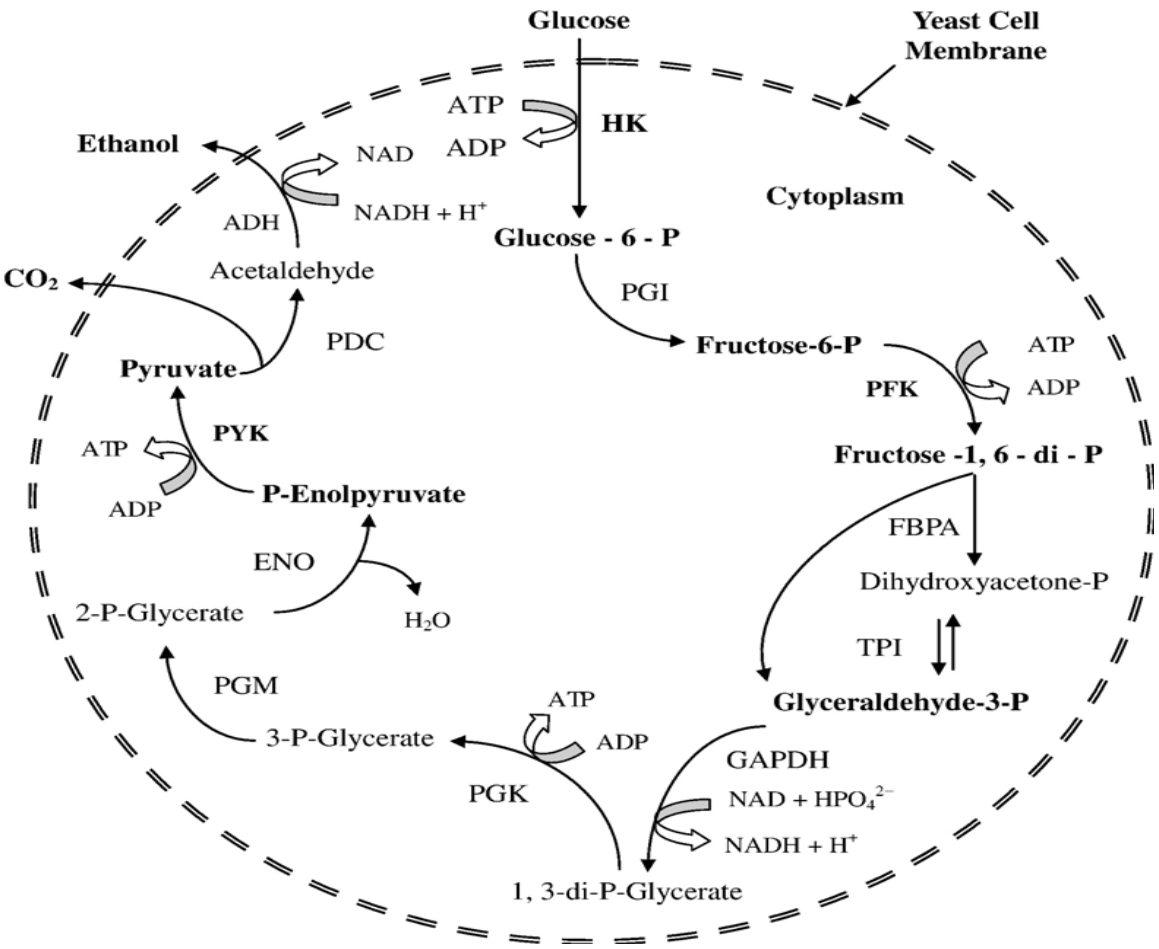


Figure 1.4: Ethanol fermentation via Embden-Meyerhoff-Parrnas pathway in *S. cerevisiae*. Abreviations: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase,PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase (Bai et al., 2008).

1.4.2 Bacteria

Bacteria are found in the air, soil, food and other organisms (Huang and Tang, 2007). They play a major role in the environment and have many industrial applications. Their industrial uses include bioremediation, bio-energy production and chemical production. They have been used in bioremediation for waste clean-up in wastewater treatment plants and have also been applied in the production of energy in the form of H_2, methane and ethanol. In addition to this, they are

employed in the production of important chemicals such as organic acids, butanol, and 1,3-propanediol (Table 1.4) (Linko, 1985).

Pseudomonads such as *Pseudomonas aeriginosa* have been used in bioremediation of toxic chromium from electroplating effluent. *Bacillus* species (*Bacillus subtilis*) have been used in the industrial production of chemicals such as vitamin B2 (riboflavin) (Ganguli and Tripathi, 2002; Tannler et al., 2008). *Clostridium* uses a variety of sugars and celluloses as carbon source and converts these substrates into alcohols such as ethanol and organic acids such as acetic acid (Huang and Tang, 2007). Members of this genus include industrially important *Clostridium acetobutylicum*, which is used mostly for production of ethanol, acetone and butanol (Lin et al., 2007). On the other hand, lactic acid bacteria (LAB) produce lactic acid from simple sugars. These include *Lactobacillus* species, which are widely used in production of fermented food such as yoghurt. Lactic acid bacteria are mainly used in vegetable fermentation such as carrots and beets (Eom, 2007).

Table 1.4: Industrial applications of bacteria (Huang and Tang, 2007)

Applications	Examples
Bio-energy	Hydrogen, electricity, methane, ethanol
Biocatalysis	Enzymes, organic solvent tolerant bacterial cells
Bioleaching	Heavy metals extraction from ores or crude oil
Bioremediation	Pollution control, toxic waste clean-up, wastewater treatment
Chemicals	Organic acids, bio-surfactants, butanol, 1,3-propanediol
Food and beverages	Dairy products: yogurt, cheese; beverages: cider, wine; vinegar
Health-care	Human therapeutic proteins, antibiotics

a) Zymomonas mobilis

Zymononas mobilis is a Gram-Negative, facultative anaerobic bacterium, known for its potential to produce a variety of substances such as ethanol, levan, oligosaccharides and sorbitol (Cazetta et al., 2007; Bai et al., 2008; Yamashita et al., 2008). Its importance in ethanol production has been increasing for a number of reasons; it is highly efficient in ethanol production due to its high glucose uptake capabilities (Swings and Deley, 1997; Ruanglek et al., 2006; Cazetta et al., 2007; Yamashita et al., 2008). It also has the ability to tolerate high ethanol concentrations of up to 16% v/v (Davis et al., 2006; Ruanglek et al., 2006; Cazetta et al., 2007). In addition to this, it is known to be tolerant to high sodium concentrations (Cazetta et al., 2007; Yamashita et al., 2008). It has nutritional requirements that are easily found in industrial wastes (Ruanglek et al., 2006). Furthermore, it has the potential to break down raw sugar, sugarcane juice, and sugarcane syrup to ethanol (Cazetta et al., 2007; Yamashita et al., 2008).

Z. mobilis produces ethanol via the Entner-Doudoroff pathway which occurs under anaerobic conditions (Cazetta et al., 2007; Wilkins, 2008; Yamashita et al., 2008). This pathway produces one mole of ATP per mole of glucose. This means that compared to *S. cerevisiae*, *Z .mobilis* consumes more energy during ethanol production; however, studies show that *Z. mobilis* produces five times more ethanol than other ethanol producing microorganisms including the brewer's yeast, *S. cerevisiae* (Wilkins, 2008; Yamashita et al., 2008).

b) Clostridium

Clostridium bacteria are rod shaped, Gram-Positive anaerobic bacteria (Byrne et al., 2008; Thompson III and Crawford, 2008). They are spore-forming bacteria that are mostly isolated from potatoes, soil, water, air and dust (delMarGamboa et al., 2005; Byrne et al., 2008; Thompson III and Crawford, 2008). They are known for producing H_2, ethanol and other solvents such as butanol and acetate (Mitchell, 1997; Lin et al., 2007). As a result they are named Acetone-butanol-ethanol (ABE) producing clostridia (Shinto et al., 2008).

ABE producing clostridia carry out two phases. The first phase is associated with acetate and bytarate production while the second phase is characterised by production of solvents - acetone, butanol and ethanol (Matta-el-Ammouri et al., 1986; Sillers et al., 2008). The solvents are produced as a result of acid accumulation during the first phase which then favours solvent production (Sillers et al., 2008).

Clostridium acetobutylicum and *Clostridium beijerinckii* are widely used for ABE production and are known to grow on a wide range of substrates (Jones and Woods, 1986; Qureshi and Maddox, 1992; Qureshi and Blaschek, 2000; Qureshi et al., 2008a; Qureshi et al., 2008b). These

substrates could be anything from domestic wastes, agricultural wastes and industrial wastes (Shinto et al., 2008).

Clostridia that have the ability to use cellulosic biomass as a sole carbon source such as *Clostridium thermocellum* usually use a number of cellulolytic enzymes (Balusu et al., 2005; Levin et al., 2006). These enzymes are found on cellulosome which is found on the cell surface (Lynd et al., 2002; Demain et al., 2005; Levin et al., 2006). The enzymes break down cellulose into glucose which is readily metabolized into useful products.

ABE producing *Clostridium* species achieve this via the Embden-Meyerhof-Parnas (EMP) pathway (Fig 1.5). This results in production of two moles of ATP per mole of hexose sugar. Pentose sugars are broken down through the pentose phosphate (PP) pathway which produces fructose-6-phosphate and glyceraldehyde-3-phosphate (G3P). The two intermediates then join the glycolytic pathway (Shinto et al., 2008). ABE producing clostridia have the ability to utilize both hexose and pentose sugars giving them an advantage over other ethanol producing bacterial species (Qureshi et al., 2008a).

Apart from ABE production, Clostridia have also been used in a number of studies for H_2 production (Levin et al., 2006). Zhang et al. (2006) produced up to 27.2 ml/h of H_2 for every 10 g/l of glucose using *C. acetobutylicum* in an unsaturated flow reactor. *C. acetobutylicum* has also been reported to produce lactate and acetic acid (Balusu et al., 2005).

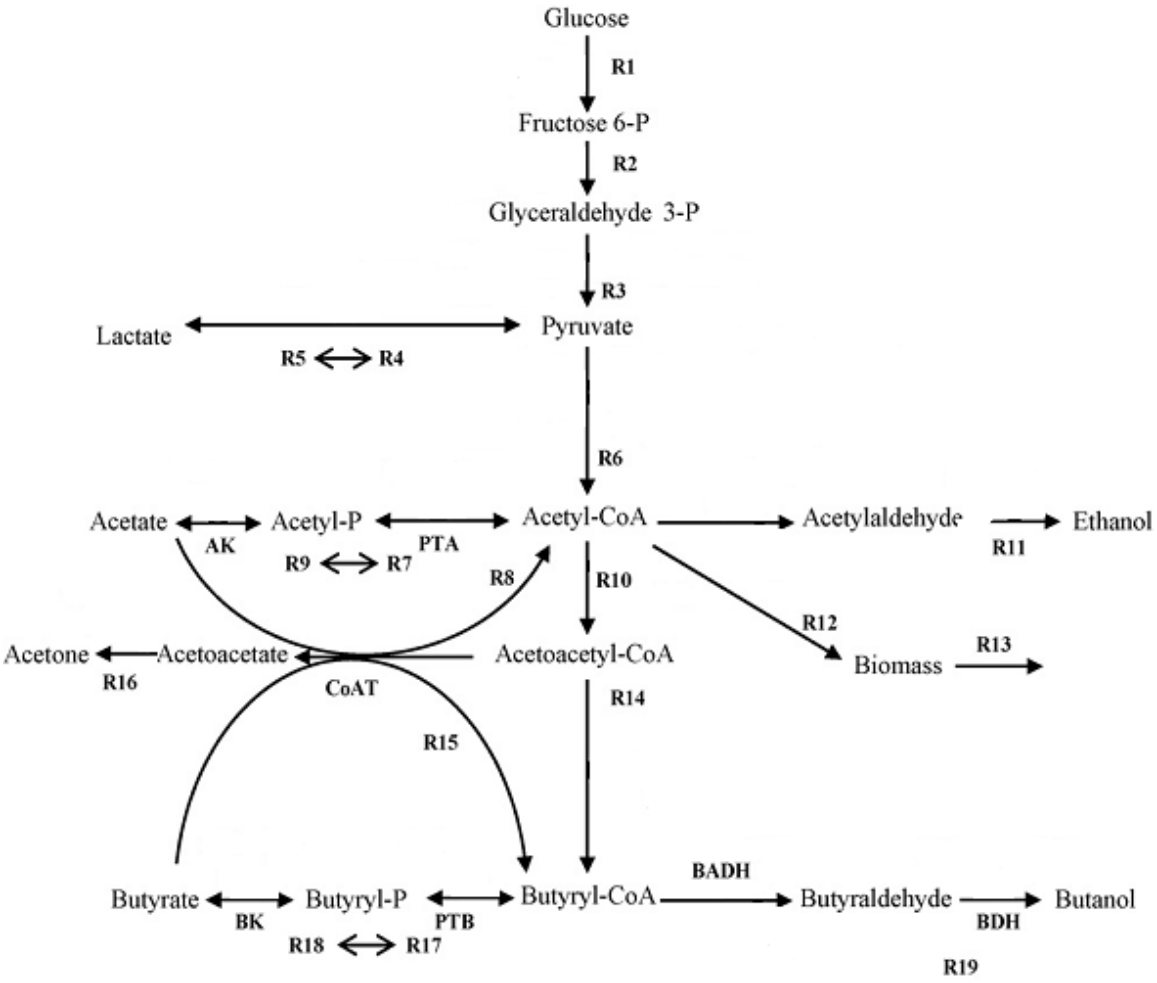


Figure 1.5: Metabolic pathway of glucose in *C. acetobutylicum* ATCC 824T. Enzymes are indicated in bold and abbreviated as follows: PTA, phosphotransacetylase; AK, acetate kinase; CoAT, CoA transferase; PTB, phosphotransbutyrylase; BK, butyrate kinase; BADH, butyraldehyde dehydrogenase; BDH, butanol dehydrogenase. (Adapted from Shinto et al., 2008).

c) Escherichia coli

Escherichia coli is a rod shaped Gram-Negative bacterium that belongs to the family *Enterobacteriaceae* (Weintraub, 2007). It is a facultative anaerobe that has been studied the most and has a complete genome sequence (Warnecke and Gill, 2005). Compared to other microorganisms, *E. coli* is advantageous because of its ability to ferment all the major sugars producing a mixture of ethanol and organic acids (Fig 1.6). Its capacity lies in its genetic nature (Alterthum and Ingram, 1989; Fortman, 2008).

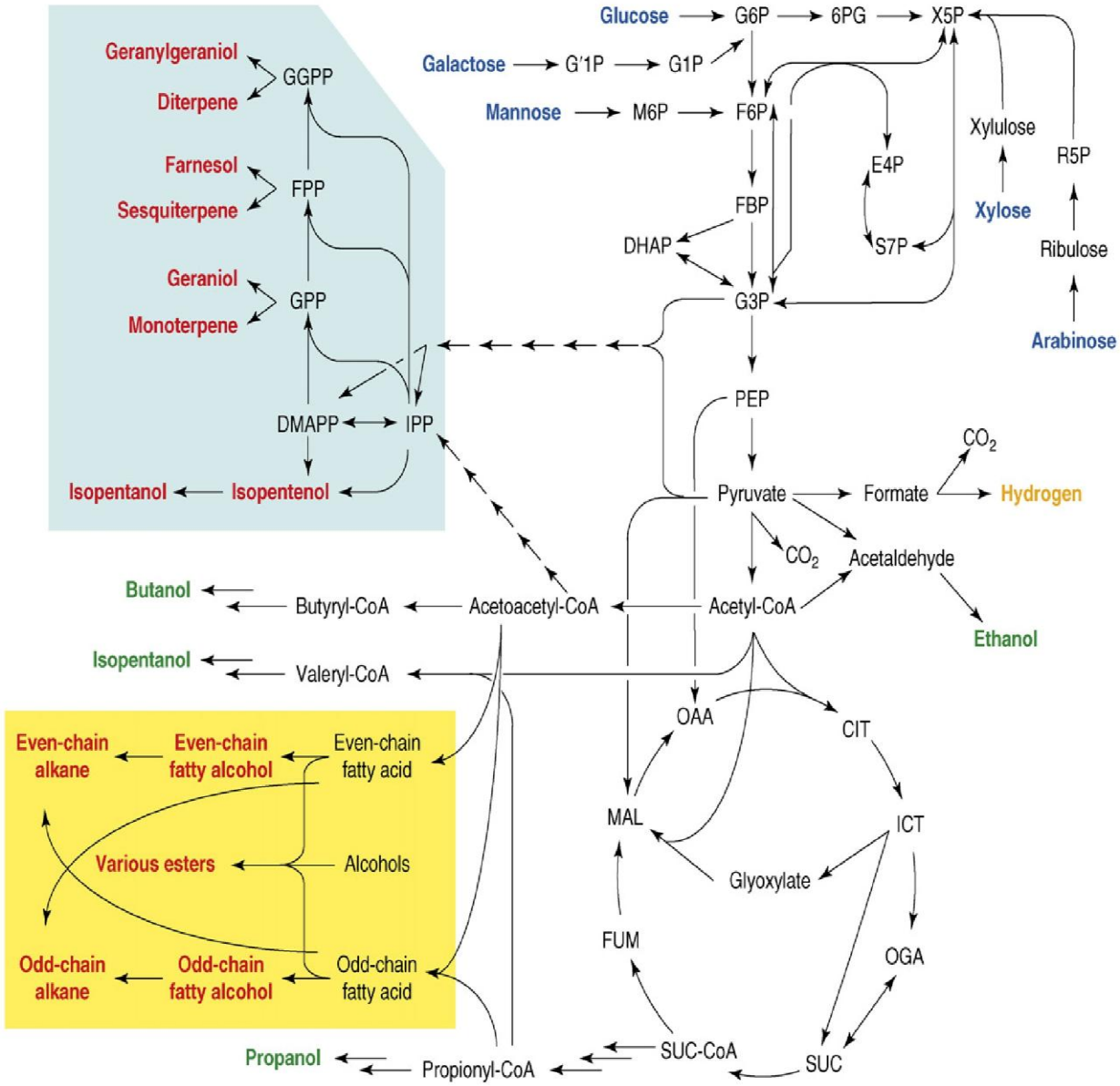


Figure 1.6: ***E. coli*** **main metabolic pathways and correlating fuel molecules**. The blue box indicates isoprenoid pathways and isoprenoid-derived molecules. Fatty acid pathway is shown in a yellow box. Short-chain alcohols are shown in green text. Main sugars are shown in blue. 6P,G, 6-phosphogluconate; CIT, citrate; DHAP, dihydroxyacetone phosphate; DMAPP, dimethylallyl pyrophosphate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; FPP, farnesyl pyrophosphate; FUM, fumarate; G01P, galactose-1-phosphate; G1P, glucose-1-phosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; ICT, isocitrate; IPP, isopentenyl pyrophosphate; M6P, mannose-6-phosphate; MAL, malate; OAA, oxaloacetate; OGA, 2-oxoglutarate; PEP, phosphoenolpyruvate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; SUC, succinate; SUC-CoA, succinyl coenzyme A; X5P, xylulose-5-phosphate (Fortman, 2008).

1.5 Fermentation products

Bacteria produce a variety of value-added products as a result of different metabolic routes (Fig 1.7). Factors affecting the type of end-product include the bacterial culture used, media composition/substrate (carbon source), and fermentation conditions such as temperature and pH (Huang and Tang, 2007). Products include alcohols, gases and carboxylic acids (Linko, 1985).

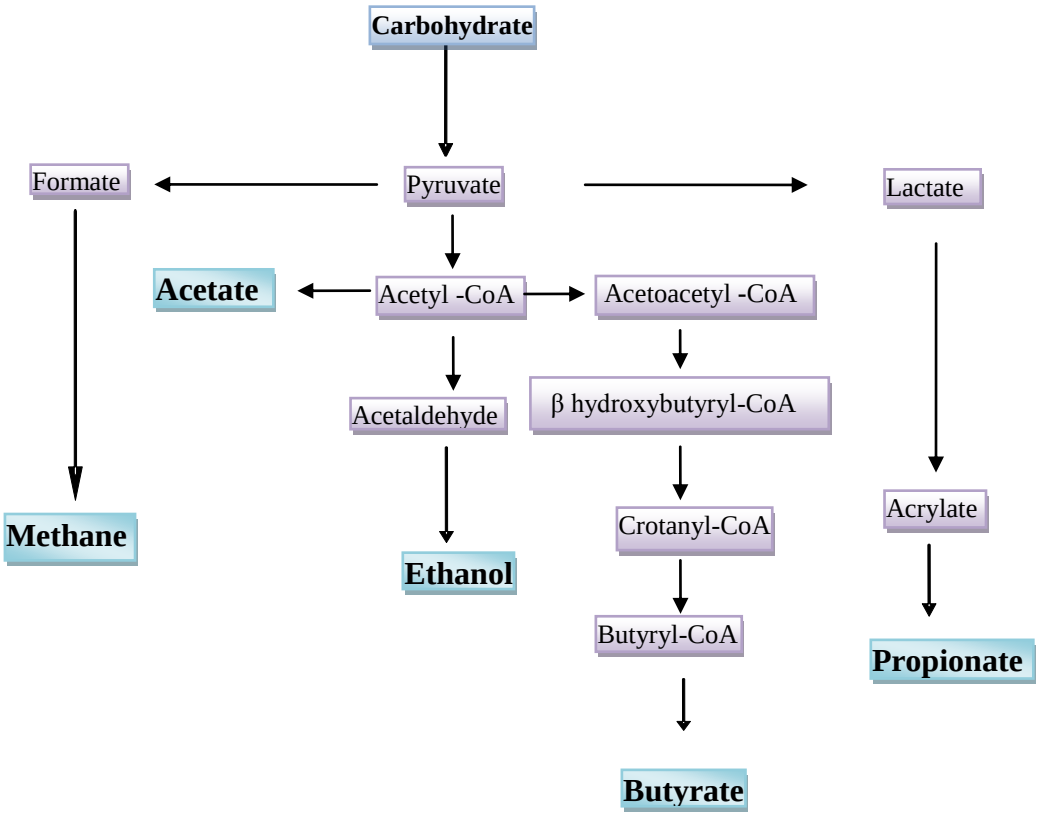


Figure 1.7: Carbohydrate metabolic pathways in bacteria leading to variety of products - methane, acetate, ethanol, butyrate and propionate (Adapted from Hungate, 1966).

1.5.1 Ethanol

Ethanol is one of the major products of microbial fermentation (Cáceres-Farfán et al., 2008). It is produced industrially from lignocellulosic biomass, starch and sugar (Yuan et al., 2008). Ethanol produced from lignocellulosic material like corn stalk, is referred to as second generation ethanol while ethanol produced from starch, is referred to as first generation biofuel (Gnansounou et al., 2005; Kopsahelis et al., 2007; Yuan et al., 2008). Ethanol is widely used as both a solvent and fuel (Linko, 1985).

Ethanol fuel is used as an alternative or additive to gasoline where it is mixed with gasoline to produce gasohol (Siqueira et al., 2008). Ethanol has higher octane rating and less emission than gasoline (Sukumaran et al., 2009).

A number of microorganisms have been utilized for ethanol production (Linko, 1985; Bai et al., 2008). For efficient ethanol fermentation, the desirable characteristic microorganism should yield high ethanol concentrations and be tolerant to high temperatures and inhibitors present in the substrate material. The characteristic organism should not only have the capacity to ferment simple sugars such as glucose, it should also have the ability to break down other sugars such as D-xylose contained in substrates (Edgardo et al., 2008). As mentioned previously, these include microorganisms such as *S. cerevisiae*, *C. acetobutylicum* and *Z. mobilis* (Linko, 1985; Bai et al., 2008).

1.5.2 Acetone and Butanol

Butanol is one of the alcohol fuels that has gained international interest (Qureshi and Blaschek, 2000; Wackett, 2008). Butanol is a superior fuel to ethanol because of its exceptional fuel

characteristics (Ladisch, 1991). Unlike ethanol, butanol is more water tolerant and has higher energy content (Linko, 1985; Qureshi and Blaschek, 2000; Sillers et al., 2008; Wackett, 2008).

In ABE fermentation, Butanol is a major product with a ratio of 3:6:1 acetone: butanol: ethanol (Qureshi et al., 2008a; Qureshi et al., 2008b). However, high butanol yields are hindered by solvent product inhibition i.e. butanol toxicity (Qureshi and Blaschek, 2000). It has been reported that as much as 13 g/l butanol is enough to inhibit butanol production (Lin and Blaschek, 1983). As a result, it is very important to use strains that can tolerate higher butanol concentrations (Ladisch, 1991; Qureshi and Blaschek, 2000).

In a study performed by Lin and Blaschek (1983), a butanol-tolerant strain (*C. acetobutylicum*) was successfully developed. They reported butanol tolerance of up to 13.2 g/l which resulted in an increased butanol production using a new butanol-tolerant strain (SA-1 mutant) compared to the parental strain (7.6 g/l). Similar results were obtained by Chen and Blaschek (1999) who reported that adding sodium acetate to the medium resulted in 20.9 g/l butanol yield by *C. beijerinckii* BA101 as opposed to 6.1 g/l produced in a medium without sodium acetate.

1.5.3 Biohydrogen

Biohydrogen is one of the most important biofuels (Fortman et al., 2008). Biohydrogen combustion and conversion only produces water, making biohydrogen a clean energy source with great potential as an alternative to fossil fuel (Maintinguer, 2008; Wang et al., 2008; Mitchell et al., 2009). In addition to clean fuel, H_2 combustion has a high energy yield (122 kJ/g) which can be used to produce electricity via fuel cells (Levin et al., 2004; Mitchell et al., 2009; Venetsaneas et al., 2009). H_2 is produced mainly via physio-chemical and biological processes using carbohydrate as a main carbon source (Venetsaneas et al., 2009).

Biological production of H_2 is preferred because it occurs under ambient pressure and temperature and as such requires less energy (Wang et al., 2008; Mitchell et al., 2009; Wang and Zhao, 2009). Biological methods include biophotolysis (photosynthesis reactions) and dark fermentation processes (Chou et al., 2008; Ntaikou et al., 2008; Saxena et al., 2009). Photosynthetic production of H_2 has low efficiency and therefore requires light. On the other hand, the fermentative route is more viable and feasible because it produces H_2 without photoenergy (Wang et al., 2008; Mitchell et al., 2009). This means that the cost of H_2 production via fermentation is 340 times lower than photosynthesis route (Antonopoulou et al., 2008). Fermentation also offers advantages such as reduced volume (Wang and Zhao, 2009).

Algae, purple sulfur and non-sulfur bacteria produce H_2 through photosynthetic reaction (Equation 1.1) (Saxena et al., 2009). Anaerobic dark fermentation on the other hand generally occurs as shown in Equation 1.2 where four molecules of H_2 are produced from one glucose molecule with concomitant production of acetic acid via Emden-Meyerhoff-Parnas (EMP) pathway (Kengen et al., 1996; Ntaikou et al., 2008; Saxena et al., 2009).

$$H_2O + CO_2 \xrightarrow{\text{sunlight}} \text{Carbohydrates} + \mathbf{H}_2 + O_2 \quad (1.1)$$

$$C_6H_{12}O_6 + 4H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4\mathbf{H}_2 \quad (1.2)$$

1.5.4 Carboxylic acids and organic acids

Organic acids have important industrial uses which include finish on food products (Fernandez-Garcia and Mcgregor, 1994; Goldberg and Rokem, 2009). This is because they are used as food preservatives/additives. Lactic acid for instance has been used as pathogenic bacteria inhibitors during yoghurt production (Fernandez-Garcia and Mcgregor, 1994; Arzumanov et al., 2002).

Though these acids are mainly produced via petrochemical routes, there has been an increasing interest on producing these acids from renewable resources (Huang and Tang, 2007; Goldberg and Rokem, 2009). Most of these acids are produced by microbial processes as they are natural products or intermediates of microbial pathways (Amaral et al., 2009). However, product inhibition due to inhibited cell growth and substrate utilization limits yield and productivity of these acids (Arzumanov at al., 2002). Simple organic acids produced by different microbial pathways include acetic acid and propionic acid (Sauer et al., 2008; Goldberg and Rokem, 2009).

a) Citric acid

Citic acid is one of the most important fermentation products by tonnage (Yalcin et al., 2010).
It is natuaraly found in various citrus fruits, pineapple, pear and peach .It is widely used in food and pharmaceutical industries (Imandi et al., 2008). In food, it is used as a preservative or emulsifier (Kamzolova, 2005). One of the main advantages of citric acid compared to other acids is its low toxicity (Yalcin et al., 2010). It is commonly produced by *Aspergillus, Citromyces* and *Penicillium* species.

b) Acetic acid

Acetic acid is a common and popular product of alcohol fermentation. It is used as vinegar in the food industry for many years (Crueger, 1984; Sano et al., 1999). It is produced as a result of incomplete oxidation and is mainly used as a solvent for production of pure terephthalic acid (PTA), vinyl acetate, acetic anhydride, cellulose acetate and acetates (Sano et al., 1999). Its derivatives are widely used in chemical sectors, textiles, pharmaceuticals and printing/dyeying.

c) Propionic acid

Propionic acid is also an important organic acid as it has various uses in process industries such as food and phamaceuticals (Keshav et al., 2009). It acts as an esterifying agent used in plasticizers. It is also used in the manufacture of artificial food flavours, perfumes bases, vitamin B_{12} (cobalamin), thermoplastics and herbicides (Czaczyk et al., 1995; Huang and Tang, 2007; Keshav et al., 2009; Zhang and Yang, 2009). Its salts (sodium, potassium and calcium) are used as food preservatives (Zhang and Yang, 2009).

Propionic acid is one of the weak acids that is predominantly produced via petrochemical routes; however, as previously mentioned, increasing oil prices have encouraged scientists to explore alternative ways of producing value-added commodity chemicals from renewable resources, hence its production from carbohydrate rich materials via fermentation (Huang and Tang, 2007; Zhang and Yang, 2009).

1.6 Biomass as a source of energy

Biomass refers to all plant and animal material and also includes organic waste products, energy crops, agricultural and industrial residues (Levin et al., 2007; Antonopoulou et al., 2009). It is a promising source of energy and provides about 14% of the world's energy (Levin et al., 2007). Biomass may be used directly as an energy source (direct combustion). It can also be converted into energy via thermochemical conversion processes (gasification, pyrolysis) and biochemical conversion process biological methods (Antonopoulou et al., 2009). Carbohydrate content found in the biomass is the building block of all different products formed during fermentation (Silva et al., 2008).

1.6.1 Carbohydrate

Carbohydrate is a class of chemical compounds that consists of carbon, oxygen and H_2 (Kim et al., 2007). It includes sugars, starch and cellulose. These compounds are classified as monosaccharides (e.g. glucose, fructose), disaccharides (e.g. sucrose, lactose) or polysaccharides (e.g. starch, cellulose) (Kim et al., 2007). All carbohydrate compounds have been used as a source of biomass and a large number of microorganisms use them as energy source. For example, chemoheterotrophs use glucose for their growth (Huang and Tang, 2007). As a result, carbohydrates are essential for maintaining life (Kim et al., 2007). Glucose is the key compound as most life systems are built around it.

a) Starch

Starch is a carbohydrate consisting of glucose compounds joined to form a polysaccharide (Dias et al., 2008). It is a plant natural energy source that is most abundant and valuable which needs to be converted to simple sugars before it can be utilized as a carbon source (Fig 1.8) (Yoo and Jane, 2002; Mosier et al., 2005; Yang et al., 2006; Gray et al., 2006). It consists of two α-glycan bipolymers, namely, amylose and amylopectin (Yang et al., 2006; Dias et al., 2008; Shariffa et al., 2009). Amylose is a more linear glucose polymer consisting of 200 to 20 000 glucose units forming a helix shape while amylopectin is a highly branched molecule of 10-15 nm in diameter and 200-400 nm long (Yoo and Jane, 2002; Yang et al., 2006; Shariffa et al., 2009).

Amylopectin comprises of D-glucopyranose monomers linked to either α-(1,4) or α-(1,6) glucosidic bonds (Yang et al., 2006). The joined monomers of α-(1,4) results in a linear chain; however, α-(1,6) bond serves as a glue that joins together the linear chains (Yang et al., 2006). Amyloses consist of linear glucan connected via α-(1,4) bonds (Lesmes et al., 2009). Most

starches contain about 17% to 28% of amylose (Matveev et al., 2001). These bonds are easily hydrolyzed by microbial enzymes (Weng et al., 2008).

Potato starch has been reported to contain about 1 in 500 phosphorylated glucose residues (Absar et al., 2009). The phosphate groups are found mostly in the amylopectin's β-chain and are joined to C-6 and C-3 of the glycosyl group (Hizukuri et al., 1970; Takeda and Hizukuri, 1982). This phosphorylation protects the phosphorylated residues from hydrolysis by amylolytic enzymes (Absar et al., 2009). Hydrolysis of potato starch results in production of commercially important saccharides such as raffinose, maltose and galactose (Absar et al., 2009).

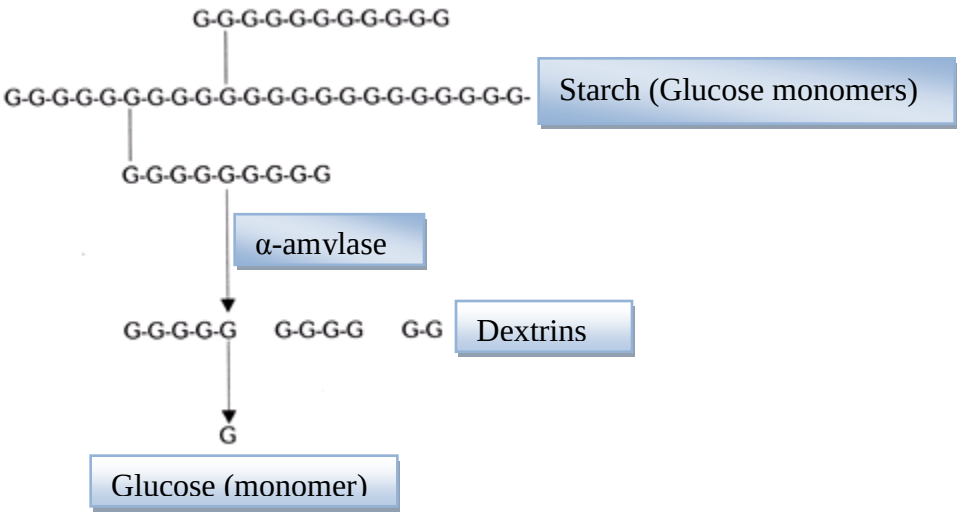


Figure 1.8: Enzymatic break down of starch molecule to simple glucose molecules (Adapted from Mosier et al., 2005).

b) Lignocellulosic biomass

Lignocellulosic biomass is one of the most inexpensive and abundant renewable materials (Lee, 1997; Wackett, 2008; Zhao et al., 2008). It consists of cellulose (30-50%), hemicellulose

(20-35%) and lignin (10-25%) (Lee, 1997; Gray et al., 2006; Weng et al., 2008; Zhao et al., 2008; Gupta et al., 2009).

Cellulose is a polymer of glucose linked via β-(1,4) glycosidic bonds and unlike starch its $-CH_2OH$ groups alternate above and below the plane of the cellulose molecule thus producing long, unbranched chains (Lee, 1997, Gray et al., 2006) Hemicellulose is a compound that consists of five-carbon sugar xylan linked to six-carbon sugars such as galactose through β-(1,4) linkage and lignin is a polymer made up of phenyl propane units connected by C-C and C-O-C links (Lee, 1997, Gray et al., 2006; Wackett, 2008, Gupta et al., 2009).

Lignin is recalcitrant to degradation and its resistance is due to its heterogenous structure (Wackett, 2008; Weng et al., 2008; Zhao et al., 2008). It is composed of distinct subunits and less reactive monomers making degradation of these polysaccharides tough (Weng et al., 2008). As a result, lignin has to be broken down to make cellulose and hemicellulose easily accessible for conversion into biofuels and bio-based chemicals (Fig 1.9) (Mosier et al., 2005). This is achieved by pretreatments methods such as physiochemical, physical (mechanical) or biological (enzymes) methods (Lee, 1997; Weng et al., 2008).

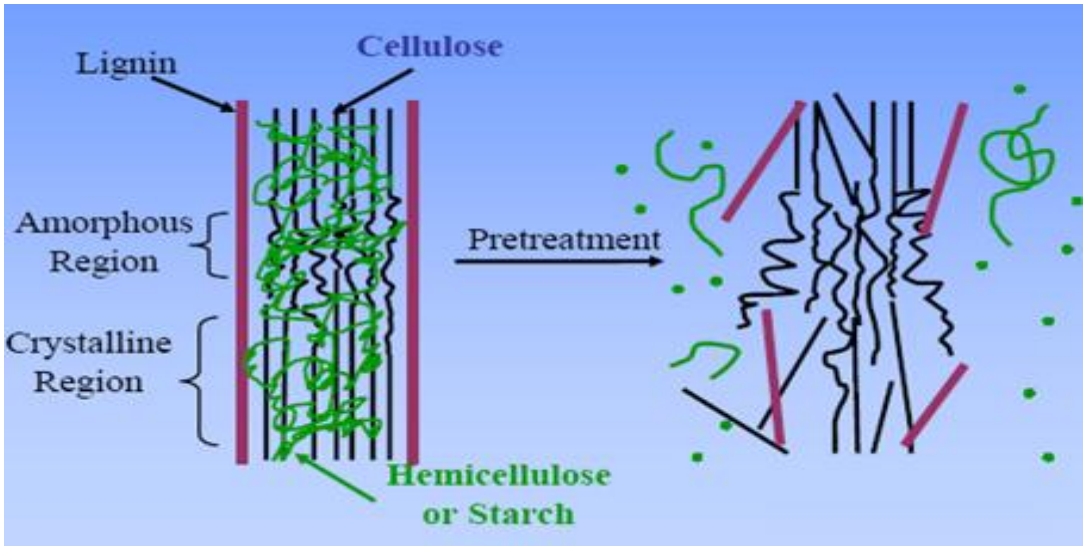


Figure 1.9: Lignocellulosic biomass pre-treatment (Mosier et al., 2005).

Delignification is therefore the first most important step in fermentation of lignocellulosic material (Lee, 1997; Gupta et al., 2009). This should be followed by depolymerization of lignin into polyphenols (Fig 1.10) (Lee, 1997). Acid treatment and enzymatic hydrolysis are the most popular pretreatment methods used to remove lignin in lignocellulosic biomass (Lee, 1997; Zhao et al., 2008; Gupta et al., 2009).

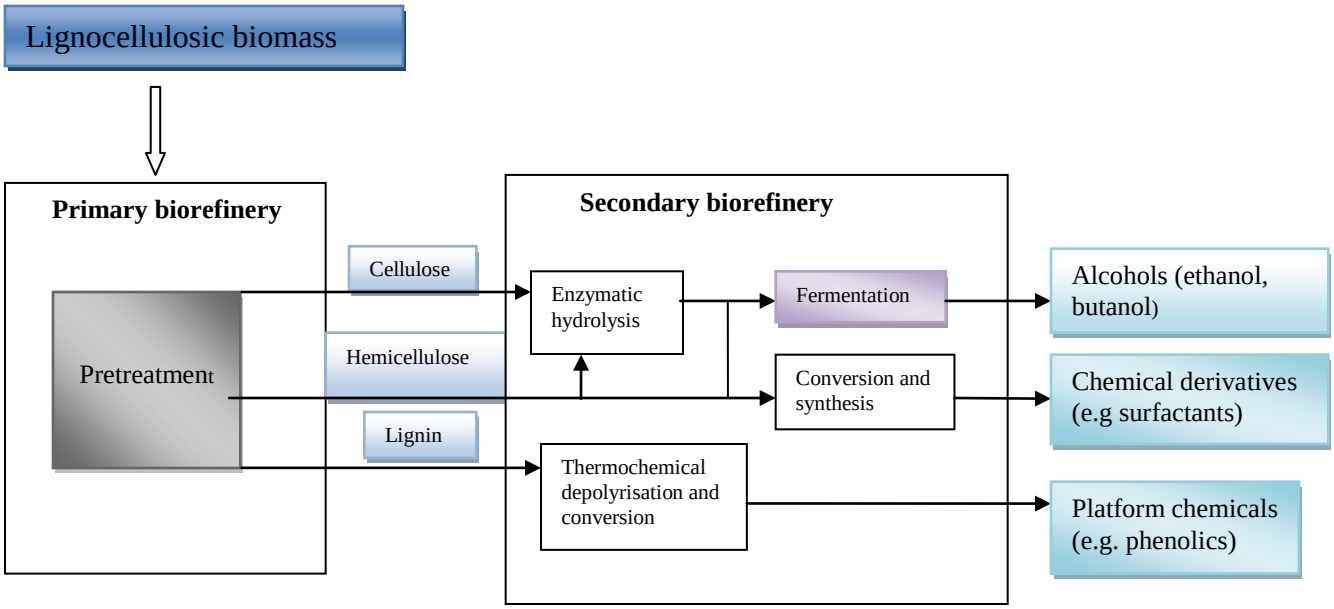


Figure 1.10: Lignocellulosic biomass pretreatment for conversion into biofuels and other chemicals (Adapted from Lee, 1997)

The breakdown of cellulose results in the formation of glucose molecules which are easily used by microbes (Ladisch and Svarczkopf, 1991). Hemicellulose is broken down to yield mostly xylose monomers and a number of other monomers such as, mannose, arabinose and galactose (Ladisch and Svarczkopf, 1991; Gray et al., 2006). Lignin is linked to carbohydrate polymers, cellulose and hemicellulose, resulting in a more complex structure hence recalcitrant compound (Lee, 1997). In addition to this, In addition to this, it is crystalline and has more inaccessible glucose-glucose bonds (Lee, 1997). Owing to these, lignocellulose biomass is more resistant to degradation compared to starch (Gray et al., 2006).

1.7 Sweet potato as a biomass source

As Richter and Berhold (1998) and Antonopoulou et al. (2008) stated, bio-conversion of renewable resources into useful chemicals is of great interest in scientific research. This can be

attributed to their abundance and the fact that they do not increase CO_2 levels into the atmosphere (Richter and Berhold, 1998; Chew and Bhatia, 2008; Ioannidou et al., 2009).

Conversion of these residues and crops results in production of key chemicals such as ethanol, acetone, butanol and isopropanol, carboxylic acids, butanediols as well as gases such as H_2 and methane (Linko, 1985; Kruger and Grossklaus, 1991; Richter and Berhold 1998; Wronkowska et al., 2006; Antonopoulou et al., 2008; Castellarnau et al., 2008). Some of these products may be used as supplements or alternatives to fossil fuels which are currently running low (Richter and Berhold 1998; Chew and Bhatia, 2008).

In cases where crops are used as biomass, it is very important to use a crop that is suitable for production of useful products (Biopact, 2007). The crop of choice should not be an essential food crop but easy to grow and harvest (Mays et al., 1990; Biopact, 2007). Sweet potato is a resilient, fast and easily growing crop. It is resistant to unfavourable environmental conditions such as infertile soils, drought, pests and diseases (Khan and Doty, 2009). It has been reported to withstand and absorb heavy metals such as lead and as such, has been used for absorption and degradation of mixed pollutants (Khan and Doty, 2009). These characteristics make sweet potato a crop of choice, above all, sweet potato is of great potential for use in production of useful industrial chemicals because of its high starch content (Dangler et al., 1984; Yokoi et al., 2001; Biopact, 2007).

In developing countries, sweet potato is the fifth most important crop (Horton, 1988; Aregheore, 2007). It is grown mainly for production of tubers and fodder and the latter is considered a waste (Ruiz et al., 1981, Aregheore, 2004; Larbi et al., 2007). According to the International Center

for Potatoes (CIP), the annual worldwide production of sweet potatoes is 133 million metric tons which is produced in over 100 developing countries.

CIP states that sweet potato major production regions are Indonesia, Vietnam, the Philippines and China, with China accounting for about 85% of the total yield. The remaining 15% is produced by the rest of Asia (6%), Latin America (1.5%), United States (0.45%) and Africa (5%). Uganda produces the largest annual yield of about 1.7 million metric tons in Africa. Only half of the sweet potatoes produced are used as food, 36% as animal feed and the rest is used for processing or lost as waste. It is this remaining percentage of sweet potatoes that can be used for the production of fuels and useful chemicals.

Sweet potato contains moisture, ash and most importantly the basic chemical building blocks - carbohydrates (Hosein and Mellowes, 1989; Daniels-Zeller, 1999; Yokoi et al., 2001). As illustrated by Hosein and Mellowes (1989), the carbohydrate content comprises of about 20% starch and 4% sugar (Table 1.5). In addition to this, Sweet potato carbohydrate has been reported to contain pectin substances, lignin, cellulose and hemicellulose which are all converted to simple sugars and fermented (Yokoi et al., 2001).

Table 1.5: Chemical composition of sweet-potato (Hosein and Mellowes, 1989)

Component	%
Starch	20-22
Ash	1.2-1.25
Nitrogen	0.19-0.23
Moisture	68-70
Sugar	3.5-4.0

As stated earlier, not all bacteria can readily use starch as their energy and carbon source (Nigam and Singh, 1995). This means that some starches need to be broken down to simple fermentable sugars so they can be utilized by bacteria (Nigam and Singh, 1995). On the other hand, sweet potato is rich in β-amylase which converts long chained starch into readily used maltose units making it a good energy and carbon source for bacteria (Yoshida et al., 1992; Brena et al., 1993; Cudney and McPherson, 1993; Nigam and Singh, 1995).

In addition to sweet potato having higher starch content compared to other carbohydrate crops such as corn, it has high yield capacity making it a potential biomass resource for producing bio-based industrial chemicals (Dangler et al., 1984; Mays et al., 1990; Biopact 2007). This was illustrated by Mays et al. (1990), who investigated alcohol production from sweet potato, sweet sorghum, sugar beet, fodder beet and potato. Their results revealed that sweet potato yielded the highest alcohol per hectare when compared with other crops.

Sweet potato as a starch and sugar crop can be used to produce liquid fuel mainly ethanol as well as other chemicals by fermentation (Nigam and Singh, 1995; Antonopoulou et al., 2008). The

starch found in sweet potatoes can also be used in the production of acetone, butanol, brewing materials and most importantly lactic acid which can be used as a key substrate in the production of other chemicals (Richter and Berhold, 1998).

Fermentation of sweet potato may also result in the production of ammonium, ethanol, H_2 gas and organic acids such as acetic, lactic, butyric acid (Yokoi et al., 2001). In a study by Yokoi and colleagues (2001), where sweet potato starch residue was used for H_2 production, it was found that H_2 yield increased with increasing starch residue concentration indicating the importance of substrate concentration on yield.

In starch and sugar fermentation, pH of medium is an important factor as this affects the type of chemicals produced (Kapdan and Kargi, 2006). Butyric acid is produced mainly between pH 4.0-6.0 while acetate and butyrate are favoured between pH 6.5-7.0 (Fang and Liu, 2002; Kapdan and Kargi, 2006). On the other hand, ethanol is produced at a different pH range depending on conditions under which H_2 is produced (Ueno et al., 2001; Zhang et al., 2003). Hence ethanol is produced concurrently with H_2 production (Wu et al., 2003).

Economic analysis of chemical production, in particular fuel (ethanol), has been investigated on corn starch in FBBR (Harshbarger et al., 1995; Taylor et al., 2000, Krishnan et al., 1999). The three studies reported savings of 6 cents/gal, 3 cents/gal and 3.12 cents/gal respectively. Since corn is more expensive than sweet potatoes, it would be speculated that even more cents would be saved when sweet potatoes are used instead.

Very little work has been carried out on use of sweet potato on production of biofuels and bio-based chemicals. A few studies that have been done, only focus on the production of fuels such as H_2 and ethanol but not on any other industrial chemicals (Hosein and Mellowes, 1989;

Yu et al., 1996; Yokoi et al., 2001; Biopact, 2007). In addition to this, most studies that use sweet potato only use sweet potato starch and sugars that have been extracted and purified rather than the whole tuber (Yu et al., 1996; Yokoi et al., 2001). To date, no work has carried out on raw sweet potato tuber to determine the range of chemicals produced in fermentation process. This however, is the focus of this study.

1.8. Hypothesis

Sweet potato tuber contains fermentable sugars in a form of starch, cellulose, hemicellulose as well as glucose. During fermentation of the tuber, these sugars are converted into organic acids, alcohols and gases. The alcohols produced are ethanol and butanol which can be used as renewable energy (biofuels). The organic acids produced include acetic acid and butyric acid which are of industrial importance.

1.9. Objectives and motivation

The aim of this research is to use a consortium of bacteria isolated from sweet potato tuber to break down carbohydrates to simple sugars for production of useful biofuels and bio-based chemicals in FBBR.

Using a bacterial consortium in fermentation can be problematic when it comes to detectection or control of contamination. Product recovery could also be difficult when mixed cultures are used. On the other hand, mixed cultures may result in increased growth rate and productivity. In addition to these, bacterial consortium results in a wider spectrum of products compared to pure cultures. Since the main aim of this research was to produce a wider range of products from sweet potato tuber, a bacterial consortium was used.

Despite the fact that sweet potatoes have been reported as potential candidate for industrial applications, very little work has been done in converting sweet potato biomass into value-added chemicals. This can be attributed to the fact that potatoes are a food source and their conversion into bio-products (biofuels and chemicals) is still very much a contentious issue, particularly in developing countries where there is a high rate of poverty and malnutrition. However, as stated by Annadana (2007), people do not usually go hungry due to food scarcity but inequality. Since the primary cause of hunger is poverty, it is obvious that increased production of biofuels can raise the incomes of small-scale farmers and rural labourers in developing countries which may in turn improve food security.

However, it should be taken into consideration that it is proposed that only surplus sweet potato tuber, not for food use, are converted to useful chemicals. This would be motivated by the knowledge that most small farmers, who produce sweet potatoes, after selling and separating some sweet potato for seeds and animal feeding, still have a lot of surplus that eventually is wasted. It should also be noted that, ideally, sweet potatoes that are cultivated for other purposes other than food should not be planted on land that is used for food.

FBBR provides high surface area to bacterial cells resulting in high microbial activity (Heijnen et al., 1989). This study focused on sweet potato fermentation in an FBBR with the aim of producing biofuels and bio-based chemicals. The use of sweet potatoes will serve as an alternative to the commonly used corn for production of biofuels and therefore ease pressure on using staple crops.

Chapter 2

Isolation and identification of bacteria associated with sweet potatoes

2. Introduction

Bacteria are capable of living in these diverse habitats mainly because of their versatile metabolic capabilities (Postma et al., 1993). They easily adapt to forever changing conditions as part of their survival strategy (Postma et al., 1993; Huang and Tang, 2007). Bacteria are single celled microscopic organisms that are between 0.5 - 3 µm in size and have different shapes such as cocci and bacilli (Huang and Tang, 2007).

2.1 Isolation methods

Bacterial isolation involves serial dilution methods and plate counting. These methods have shown to be efficient and are still very popular (Janssen et al., 2002). They are inexpensive, fast and accurate in giving information on active population (Kirk et al., 2004). Disadvantages include colony spreading, colony inhibition, inability to culture a huge number of species and that only species that have fast growth rates are favoured (Trevors, 1998; Kirk et al., 2004).

2.2 Identification and classification

Identification and classification are based on phenotypic methods (e.g. Gram stain method, colony morphologies, growth requirements, and enzymatic activities) and manual methods which include the use of Microbiologists' standard reference such as *Bergey's Manual of Systematic Bacteriology* or the *Manual of Clinical Microbiology* (Clarridge III, 2004; Petti et al., 2005). Generally, the most common classical methods include cultural (colony morphology), morphological (cell size), physiological (temperature range), biochemical (carbon source utilization), genotypic (DNA based ratio) and phylogenetic (DNA-DNA hybridization) methods (Table 2.1) (Busse et al., 1996).

Table 2.1: Methods used in bacterial identification and classification (Adapted from Busse et al., 1996)

Categories	Examples
Cultural	Colony morphology, colour of colonies, Fruiting bodies, Mycelia
Morphological	Cell morphology, cell size, motility, Gram stain, acid-fast stain
Physiological	Temperature range, pH range, salinity tolerance
Biochemical	Carbon source utilization, enzyme profile, carbohydrate fermentation
Inhibitory test	Selective media, antibiotics, dyes
Serological	Agglutination, immundiffusion
Chemotaxonomic	Fatty acids, polar lipids, mycolic acids.
Genotypic	DNA base ratio, random amplified polymorphic DNA
Phylogenetic	DNA-DNA hybridization, 16S rRNA sequence

2.2.1 Phenotypic -based methods

Phenotypic methods include cellular fatty acid profiles, carbon source utilization systems, colony morphologies and staining behaviour (Bosshard et al., 2004; Petti et al., 2005). However, these traits can change under harsh conditions such as stress, making phenotypic profile method inefficient, inaccurate and unreliable. In addition to this, the method can be tedious and time consuming (Clarridge III, 2004; Petti et al., 2005; Mignard and Flandrois, 2006).

2.2.2 Genotypic method

Genotypic profile method is based on the application of molecular techniques and involves DNA or RNA extraction (Badiane Ndour et al., 2008). It allows for identification of bacteria based on conserved sequences (Tang et al., 1998) i.e. stable parts of the genetic code such as 16S ribosomal RNA (rRNA) gene sequences (Bosshard et al., 2004; Clarridge III, 2004). This is considered a 'gold standard' method for prokaryote identification and classification (Schleifer, 2009). As a result, molecular methods are still considered the best methods for identifying bacteria at the species level and are therefore used as an alternative to other mentioned methods (Tang et al., 1998; Bosshard et al., 2004; Mignard and Flandrois, 2006). This is mainly because they are more reliable, objective and accurate compared to other methods (Petti et al., 2005).

a) 16S rRNA sequence

The 16S rRNA gene sequence is a component of the 30S subunit (Smaller subunit of the 70S ribosome) of prokaryotic ribosomes consisting of 1550 base pairs (bp). This sequence has both conserved and variable regions. The conserved region was discovered in the 1960s by Dubnau et al. (1965) in *Bacillus* spp. Universal primers are usually used as complementary to this region. The 16S rRNA gene is the most commonly used part of the DNA for bacteria identification and comparison purposes (Clarridge III, 2004). Some scientists refer to this gene as 16S rDNA; however, the approved name is 16S rRNA. It is the most popular gene sequence used in identifying organisms because it behaves like a molecular chronometer (Woese, 1987; Stackebrandt et al., 2002; Selvakumaran et al., 2008). This conserved region is found in all bacteria as such it allows for comparisons and differentiation among bacteria (Woese et al., 1985).

During sequencing, it is crucial to consider whether the entire gene (1550 bp) should be sequenced or just the first 500 bp. The entire sequence analysis is desirable for description of new species while short sequence analysis still provides adequate differentiation and higher percentage difference between strains. This is because of the ability of the region to show more diversity per base pair sequenced.

The 16S rRNA sequencing has become a useful tool in the studying of phylogenetic relationships between microorganisms and in identifying taxanomic position of the unknown isolate (Selvakumaran et al., 2008; Schleifer, 2009). Such relationships and taxanomic positions are normally illustrated in a phylogenetic tree (Steel et al., 2009). This tree describes how species have evolved from a common ancestor or how the evolutionary relationships among a group of organisms came about during evolution (Du et al., 2005). It is mostly used in biological systems for function prediction and drug design (Bull and Wichman, 2001). Most common sources of phylogenetic tree construction software include PAUP* version 4.0b10 (Swofford, 2002; Du et al., 2005). The 16S rRNA sequencing has a lot of advantages (Table 2.2) (Selvakumaran et al., 2008; Schleifer, 2009).

Table 2.2: Advantages and disadvantages of 16S rRNA sequencing (Selvakumaran et al., 2008; Schleifer, 2009)

Advantages	Disadvantages
Molecular makers properties (Non changing-conserved) are fulfilled by rRNAs	The resolution at the species level is often not sufficient, since the gene is too conserved.
rRNAs are very stable markers therefore less susceptible to lateral gene transfer	Multiple 16S rRNA genes exist
Good congruence for branching pattern of phylogenetic trees derived from conserved genes	At the phylum level, it is often difficult to organize relative branching orders
Genome-based studies are in good agreement with the rRNA data	
Facilitates identification of uncultivated prokaryotes	

2.3. Objectives

Sweet potato is a fast and easily growing crop that is capable of absorbing heavy metals. It has potential in industrial applications such as bioremediation. These attractive characteristics have been attributed to microorganisms associated with this crop (Asis Jr et al., 2005; Khan and Doty, 2009). As a result, it is important to isolate and identify bacteria associated with sweet potato so as to discover new industrial applications for this crop and optimize existing ones.

The aim of this study was therefore to isolate and identify bacteria associated with sweet potato tuber. Isolated bacteria were identified based on the 16S rRNA genes. The genes were compared to those in the NCBI Genbank in order to identify each isolate.

2.4 Materials and methods

2.4.1 Bacterial strains

Bacteria were isolated from 1 g of unpeeled sweet potato tuber. The weighed piece of sweet potato was washed in tap water for 1 minute. It was then rinsed four times with sterile distilled water. After this it was cut into small pieces of about 2 mm diameter using a sterile scalpel. All these were done in a laminar air flow hood to eliminate contamination.

The cut pieces were placed in a sterile 50 ml conical flask containing sterile distilled water and incubated for four days at 30 °C with shaking at 100 rpm. Sterile distilled water was used and not a specific medium. This was to ensure that only naturally occurring bacteria associated with sweet potato tuber grew because specific media sometimes allow for growth of contaminants. Resulting bacteria (50 µl) were sub-cultured into 50 ml nutrient broth (NB) (Biolab, Wadeville, Gauteng, RSA) and incubated overnight at 30 °C with shaking at 100 rpm. From the resulting culture, 1 ml aliquot was serially diluted to 10^{-3} and plated on nutrient agar (NA) (Biolab, Wadeville, Gauteng, RSA). Plating was carried out in dulicates and incubated overnight at 30 °C. Single individual colonies were selected based on differences in colony colour and shape. Individual colonies were plated on separate agar plates and stored on Tryptone Soya Agar (TSA) (Oxoid) plates at 4 °C for subsequent characterization and identification.

Bacterial identification

a) DNA extraction

DNA was extracted from each colony using a modified boiling method described by Scarpellini et al. (2004). Each colony from TSA plates was boiled for 20 minutes in 60 µl mixture comprising of 40 µl sterile distilled and 20 µl chloroform. The resulting mixture was then centrifuged at 12000 rpm for 5 minutes. The supernatant was collected and used as a DNA template for PCR (Table 2.3).

Table 2.3: Reagents and volumes used in preparation of the PCR master mix

PCR Mix	Volume (µl)
PCR master mix 2X (Fermentas Canada)	25
Forward primer (10 µM)	2.5
Reverse primer (10 µM)	2.5
DNA template (500 ng/ml)	2.5
Nuclease free water	17.5
Total	50

b) 16S rRNA amplification and sequencing

16S rRNA was amplified using forward primer 5'-AGA GTTTGATCCTG GCT CAG-3' and reverse primer 5'-GCT ACC TTG TTA CGA CTT-3'. DNA and reagents were prepared according to manufacturers instructions (Table 2.3) (Fermentas Life Sciences, www.fermentas.com). PCR amplification was performed using the following conditions: initial denaturation of template DNA at 94 °C for 3 minutes; 35 cycles consisting of denaturation

(94 °C, 30 seconds), annealing (60 °C, 45 seconds), extension (1 minute 30 seconds, 72 °C), and a final extension at 72 °C for 7 minutes. The PCR products were sent to Inqaba biotech (Pretoria South Africa) for sequencing. The resulting sequence data were compared with the 16S rRNA sequence database via the National Centre for Biotechnology (NCBI) site (http://www.ncbi.nlm.nih gov) (Altschul et al., 1990). All the sequences were then submitted to NCBI GenBank using BankIt submission tool to obtain accession numbers. Unique names or laboratory designations were given to each isolate to distinguish the sequence submissions.

c) Phylogenetic analysis

Phylogenetic analysis was done in order to determine relationships between identified isolates hence predict their functions and industrial applications (Bull and Wichman, 2001). Parsimony analysis of the nuclear DNA data set was performed using PAUP* version 4.0b10 (Swofford, 2002). The phylogenetic tree was rooted using an outgroup *E. coli* strain ATCC 25922 (DQ360844), which is a sister genus to *Enterobacter* family and *Bacillus clausii*. A heuristic search comprising 10 random repetitions holding one phylogenetic tree at each step was performed. The maximum number of phylogenetic trees was set at 100. Swopping on best trees was used with Tree Bisection Reconnection (TBR) branch swopping, saving multiple trees.

2.5 Results

2.5.1 Colonies isolation and DNA sequence analysis

Eight different isolates exhibiting different colours and shapes were selected and designated as isolate 1, isolate 2, isolate DY4, isolate 1A, isolate 1B, isolate 2A, isolate 2B and isolate 2C. This was done in order to differentiate between all the obtained sequences. The sequences were submitted to NCBI GenBank using BankIt submission tool to obtain accession numbers (Table

2.4). Observed colonies of different shapes (round and irregular) and colour (white, gray, pale to bright yellow) are shown in Fig 2.1. DNA was extracted after which electrophoresis was run on 1% agarose gel to confirm the presence of PCR products (Fig 2.2).

Table 2.4: Colony colours of isolates from sweet potato tuber and allocated accession numbers

Isolate's Unique name	Accession No.	Colony Shape and colour on NA plates
Isolate 1	JF920410	Round, white
Isolate 2	JF920411	Irregular, white
Isolate DY4	JF920417	Round, yellow
Isolate 1A	JF920412	Round, cream white
Isolate 1B	JF920413	Irregular, yellow
Isolate 2A	JF920414	Irregular, cream white
Isolate 2B	JF920415	Round, pale yellow
Isolate 2C	JF920416	Irregular pale yellow

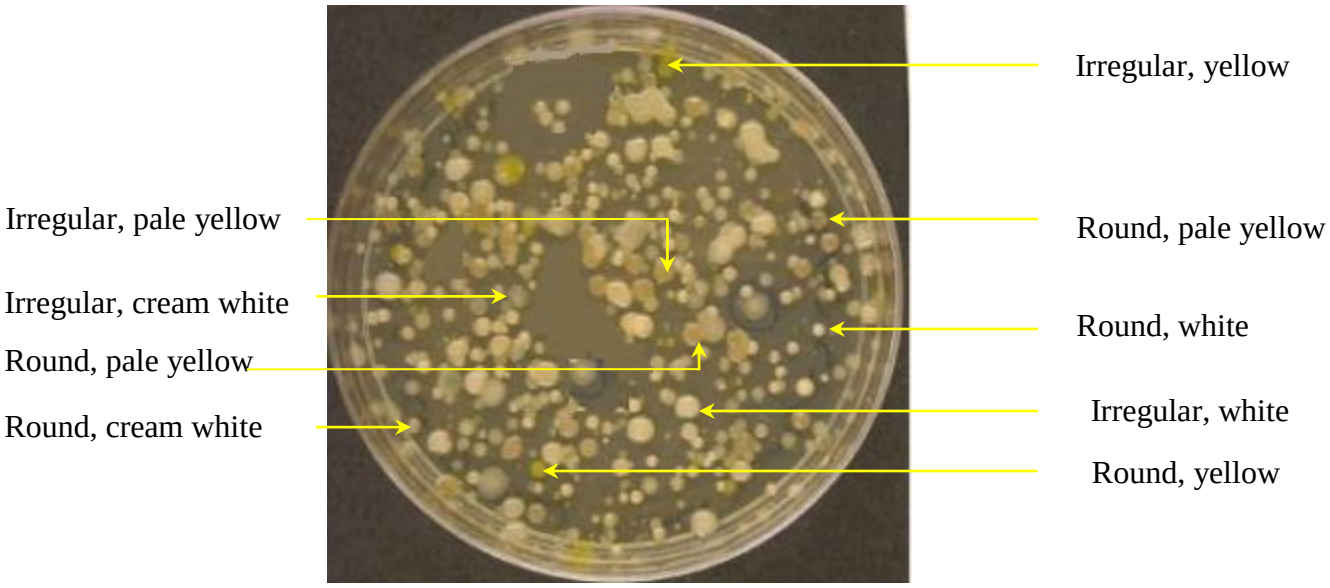


Figure 2.1: Different colours and shapes of isolates from sweet potato tuber plated on NA.

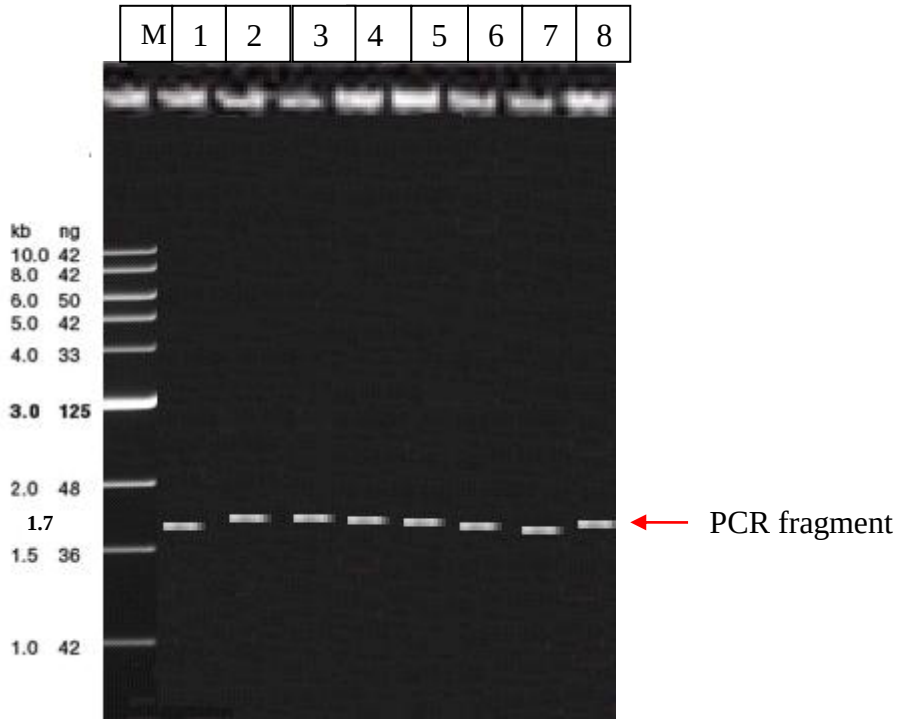


Figure 2.2: Agarose gel (1%) electrophoresis of PCR products of the eight isolates. Lane M contained 7µl of the 1kb DNA ladder marker. In lanes 1- 9 5µl isolates - isolate 1, Isolate 2, isolate DY4, isolate 1A, isolate 1B, isolate 2A, isolate 2B and isolate 2C were loaded respectively. The PCR yielded fragments of about 1.7 kb.

2.5.2 Phylogenetic tree analysis

Eight bacterial isolates were analyzed by comparative sequence analysis of the 16S rRNA. NCBI results revealed that three isolates (Isolate 1, isolate 2 and Isolate DY4) clustered within the *Enterobacterceae* family, a class of facultative anaerobes used in production of acetic acid (Fig 2.3).

Isolate 1 was closely related to an ethanol producer *Klebsiella Oxytoca* strain PYR-1 (GU253335) with a 100% identity. *Klebsiella* strains are normally found in plants. Isolate 2 strain clustered within the *Enterobacter* group and was genetically similar (100%) to *Enterobacter cloacae* (GU191924), a previously isolated biohydrogen producer strain. Isolate DY4 had a 99% genetic similarity to H_2 producing *Citrobacter amalonaticus* (GU185859) which was originally isolated from contaminated soil near Hangzhou (China) steel plant.

Isolates 1A, 1B, 2A, 2B and 2C were clustered with *Bacillus* species, a well known industrial enzyme producer, popular for production of enzymes such as β-amylase (Fig 2.4). The isolates also clustred with *Alcaligenes* and *Bordetella* species, both of which, are used in bioremediation studies (Schallmey et al., 2004; Wang et al., 2007).

Isolates 1A and 2C in particular, were found 100% genetically identical to *Bacillus clausii* strain D1 (HM560954). On the other hand, isolates 1A, 1B, 2A and 2B shared a 100% similarity to *Alcaligenes* sp (GU362711) while isolate 2C was 100% genetically similar to *Alcaligenes* sp. DF18SC (HQ163792). Isolate 1A was also found to be 100% similar to *Bordetella* sp. AC3 (EU043370). Isolates 1B and 2A were both 100% similar to *Beta proteobacterium* strain, a class known for H_2 gas production (Kersters et al., 2001).

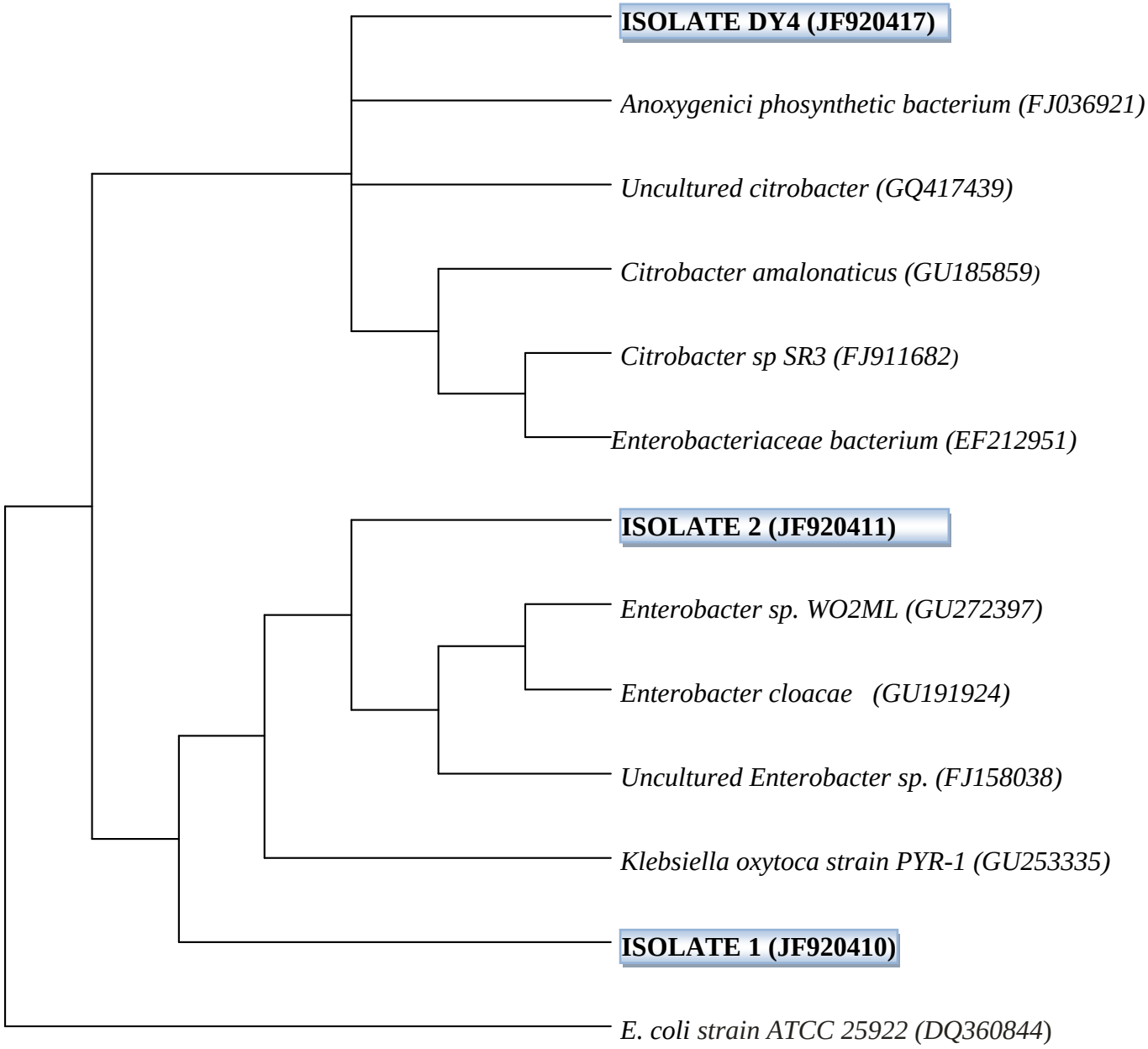


Figure 2.3: A phylogenetic tree showing relationships of isolate and related species from NCBI. Isolates from sweet potato tuber are shown in blue.The phylogenetic tree was rooted by outgroup (*E.coli*) and constructed using PAUP* version 4.0b10 (Swofford 2002) which was based on partial 16S rRNA sequences. GenBank accession numbers are shown in parentheses.

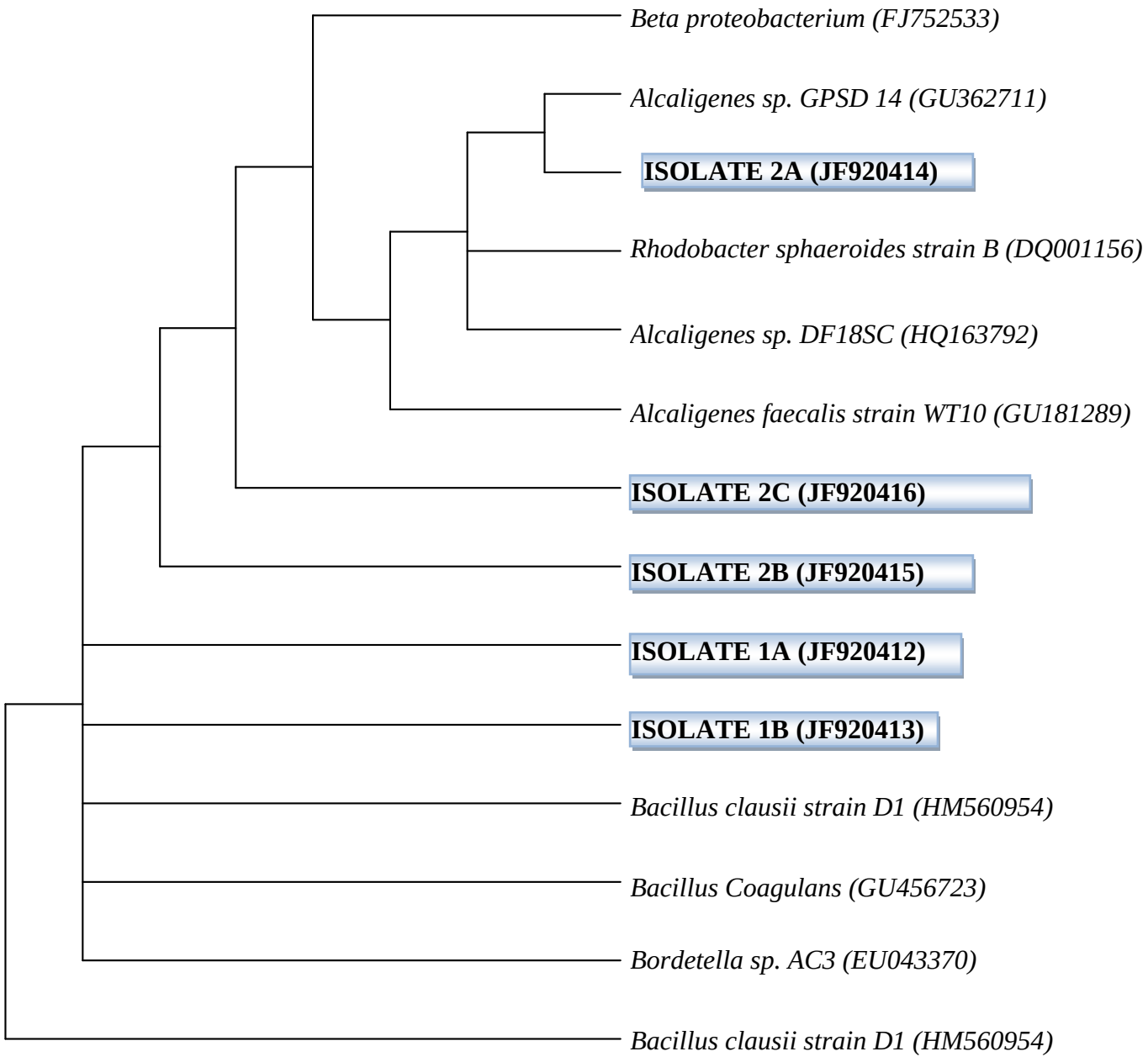


Figure 2.4: A phylogenetic tree showing relationships of isolate and related species from NCBI. Isolates from sweet potato tuber are shown in blue**.** The phylogenetic tree was rooted by outgroup (*Bacillus clausii*) and constructed using PAUP* version 4.0b10 (Swofford 2002) which was based on partial 16S rRNA sequences. GenBank accession numbers are shown in parentheses.

2.6 Discussion

Bacteria were isolated from sweet potato tuber. The isolates were adapted to sweet potato environments resulting in them acquiring new genetic information as would be expected of microorganisms isolated from specific environments (Leahy and Colwell, 1990). It is believed that the new genetic variation gives the bacteria the ability to break down tuber compounds to useful chemicals (Ma et al., 2006). This suggests that the isolates may use sweet potato carbohydrate as a carbon source. Bacteria can adapt to the environment by changing their genetic code to suit the new environment by receiving genes from their closely related or even distant relatives by Horizontal Gene Transfer (HGT) - a process of genetic material transfer from one cell to a different cell that is not its ancestor (de la Cruz and Davies, 2000).

Facultative and obligate anaerobes such as those belonging to *Escherichia*, *Citrobacter*, *Enterobacter* and *Bacillus* genera are capable of biohydrogen production (Kumar and Das, 2000; Kotay and Das, 2007; Redwood et al., 2008; Thompson et al., 2008; Khanna et al., 2011). Some of the identified isolates belonged to the *Enterobacteriaceae* family (*Enterobacter*, *Klebsiella* and *Citrobacter* species) - a group of bacteria normally found in soils, water, fruits, vegetables, grains, trees, crops and plants (Asis Jr et al., 2005; Khan and Doty, 2009; Le Bouguénec and Schouler, 2011). This was expected as sweet potato is a root plant.

Isolate 2 was 100% identical to *Enterobacter cloacae*, a Gram-Negative facultative anaerobe popular for H_2 production (Le Bouguénec and Schouler, 2011). This is mainly because unlike strict aerobes, facultative anaerobes are less sensitive to oxygen. Their decreased susceptibility to oxygen relate to their ability to absorb any oxygen present, thereby recovering the activity of Fe-hydrogenase (which is depleted if exposed to oxygen for longer periods) (Khan and Doty, 2009).

This results in high bacterial growth and H_2 production rates (Kumar and Das, 2000). *E.cloacae* has been identified in rice plants with the ability of nitrogen fixation (Khan and Doty, 2009).

Isolates 1 and 2 were 100% genetically homogeneous to *Klebsiella oxytoca*. *Klebsiella* species are Gram-Negative nitrogen fixing facultative anaerobes found in drinking water, soils, surface waters, industrial effluents, and vegetation (Chen et al., 2006). They use a wide variety of substrates as carbon source leading to production of various acids, gases and solvents (Chotani et al., 2000). However, due to inhibitory effects of these products, only limited concentrations can be produced.

Reportedly, under suitable conditions, *Klebsiella* species have been used for laboratory scale production of gas (H_2) and liquid (ethanol) biofuels as well as industrial bio-based chemicals such as 2,3-butanediol (2,3-BDO) (Harden and Walpole, 1906; Menzel et al., 1996; Wu et al., 2008). The most common species used is *Klebsiella oxytoca* (Jansen et al., 1984).

Generally *Klebsiella* species use pentose as their carbon source resulting in production of chemicals such as 2,3-butanediol, acetone, isopropanol, butanol, and H_2 as soluble or gaseous metabolites (Rosenberg, 1980). This species also break down glycerol to 2,3-BDO (Deckwer, 1995; Biebl *et al*., 1998). Champluvier et al. (1989) and Wu et al. (2008) also reported that *K. oxytoca* utilized lactose as carbon source for 2,3-BDO production using permeabilized-cell system. This therefore means that *Klebsiella* species are capable of bioconversion of a variety of biomass substrates to liquid fuels and chemical feedstocks (Cao et al., 1997; Wu et al., 2008).

Facultative anaerobes such as the genera *Enterobacter*, *Klebsiella* and *Bacillus* utilize $NADH_2$ as a reducing agent for the production of reduced metabolites such as 2,3-BDO, ethanol and lactate

from pyruvate (3-carbon intermediate) via the EMP glycolytic pathway, but not for H_2 production (Chotani et al., 2000; Nakashimada et al., 2002; Chen et al., 2006). However, facultative bacteria that are nitrogen fixing such as *K.oxytoca* are capable of producing H_2 at significantly high quantities. H_2 production by these bacteria is mainly with the activity of nitrogenase (Vignais et al., 2001). Nitrogenase cannot only reduce N_2 to NH_3, but also catalyze H_2 production in the absence of molecular nitrogen (Das and Veziroglu, 2001). H_2 production by nitrogenase uses a large amount of ATP as shown in Equation 2.1.

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4Pi \quad \text{(2.1)}$$

In the nitrogen-fixing bacteria, $NADH_2$ acts as an electron and proton donor (Equation 2.2) (Koku et al., 2002; Chen et al., 2006).

$$NADH_2 \rightarrow NAD^+ + 2H^+ + 2e^- \quad \text{(2.2)}$$

Combining Equations 2.1 and 2.2 produces an overall Equation 2.3 where nitrogen fixing bacteria produces H_2.

$$NADH_2 + 4ATP \rightarrow H_2 + NAD^+ + 4ADP + 4Pi \quad \text{(2.3)}$$

The diversity of fermentation products is determined mainly by the intracellular redox state. The state is controlled by nicotinamide adenine dinucleotide coenzymes (NADH and NADPH) as

their roles in many anabolic and catabolic reactions have a wide range of uses in biological systems (Foster and Moata, 1980).

Isolates similar to *Citrobacter* species which are facultative anaerobes that are normally found in soil, water and food were identified. These bacteria are used mainly for H_2 production (Oh et al., 2003). Maximum yields of up to 2.49 mol H_2/mol glucose have been reported for *Citrobacter* sp. Y19 (Oh et al., 2003). Other industrial applications of *Citrobacter* sp. include metal remediation, dye decolourization and flocculant production (Oh et al., 2011).

Isolates 1A, 1B, 2A, 2B and 2C were clustered with *Bacillus* and *Alcaligenes* species (Fig 2.3). *Bacillus coagulans* is a Gram-Positive facultative anaerobe common in acidic food spoilage (Kotay and Das, 2007; Karadag and Puhakka, 2010). Its close relatives are ideal biocatalysts for fermentation of lignocellulosic biomass to fuels and bio-based chemicals. It is known to be a lactic acid producer (Karadag and Puhakka, 2010). This group of bacteria is quite flexible in its carbon source and energy source and so grow well in minimum mineral. This was evident in a study done by Das and Kotay (2007) where these bacterial species broke down all the 9 substrates (glucose, maltose, sucrose, D-xylose, lactose, starch, galactose, mannose and glycerol) into H_2 gas with sucrose break down yielding most H_2. This results highlight on the potential of this species in H_2 production. However, very brief study has been reported on *Bacillus* sp. for H_2 fermentation.

Alcaligenes faecalis is important because it converts the most pathogenic compounds of arsenic, arsenite, to its less dangerous form, arsenate. *Alcaligenes* species have been used for production of polyhydroxyalkanoates (PHA). Isolates of this genus are found in soil or water (Chen, 2010). *Bordetella* sp. has been used for biodegradation and bioremediation studies. Isolate 2A had a

100% identity to *Beta proteobacterium* strain. The *Beta proteobacterium* belongs to proteobacteria genus which is normally used for biohydrogen production and is found in plants such as strawberry (Kersters et al., 2001; Khan and Doty, 2009).

All isolates identified were facultative with the exception of *Alcaligenes* sp (aerobic). Facultative environments were favoured for the reasons mentioned earlier.

2.7 Conclusion

In this study, potential H_2, ethanol and lactic acid producing microorganisms were successfully isolated from sweet potatoes. These isolates belonged to a diverse array of bacterial species including *Klebsiella*, *Enterobacter*, *Rhodobacter*, *Bacillus*, *Citrobacter*, *Alcaligenes* and *Bordetella*. These strains have the potential to be further used in biofuel and bio-based chemical production as well as biodegradation studies as they are closely related to species that have been used for such studies.

Chapter 3

Fermentation of sweet potato under batch operation

3. Introduction

3.1 Batch systems

Batch operation represents a closed system in which all reactants are put into a reaction vessel, mixed together and left to react for a certain period with no addition of substrate or removal of products (Minihane and Brown, 1986; Johnson, 1987; Aziz and Mujtaba, 2002).

In general, Batch configurations are used for small scale experiments that have not been fully developed, production of very expensive products, processes that cannot be run under continuous mode of operation or chemical processes that involve multiple steps (Aziz and Mujtaba, 2002). These systems are also used in cases where there is low production volume or where isolation is required for reasons of safety or sterility (Srinivasan et al., 2003). In addition to these, batch systems are useful in production of seasonal products and products that have short life span. Moreover, they are used in production of chemicals whose reactions are so slow that continuous production would be impractical as well as for materials that taint equipment quickly (Goršek and Glavič, 1997).

Batch production is performed in flexible equipment that can easily be adapted for production of diverse products (Aziz and Mujtaba, 2002). As a result of their flexibility, batch configurations are popular in many industries that produce specialty chemicals and fine chemicals such as pesticides/biocides, pharmaceuticals, food products, antibiotics and polymers (Minihane and Brown, 1986; Aziz and Mujtaba, 2002; Ramaker et al., 2002; Srinivasan et al., 2003; Cavin et al., 2005). Processes for production of such chemicals usually involve long complex reactions and continuous operation is therefore not ideal (Goršek and Glavič, 1997).

Products manufactured under batch systems normally have significantly different characteristics in terms of viscosity, enthalpy and conversion. As such different parameters may be critical depending on the product. For example, heat removal could be vey important where safety is an issue (Friedrich and Perne, 2003). Batch modes are known for their duration of operation and high conversions (Ramaker et al., 2002).

Batch systems are not only favoured by the nature of the product to be manufactured but also by the way the end- product is to be delivered to the customer (Rippin, 1993). It has been shown that customer specification in terms of delivery and quality in most cases can only be achieved and met under batch operation (Rippin, 1993).

In batch systems, high cell concentrations are achieved by supplying a high concentration of nutrients. Most batch processes are economically efficient under such high cell concentrations. Nonetheless, the high cell concentrations may not be feasible due to substrate inhibition (Minihane and Brown, 1986).

3.2 Objectives

Reportedly, unstable conditions such as exposure time usually experienced in other modes of operation can easily be controlled under batch operation resulting in high bacterial activity (Mohan et al., 2007). Batch cultures result in constant circulation of biomass leading to increased resistance to substrate shock (Kaballo et al., 1995).

Reports show that sweet potato is a fast growing crop with high starch content capable of tolerating harsh conditions. It has characteristics that make it very superior and more attractive compared to other crops.

The aim of this study was therefore to use sweet potato tuber to produce a wide spectrum of biofuels and bio-based industrial chemicals under batch system. Eventhough a mixed culture is likely to be less reproducible and is disadvantaged when it comes to product recovery compared to a pure culture, reports indicate that mixed bacterial culture has higher growth rate and productivity. Mixed cultures are also known to produce a wider spectrum of products. In light with these disadvantages, a mixed culture was used as it would enable formation of a wider range of products.

3.3 Materials and methods

3.3.1 Bacterial preparation

A mixed culture of bacterial strains isolated and identified from a sweet potato tuber as described in chapter 2, was used in the fermentation of sweet potato tuber. Prior to inoculation, the mid-log phase of the consortium was determined as described below.

3.3.2 Determination of bacterial consortium mid-log phase

The growth curve of the consortium was generated in order to determine the species mid-log phase where cell growth is linear. This was achieved by standardizing overnight culture of the consortium grown in 50 ml nutrient broth (NB) (Biolab, Wadeville, Gauteng, RSA). The overnight inoculum was diluted 10^{-2} from which 0.5 ml sample was transferred into 50 ml of NB and grown at 30^{o}C with shaking at 100 rpm overnight. Duplicate optical density (OD_{600nm}) readings were measured every 2 hours over a 16-hour period using cuvettes of a light path of 1 cm. The obtained OD_{600nm} readings were averaged and used to construct a growth curve by plotting the $OD_{600\ nm}$ readings against time.

3.3.3 Initial batch experiments to determine fermentation products

Preliminary batch experiments were carried out with the aim of identifying and determining products of sweet potato fermentation using identified bacterial isolates. In these experiments, products formed during sweet potato fermentation were only identified and not quantified.

a) Experimental set up

Experiments were performed in four separate fermentation bottles. The first bottle contained all fermentation reagents which included: bacteria mixed cultures, sweet potato tuber cut into small pieces (1 g) and the M9 minimal growth medium (64 g $Na_2HPO_4.7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5.0 g NH_4Cl combined with 0.5 ml of 1M $MgSO_4$ and 25 µl of 1M $CaCl_2$/l). The other three bottles were controls and did not have one or more of the reagents that were in the test experiment bottle as summarized in Table 3.1.

Table 3.1: Summary of contents of each fermentation bottle

Fermentation reagent	Bottle 1	Bottle 2 (C1)	Bottle 3 (C2)	Bottle 4 (C3)
M9 minimal medium	√	√	√	√
Bacteria mixed culture	√	-	√	-
Sweet potato tuber	√	√	-	-

The experiments were performed in a serum bottle containing a total volume of 100 ml (95 ml medium + 5 ml inoculum). Anaerobic conditions were maintained by sealing bottles with a rubber septa and a cap (Fig 3.1). The culture was grown at 30 ^oC with shaking at 100 rpm for 7 days. Product analysis was done after 7 days of incubation using gas chromatography (GC) as

described later in section 3.3.9. Gas samples were taken before liquid samples to avoid gas loss during sampling.

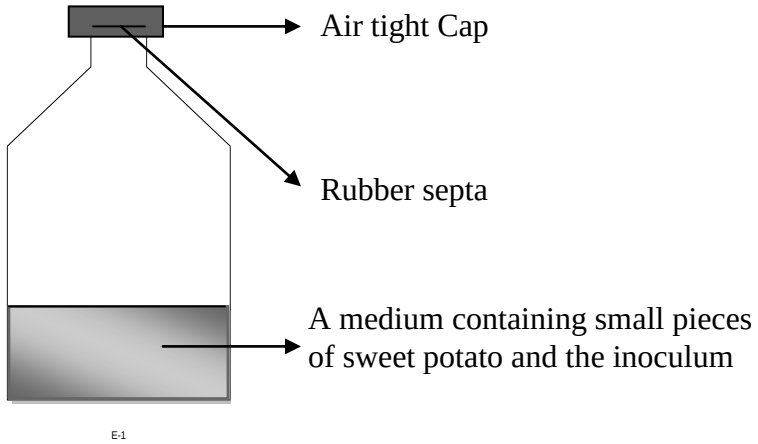


Figure 3.1: Batch fermentation experimental set-up.

3.3.5 Quantification of fermentation products using the M9 minimal growth medium

Based on the results obtained from initial batch experiments, a wide spectrum of products was obtained in the presence of all fermentation reagents (the inoculum, sweet potato and the M9 minimal growth medium). Therefore an experiment consisting of all the reagents was carried out in order to quantify all products.

A medium of 1140 ml was prepared and sterilized by autoclaving at 121 °C, 15 psig, for 20 minutes. An inoculum (60 ml) was added to the sterile medium giving a final volume of 1200 ml. The resulting mixture was equally distributed into 12 fermentation bottles with each bottle containing 100 ml of the mixture and 20 g of sweet potato tuber. Liquid and gas analysis was done every second day from each fermentation bottle over a 24 day period. Bacterial cell density was also determined as described in section 3.3.8. Fermentation was allowed to proceed without controlling the pH. pH was not controlled so as to determine how it varied during operation. For

reasons of product distribution comparison, an experiment was performed using GP medium in place of the M9 minimal growth medium.

3.3.6 Quantification of fermentation products using the GP medium

The GP medium was previously used in H_2 production from sweet potato starch (Yokoi et al. 2001). Based on this, a modified GP medium which consisted of 0.2 g/l polypepton, 0.02 g/l KH_2PO_4, 0.005 g/l yeast extract and 0.005 g/l $Mg_2SO_4.7H_2O$ and 20 g of sweet potato tuber cut into small pieces was used.

In this experiment, a series of 12 fermentation bottles (100 ml) was prepared as previously described. Liquids and gas samples were analyzed and bacterial cell density monitored from each bottle every second day over a 24 day period. pH was also measured but not controlled.

3.3.7 Quantification of fermentation products using the M9 minimal growth medium at pH 6.0

Based on the results obtained from the M9 minimal growth medium and the GP medium experiments, it was observed that the largest increase in production occurred at pH 6.0 in both experiments. However, relatively higher product concentrations were recorded in an experiment that used the M9 medium compared to the one that used the GP medium. An experiment was therefore carried out using the M9 medium at a constant pH of 6.0. pH 6.0 was maintained by adjusting at the beginning of the fermentation with the use of HCl.

This experiment was carried out in the same manner as above (Section 3.3.6) with each fermentation bottle containing a 100 ml (95 ml medium + 5 ml inoculum) solution and 20 g of sweet potato. Liquid and gas analysis was done every second day in each fermentation bottle over a 24 day period. All the media were sterilized by autoclaving at 121 °C, 15 psig, for 20

minutes. The gas and liquid products were quantified and cell density determined as described below.

3.3.8 Bacterial cell density determination

Bacterial cell growth was determined so as to reflect on bacterial metabolic activity (Kogure et al., 1979). Bacterial concentrations were determined by preparing serial dilutions of up to 10^{-5}. These were then plated onto Nutrient Agar (NA) plates using standard drop plate method. Standard drop method was used because it is faster and less laborious than the spread plate method (Herigstad et al., 2001). Plates were incubated at 30 ^{o}C overnight and growth was reported as colony-forming units per ml (Cfu/ml).

3.3.9 Analytical methods

a) Liquid analysis

Soluble metabolites were analyzed in order to identify and quantify each product. Prior to analysis, all liquid samples were filtered through a 0.45 µm membrane filter. The aqueous products (alcohols and volatile fatty acids) were analyzed using an Agilent 6820 gas chromatograph (Agilent Technologies, Supelco, 24107, USA) equipped with a thermal conductivity detector (TCD). The operational temperatures of the oven and the detector were 185°C and 155°C, respectively. The samples were separated on a fused silica capillary column (30 m × 0.25 mm, 0.25 µm film thickness) using helium as a carrier gas at a flow rate of 20 ml/0.01 mins. Liquid samples (1 ml) were collected from the fermentation bottle using a sterile syringe (10 ml) and needle. Filtered liquid sample (0.2 µl) was injected manually into the GC. Peak identification of products was based on retention times of reference standards. A

standard curve of area % which represents the concentration of each component and corresponding concentrations (%) was constructed and used to quantify products.

b) Gas analysis

Gas products were analyzed for purposes of identification and quantification. Gas samples (10 ml) were collected from the top of the fermentation bottle using an airtight gas syringe. The samples were then analyzed using a Dani GC (Dani 1000) with pora pack Q column equipped with a TCD detector. The oven temperature was at 200 °C. Argon was used as the carrier gas. Injection port temperature was kept at ambient while the detector temperature was 120 °C. Peak identification of products was based on retention times of reference standards. Gas samples (10 ml) were collected from the fermentation bottle using a gas syringe and analyzed. A standard curve of area % which represents the concentration of each component and corresponding concentrations (%) was constructed and used to quantify gas products.

c) Glucose concentration

Bacteria need to break down starch or other more complex carbohydrate into simple glucose molecules before utilizing it as carbon source. To determine how much glucose was produced from other complex carbohydrates such as starch and cellulose or how much was consumed during fermentation, glucose concentration was measured.

Glucose concentration was determined using high-performance liquid chromatography (HPLC). Refractive index detector (RID) was used as a detector while ultron PS-80H separation column was used for separation of all carbohydrates (Shinwa Kakoh Co., Ltd). The column was maintained at 40 °C and the flow rate of carrier liquid (0.1 wt% solution of perchloric acid, pH 2.1) was set at 1.0 ml/min. Components were eluted with 0.006 NH_2SO_4 from a cation-exchange

resin in the H_2 form. Eluted components were detected by means of a differential refractometer, plotted on a recorder and quantified using an electronic integrator. The area under the curve which represents the concentration of each component was reported as a percentage of the total area. The separations were made on a 1-foot HPX-87 column available from Bio-Rad Laboratories (California, USA).

3.3.10 Determination of dominating microbial populations

Microbial analysis was done every second day over a 24 day period of batch operation to check if there was any shift in microbial populations. To obtain colonies, serial dilutions (10^{-3}) were done. Only dominating colonies with the same morphology (size, colour and shape) were selected. DNA extraction, PCR and sequencing were performed as described in chapter 2.

3.3.11 Determination of the rate equation for sweet potato tuber batch fermentation

Sweet potato tuber rate equation was determined in order to calculate bacterial specific rate and productivity. Different concentrations of sweet potatoes from 1- 5 g/l were used in batch fermentation and cell concentration monitored over a 16-day period as that was the period when cell concentration started to decline. Cell density was recorded as colony-forming units per ml (cfu/ml) as described earlier in this chapter. Then plots of cell concentration versus time were constructed for each substrate concentration. The collected experimental data was then fit into Monod's Equation 3.1.

$$\mu = \mu_m \frac{S}{K_S + S} \quad \text{..... (3.1)}$$

Where

μ = Specific growth rate (h^{-1})
S = Substrate concentration (g/l)

K_S = Saturation constant (g/l)
μ_m = Maximum growth rate (d^{-1})

3.4 Results

3.4.1 Bacterial growth curves

During the log phase of bacterial growth, bacteria are known to be most active, hence easily adapt to new environments (Stanbury, 2000). A growth curve of the bacterial culture was constructed in order to determine the log phase period (exponential growth). The mid-log phase which is a stage where bacteria are most competent was achieved after four hours of initial growth (Fig 3.1). A two hour lag phase was observed with a stationary and death phase of eight hours and two hours respectively. The seed culture was allowed to reach mid-log phase before inoculating into fermentation bottles.

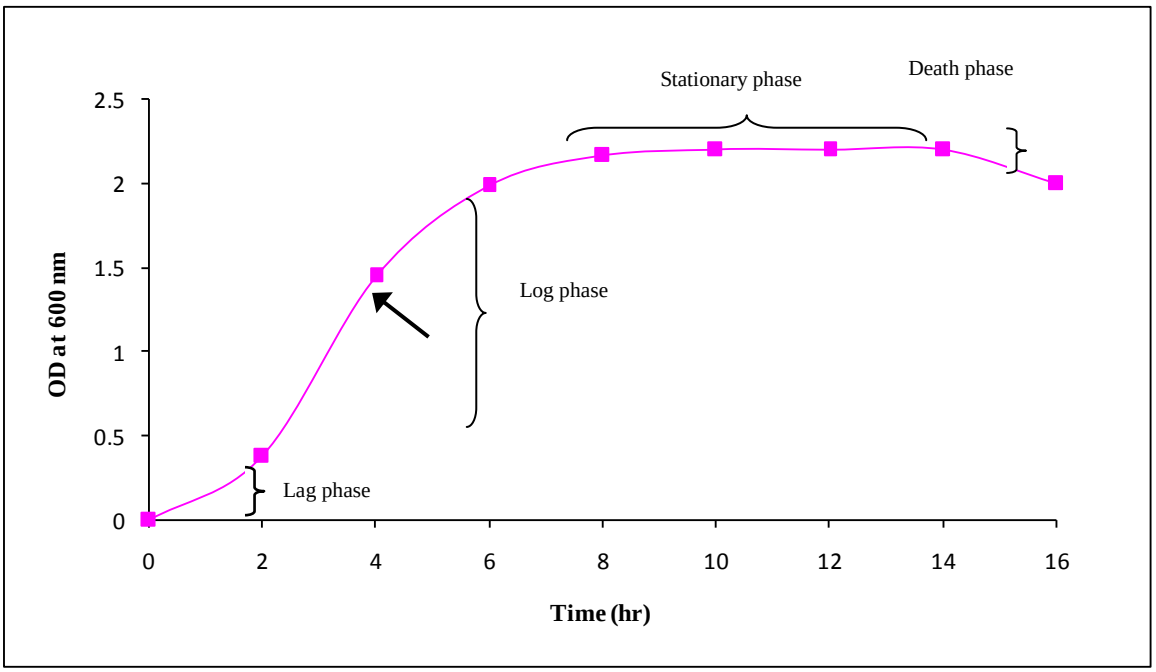


Figure 3.2: Growth curve of bacterial consortium isolated from sweet potatoes. The consortium was grown in NB; $OD_{600\ nm}$ was determined every two hours over a 16 hour period. Arrow indicates the stage of the growth at which the consortium was used as an inoculum.

3.4.2 Initial batch experiments to determine fermentation products

It was not known what fermentation products would be formed from sweet potato tuber fermentation using mixed cultures of the bacteria isolates. As a result, initial batch experiments were conducted to determine product spectrum. Controls C1, C2 and C3 were included in order to eliminate any false results. Liquid and gas samples were collected from the fermentation bottles, analyzed and identified.

a) Liquid analysis

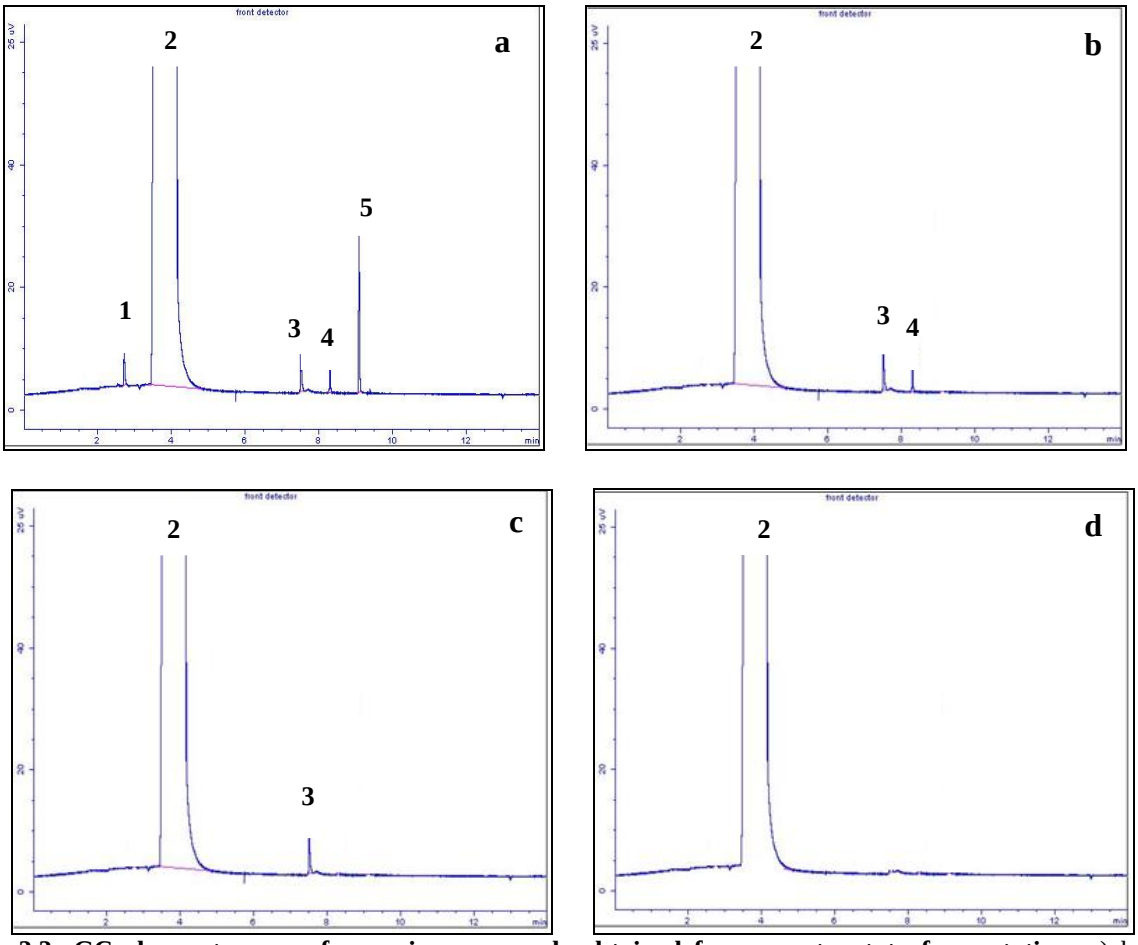


Figure 3.3: GC chromatogram of organic compounds obtained from sweet potato fermentation. a) batch experiment that had all fermentation reagents, b) C1 which was operated without the inoculum, c) C2 which contained no substrate (sweet potatoes) and d) C3 which was conducted using the M9 minimal growth medium only Peaks 1-5 were identified as ethanol, water, acetic acid, propionic acid and butyric acid respectively from a standard profile (data not shown).

A large water peak was noted in all experiments since the fermentation process occurred in a liquid medium. Butyric acid had the highest peak, while ethanol, acetic and propionic acid had relatively low peaks. An experiment containing all reagents showed production of ethanol, acetic acid, propionic acid and butyric acid (Fig 3.3a). In the absence of bacteria (C1), acetic acid and propionic acid were detected (Fig 3.3b), while only acetic acid was produced when no sweet potato (C2) was added (Fig 3.3c). No products were detected when only M9 minimal growth medium (C3) was used (Fig 3.3d).

b) Gas products

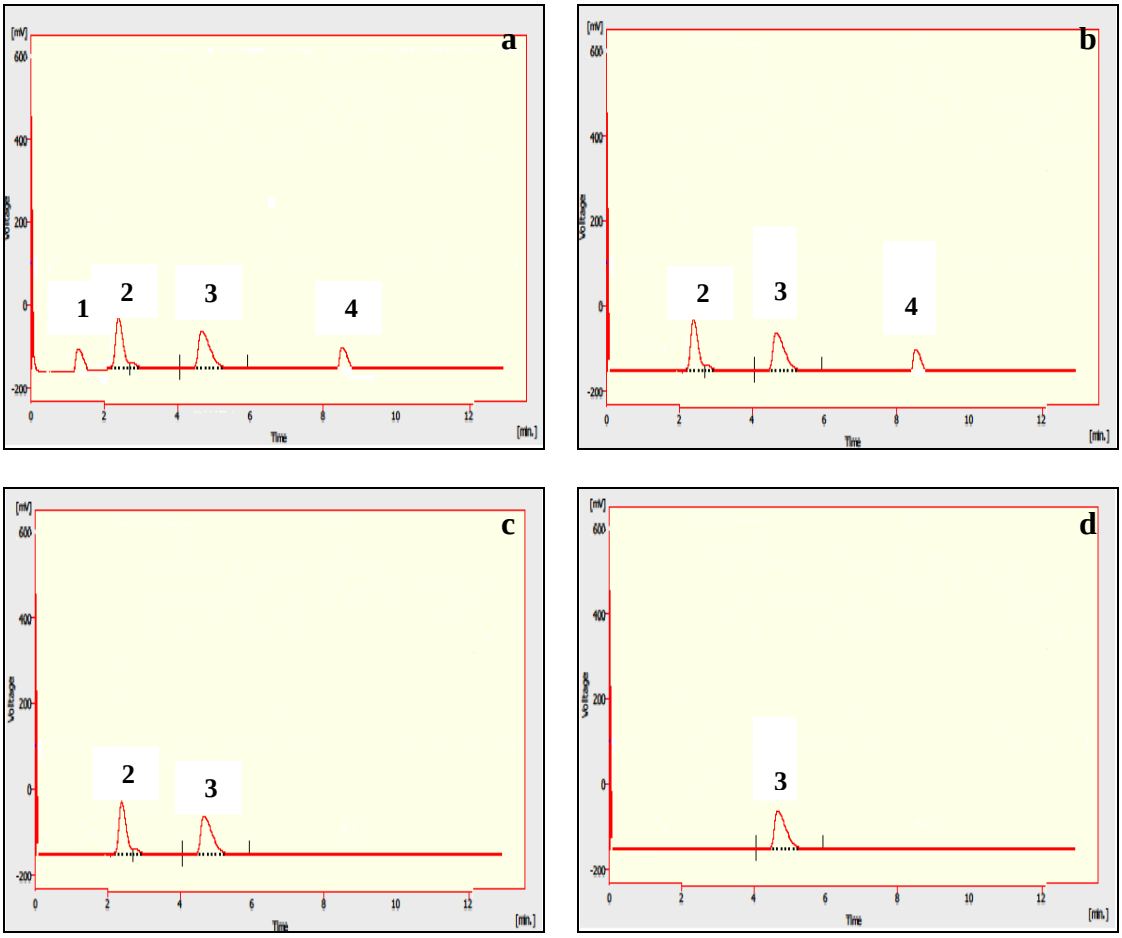


Figure 3.4: GC spectra of gases produced. a) A batch fermentation bottle which contained bacterial consortium, sweet potato and the medium b) CI operated without bacterial consortium, c) C2 operating without sweet potatoes, and d) C3 containing the medium only. Peaks 1-4 were identified as hydrogen (H_2), carbon dioxide (CO_2), nitrogen (N_2) and methane (CH_4) respectively.

Fermentation bottle containing bacterial consortium, sweet potato and medium produced H_2, CO_2, N_2 and CH_4 (Fig 3.4a). Nonetheless, no H_2 was detected in control C1 which was operated in the absence of the bacterial inoculums (Fig 3.4b). In a Control containing no sweet potato (C2), CO_2 and N_2 were detected (Fig 3.4c). In the presence of medium only (C3), only N_2 was detected (Fig 3.4d). Notably N_2 was detected in all the experiments.

3.4.3 Quantification of fermentation products using the M9 minimal growth medium

Concentrations of soluble products and gases were determined every second day. pH was not controlled and therefore varied over time. Glucose concentration and bacterial cell density were also determined.

a) Liquid products

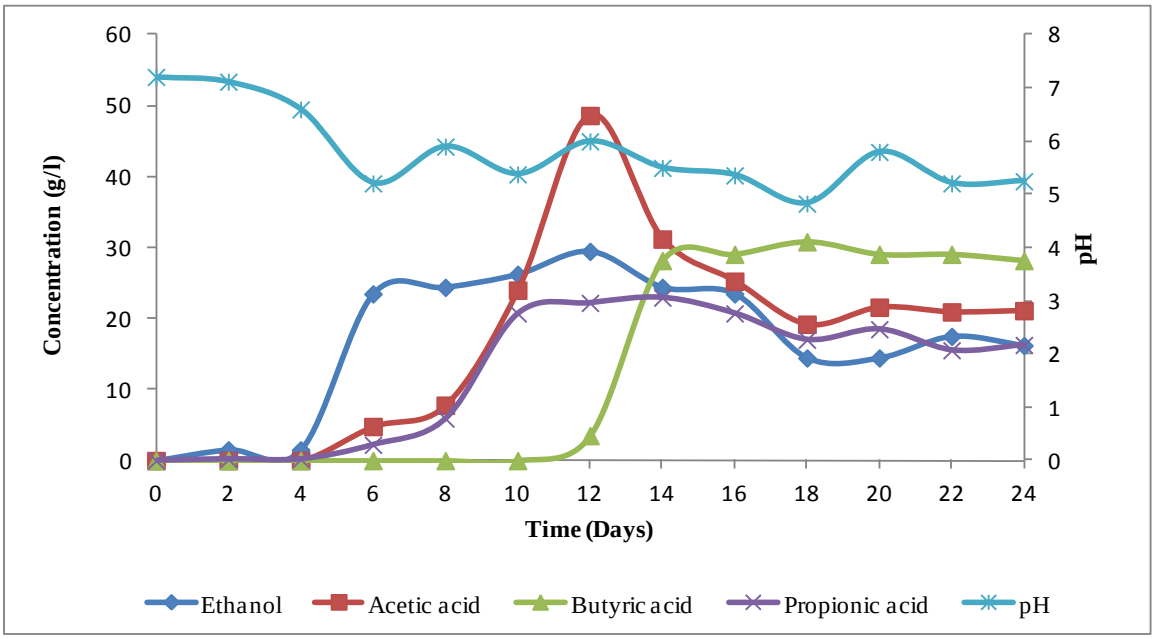


Figure 3.5: Detected liquid products over a 24 day period. Liquid analysis was done every second day and each detected product quantified. pH was also recorded.

During operation, pH varied from 4.83 to 7.21 with 7.21 recorded at the beginning. The pH fluctuated considerably throughout (Fig 3.5).

During the first four days of fermentation, no products were detected. On day 6, ethanol was detected producing a maximum of 29 g/l on day 12 after which production decreased to 16 g/l at the end of fermentation (day 24).

Acetic acid was produced at the highest rate of 10.2 g/l/d, yielding a maximum of 48.6 g/l on day 12. Concentrations had dropped to 21 g/l on the last day of fermentation. Propionic acid was produced at a rate of 4.6 g/l/d with a maximum concentration of 22.9 g/l recorded on day 14. Concentrations of most metabolic products were lowest on day 18 when the pH was lowest (4.83) except for butyric acid which showed a slight increased from 29 g/l to 31 g/l. Butyric acid was only detected on day 12 of fermentation at a rate of 6.4 g/l/d. The concentration slightly decreased to 29 g/l from day 20 and was maintained until end of fermentation. Butyric acid and acetic acid were the most dominant products with acetic products being the most dominant.

b) Gas products

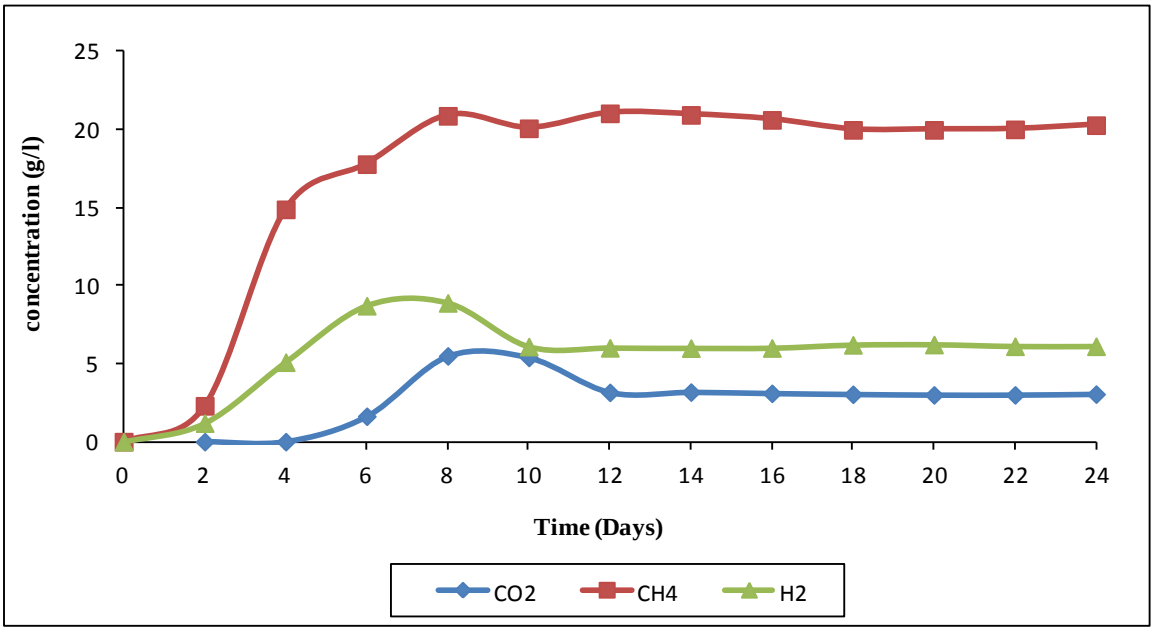


Figure 3.6: Concentration of gases produced over a 24 day period. Gas analysis was done every second day and the concentrations determined using standards curves.

Gaseous products collected during fermentation of sweet potato tuber in M9 minimal growth medium were analyzed (Fig 3.6). Considerable amounts of gases were detected after day 2, with CH_4 dominating. The highest concentration of 21.1 g/l was observed with production rate of 1.5 g/l/d. H_2 was produced at a rate of 1.28 g/l/d resulting in maximum concentration of 8.9 g/l. CO_2 was produced the least produced at a rate of 0.94 g/l/d reaching a maximum concentration of 5.5 g/l.

c) Glucose depletion/production

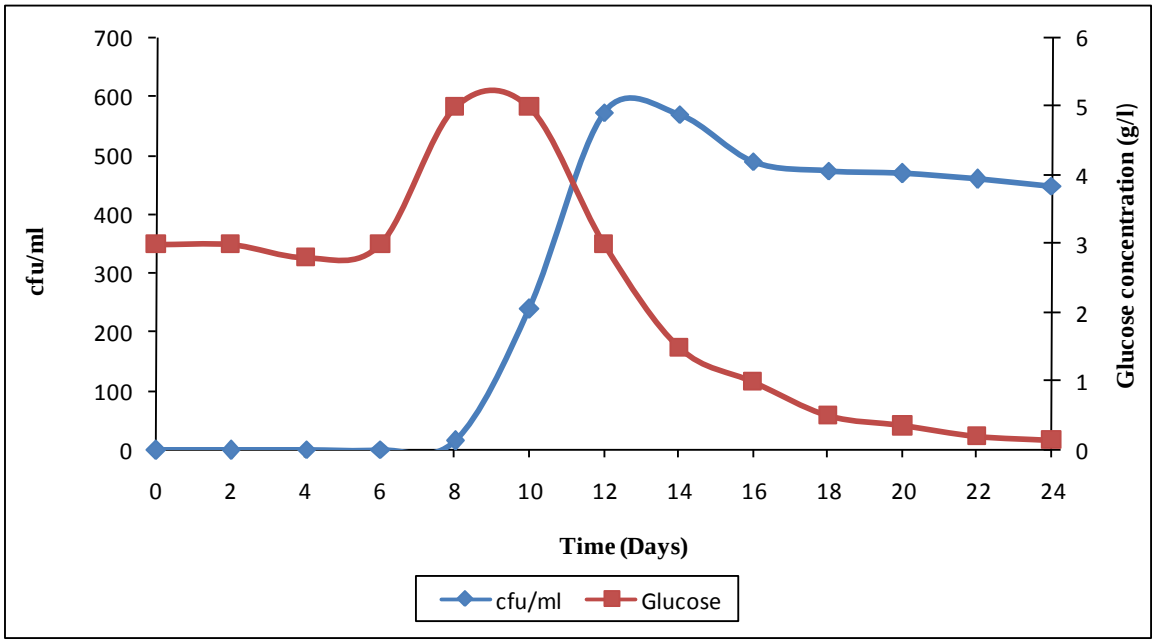


Figure 3.7: Glucose production/depletion and bacterial cell density over time. Glucose concentrations were measured every second day using HPLC with concurrent determination of cell density.

Glucose concentrations were determined during fermentation and a remarkable increase was observed from day 6 to 8 where the concentration increased from 3 g/l to 5 g/l. The increase was at a rate of 1.1 g/l/d. However, a gradual decrease in glucose concentration was observed after day 10. By day 24, glucose concentrations had dropped to 0.1 g/l indicating glucose consumption or conversion (Fig 3.7).

There was no significant bacterial growth observed for the first eight days of the experiment. Growth was observed from day 10 with the highest growth observed on day 12. Glucose concentration decreased from 5 g/l-2 g/l which may be a direct indication of glucose being used up by bacterial cells. Bacterial growth rate was recorded at 139.75 cfu/ml/d.

3.4.4 Quantification of fermentation products using the GP medium

GP medium was used in order to compare product distribution and concentrations to that of the M9 minimal medium. GP medium was previously used for H_2 production (Yokoi et al., 2001). Liquids, gas products as well as glucose concentration were determined and recorded over time. Cell density was also monitored.

a) Liquid products

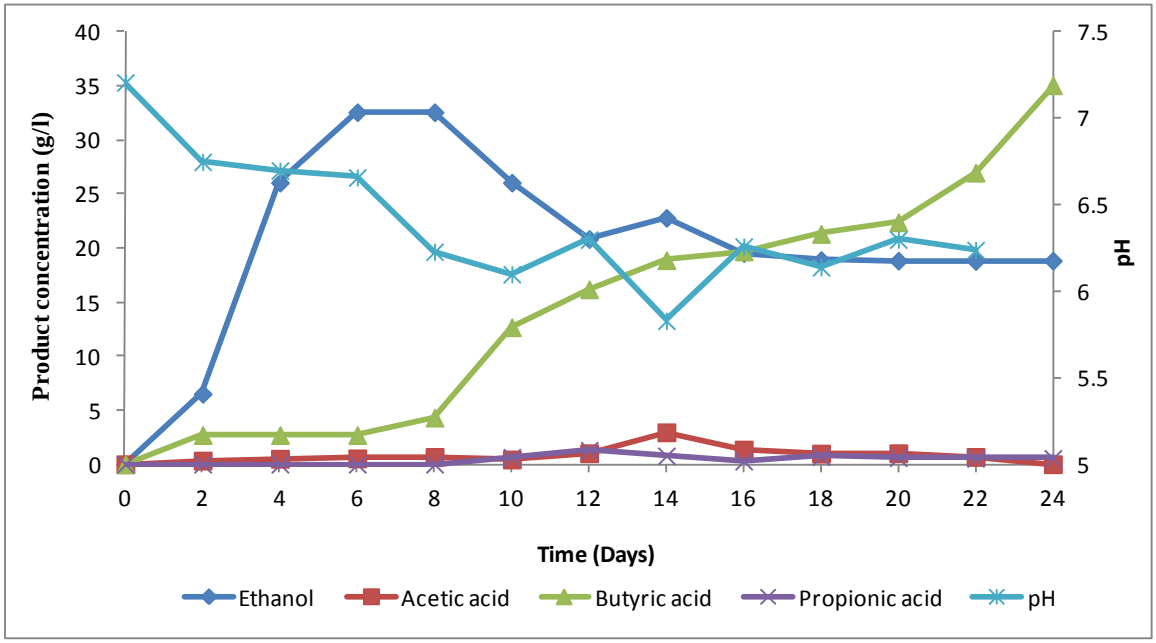


Figure 3.8: Detected liquid products over a 24 day period. Liquid products were detected using GC and quantified using standards curves. Recorded pH is also shown.

During batch fermentation of sweet potato using GP medium, pH ranged from 5.93-7.21. Initial pH of the medium was recorded at 7.21. As bacterial culture established within the fermentation vessel, a spontaneous decrease in pH was observed for the first 10 days (from 7.21-6.10). Thereafter, pH fluctuated between 5.83 and 6.30. A drop in pH was noted on day 14 with a pH value of 5.83. On this day, ethanol and acetic acid had a slight peak (Fig 3.8).

Ethanol and butyric acid were detected on day 2 with ethanol increasing rapidly at a rate of 6.52 g/l/d reaching a maximum concentration of 32.6 g/l on day 6. Ethanol concentration decreased after day 8 and stabilized at a concentration 18 g/l.

A relatively slow increase in butyric acid concentration was observed for the first eight days. However, a gradual increase was noted after that with maximum concentrations of 35.1 g/l recorded at the end of fermentation with production rates of 1.08 g/l/d.

b) Gas products

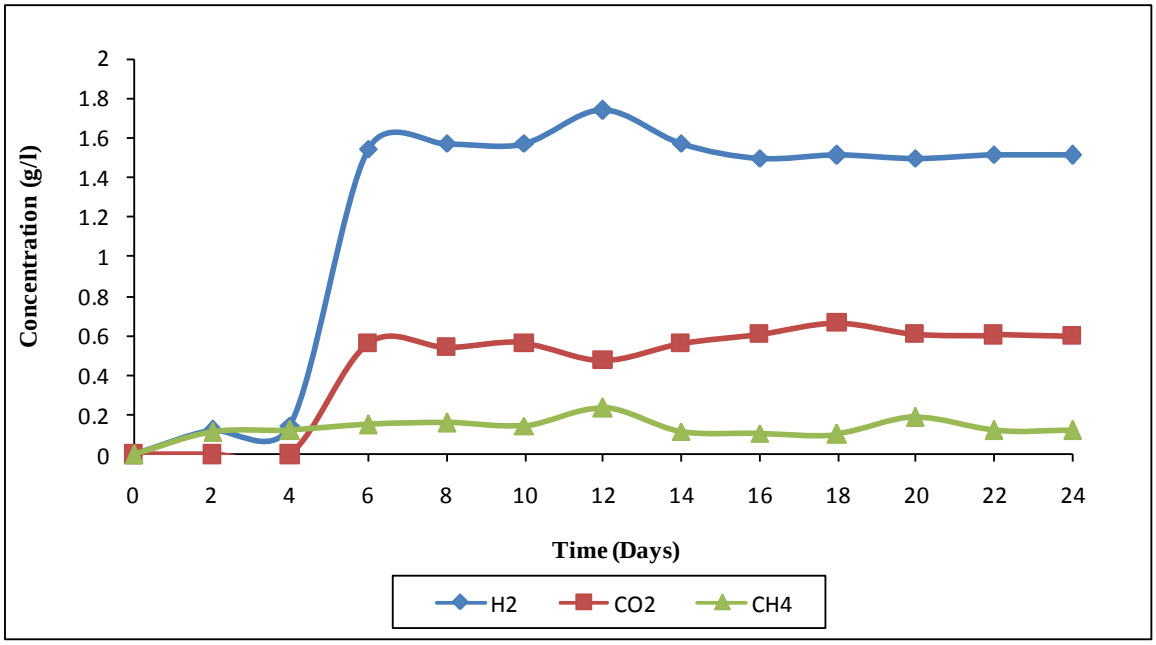


Figure 3.9: Production of gases over a 24 day period. Gas analysis was done every second day and the concentrations determined using standards curves.

Gaseous products collected during fermentation of sweet potato tuber in GP medium were analyzed. From day 4, H_2 production increased from 0.14 g/l to 1.55 g/l at a rate of 0.70 g/l/d. The highest concentration of 1.74 g/l was detected on day 12. H_2 concentrations remained constant at 1.5 g/l from day 16 until the last day of fermentation. CO_2 concentrations were higher than that of CH_4 with highest concentrations of 0.66 g/l measured on day 18. CO_2 was produced

at a rate of 0.28 g/l/d. CH_4 which was the least produced, was produced at a rate of 0.05 g/l/d. Its concentration remained relatively low at 0.1 g/l, with just a slight increase on days 12 and 20 with concentrations of 0.24 g/l and 0.19 g/l respectively (Fig 3.9).

c) Glucose production/depletion

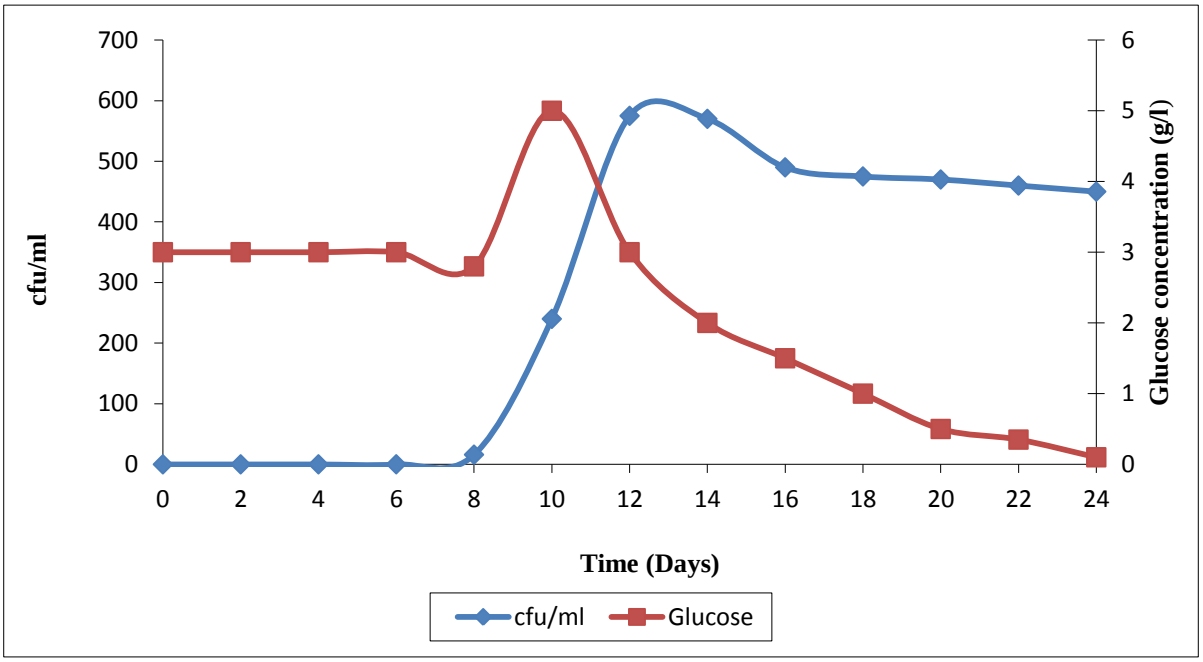


Figure 3.10: Glucose production/depletion as well as bacterial cell density over time. Glucose concentrations were measured every second day with concurrent determination of cell density.

Glucose concentrations were determined over time and there was no change in glucose concentration for the first six days. A slight decrease was observed on day 8, while a huge increase in glucose concentration was observed on day 10 (from 3 g/l to 5 g/l) at a rate of 0.5 g/l/d. A gradual decrease from day 12 was noted until day 24 when glucose concentration reached 0.5 g/l at a conversion rate of 0.31 g/l/d (Fig 3.10).

No bacterial growth was recorded until day 8 after which there was a sharp increase with maximum counts of 575 cfu/ml recorded on day 12. A slight decrease was noted on day 14 with growth stabilizing at 450 cfu/ml until day 24. Bacteria growth rates were recorded at 91.8 cfu/ml/d.

3.4.5 Quantification of fermentation products using the M9 minimal growth medium at pH 6.0

Higher product concentrations were recorded when the pH level reached 6.0 regardless of what medium was used, and relatively higher concentrations were recorded when M9 minimal medium was used. Therefore an experiment was carried out using M9 medium and culture conditions of pH 6.0. Aqueous and gas products were analyzed (Figs 3.11 and 3.12 respectively) over a 24 day period. Glucose concentration and cell growth were also measured.

a) Liquid products

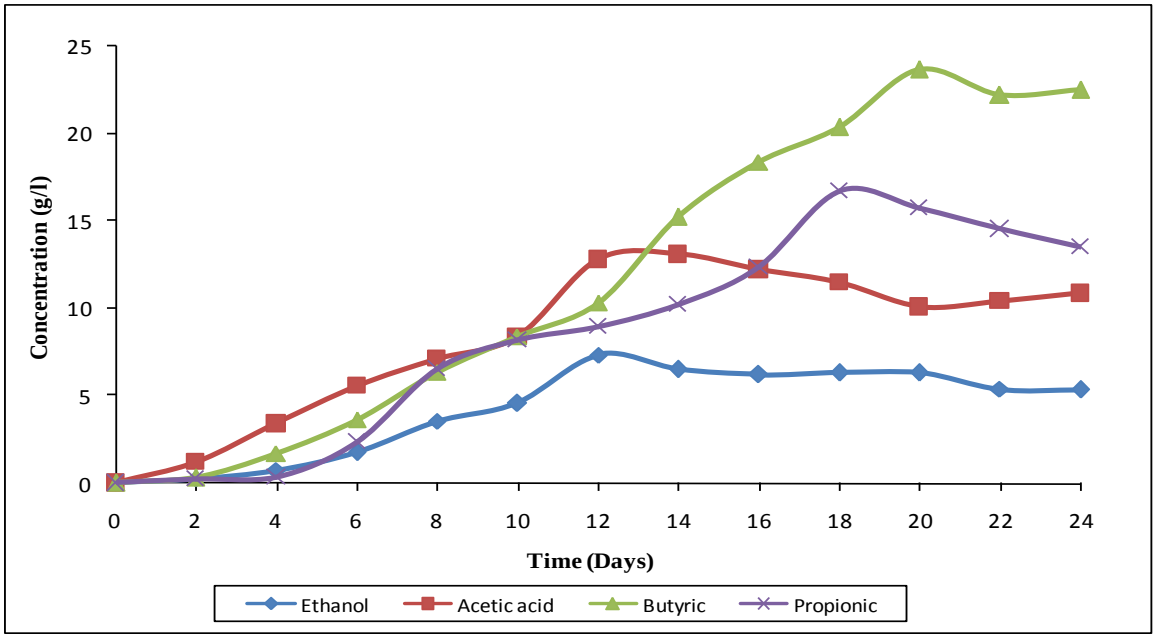


Figure 3.11: Concentrations of soluble metabolites over a 24 day period using M9 minimal medium at pH 6.0. Aqueous metabolites were analyzed every second day. Concentrations were determined using standards curves (Appendix B).

Concentrations of soluble products were determined during batch operation at controlled pH of 6.0 (Fig 3.11). Higher concentrations of volatile fatty acids (VFAs) were recorded compared to ethanol. Ethanol production was minimal while there was a shift in dominating acids after 12 days of fermentation. Initially acetic acid was dominant, followed by butyric acid and lastly propionic acid; however, this changed during the last 12 days as butyric acid took over, followed by propionic acid then acetic acid.

Ethanol and propionic acid were produced at the same rates of 0.7 g/l/d; however, higher propionic acid concentrations were recorded compared to ethanol. Acetic acid was produced at the rate of 1 g/l/d while butyric acid was produced at the rate of 1.7 g/l/d.

b) Gas products

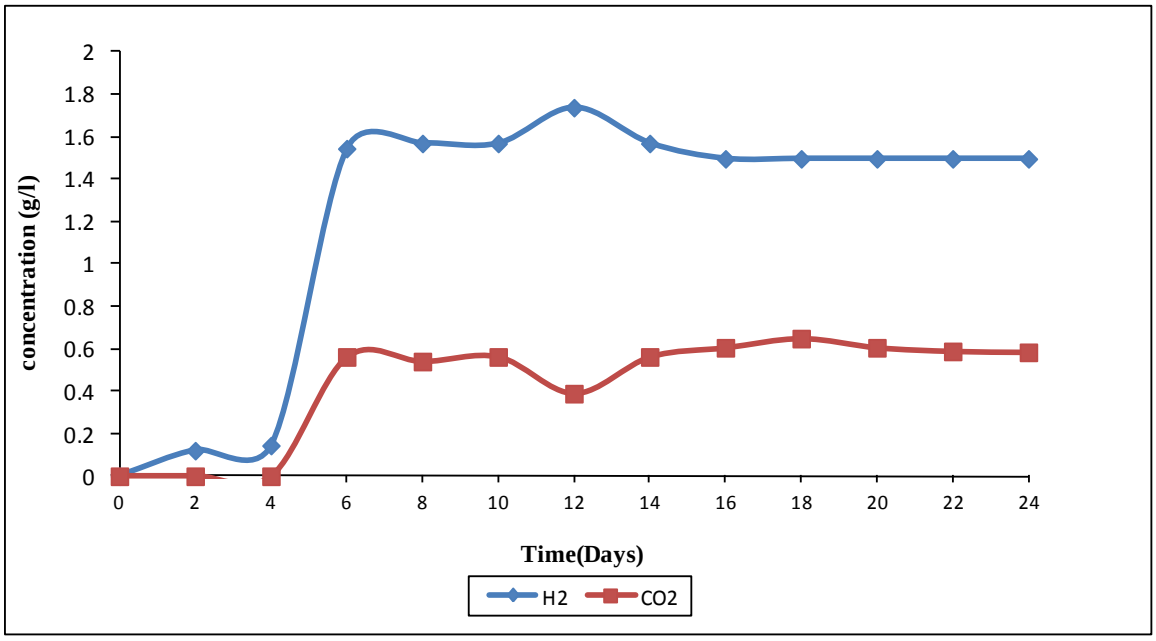


Figure 3.12: Concentration of gases detected over a 24 day period. Gas analysis was done every second day and the concentrations determined using standards curves. These concentrations were obtained when the M9 minimal medium was used under controlled pH 6.0 conditions.

When gas samples were analyzed, no CO_2 was detected during the first four days. However, H_2 was detected at low concentration during the first four days after which elevated levels were noted (Fig 3.12). Maximum H_2 concentration of 1.54 g/l was detected on day 12 with production rates of 0.7 g/l/d. On the same day, CO_2 concentrations dropped from 0.38 g/l to 0.56 g/l. CO_2 was produced at a lower rate of 0.3 g/l/d. Relatively low concentrations of gases were recorded in this experiment.

c) Glucose production/depletion

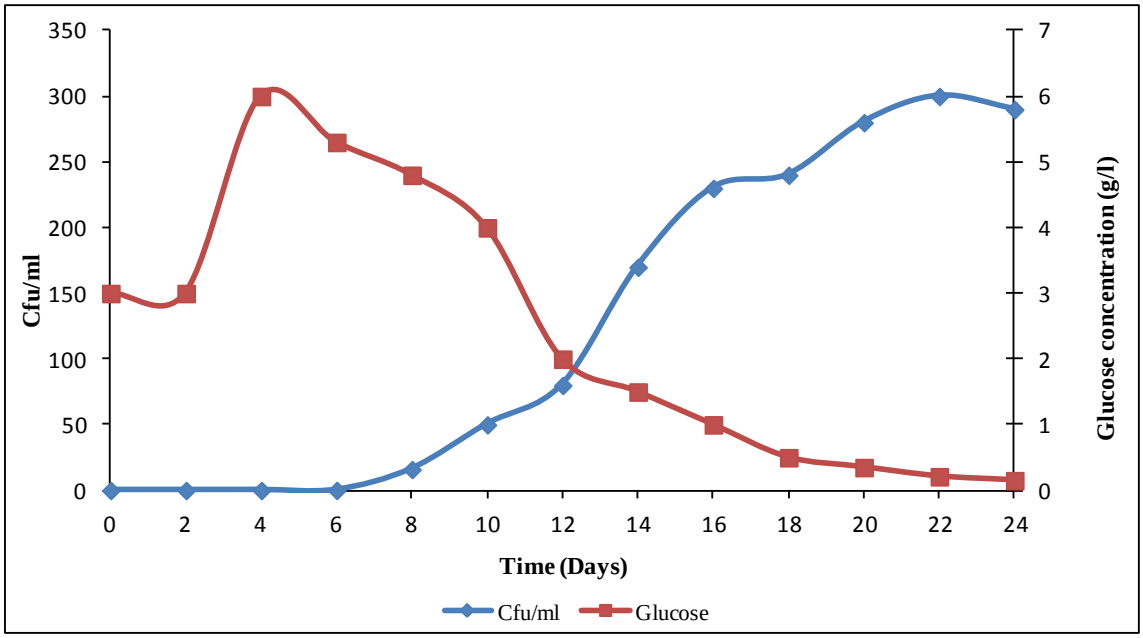


Figure 3.13: Glucose production and depletion and bacterial cell density over time. Glucose concentrations were measured every second day with concurrent determination of cell density under controlled pH conditions of 6.0 using M9 minimal medium.

Glucose concentrations and cell growth were determined (Fig 3.13). An increase in glucose concentrations was recorded on day 4 reaching concentrations of 6 g/l. Glucose concentration dropped gradually from day 6 to day 24 at a rate of 0.33 g/l/d.

No bacterial growth was observed until day 8. A gradual increase was observed in growth at a rate of 26.7 cfu/ml/d with a maximum counts of 300 cfu/ml recorded on day 22.

3.4.6 Identification of dominating microbial populations

To determine which species of bacteria dominated during fermentation, samples were collected and analyzed throughout operation. In an experiment that used M9 minimal growth medium, *Bacillus* dominated during the first four days, then *Enterobacter* dominated after day 4 until day 12 after which *Klebsiella* species prevailed. However, when GP medium was used, *Citrobacter* species dominated for the first eight days, after which *Alcaligenes* species took over. When pH level was controlled at 6.0 in M9 minimal growth medium, only *Klebsiella* species dominated (Table 3.2).

Table 3.2: Dominating bacterial species in three batch experiments over a 24 day period

Conditions	Time (Days)	Species
M9 minimal medium	0-4	*Bacillus*
	4-12	*Enterobacter*
	12-24	*Klebsiella*
GP medium	0-8	*Citrobacter*
	8-24	*Alcaligenes*
M9 medium at pH 6.0	0-24	*Enterobacter*

3.4.7 Determination of the rate equation for sweet potato batch fermentation

Data obtained from the experiment that investigated the effect of different concentrations (1-5 g/l) of sweet potato tuber on bacterial cell density was used as a basis for sweet potato rate equation determination (Fig 3.14). The rate equation was formulated in order to compare specific growth rate and productivity in all the three batch experiments.

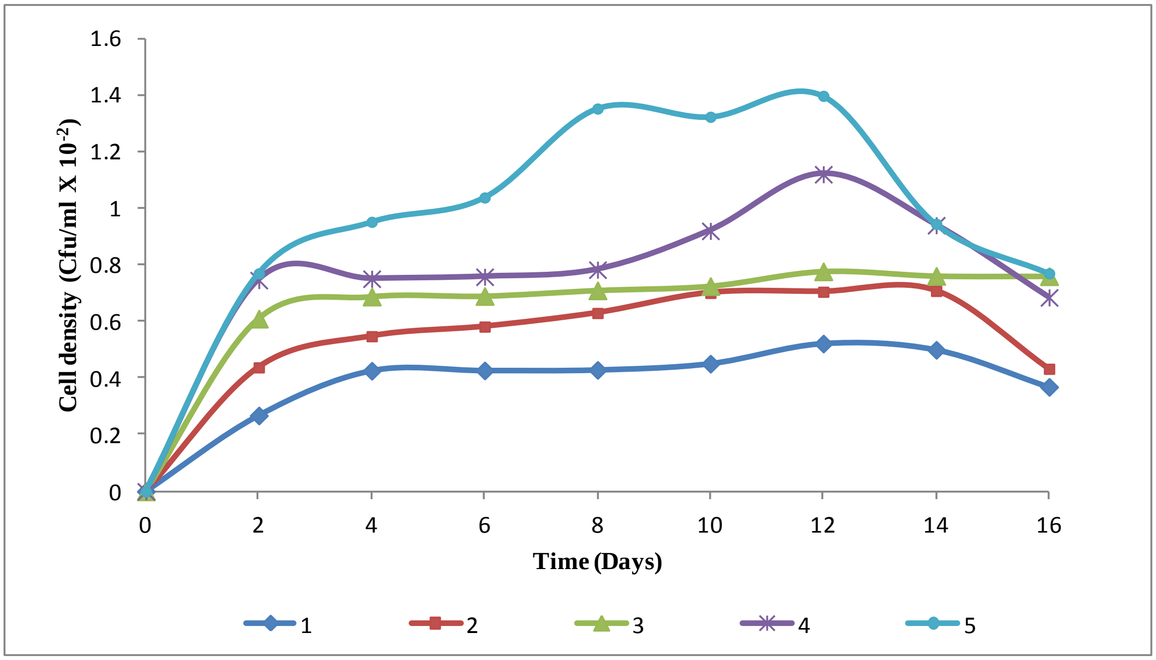


Figure 3.14: A plot of cell concentration at different concentrations (1-5 g/l) of sweet potatoes over time.

The slopes of each curve were determined as that would show the rate under each substrate concentration and their reciprocals calculated so as to construct a curve of those reciprocals (Fig 3.14).

The Monod equation can be linearized by taking the reciprocals of Equation 3.1 (page 69):

$$\frac{1}{\mu} = \frac{K_S}{\mu_m}\frac{1}{S} + \frac{1}{\mu_m} \quad \text{.......} \quad (3.2)$$

The plot of 1/μ versus 1/S results in a straight line with a slope of K_S/μ_m, an x-intercept of -1/Ks, and a y-intercept of $1/\mu_m$ (Scragg, 1988).

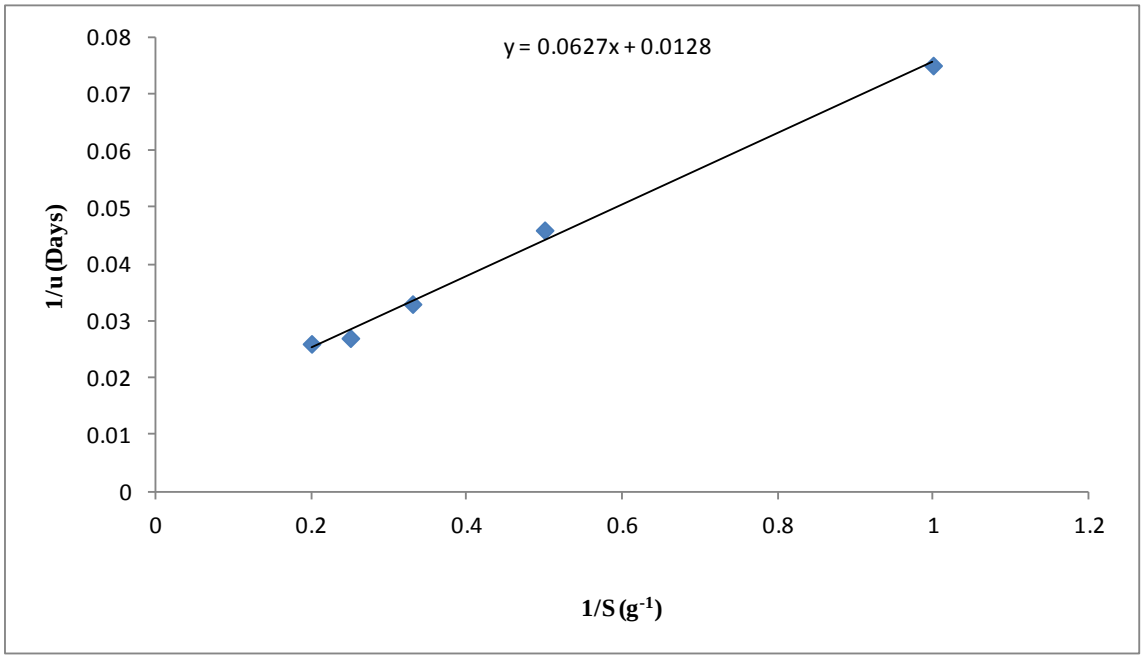


Figure 3.15: A plot of 1/μ versus 1/S. This results in a straight line with a slope of K_S/μ_m, producing in a plot (Lineweaver-Burk plot) with K_m as the saturation constant (K_S), V as the growth rate (μ) and V_m as the maximum growth rate (μ_{max}) (Shuler and Kargi, 1992).

From the graph above,

Since intercept = 0.0128, then $1/\mu_m = 0.0128$ and $\mu_m = 78.1$

Since slope = 0.0627, then $K_S/\mu_m = 0.0627$ and $K_S = 4.898$

Therefore rate equation for the sweet potato batch fermentation Equation 3.3

$$\mu = \frac{78.1S}{4.898 + S} \quad \text{.....} \quad (3.3)$$

Since, the same amount of substrate concentration was used in all experiments; maximum concentration obtained in each experiment was used to calculate μ (Table 3.3).

Since cell density increases exponentially with time, the specific growth rate is expressed by Equation 3.4.

$$\frac{dx}{dt} = \mu x \quad \ldots\ldots (3.4)$$

Integration gives the expression for cell concentration (X) with respect to time (t) (Shuler156):

$$X = X_0^{\mu t} \quad \ldots\ldots (3.5)$$

Where
μ = Specific growth rate
X = Cell concentration,
X_0 = Initial cell concentration
t = Time

Productivity in batch culture may therefore be described by Equation 3.6.

$$R_{batch} = \frac{(x_{max} - x_0)}{t_i - t_{ii}} \quad \ldots\ldots (3.6)$$

Where

R_{batch} = Output of the culture in terms of biomass concentration (d^{-1})

X_{max} = maximum cell concentration achieved at stationary phase (Cfu/ml)

X_o = Initial cell concentration (Cfu/ml)

t_i = Time during which the organism reached maximum growth (d)

t_{ii} = Time during which the organism is not growing at maximum and includes the lag phase and the deceleration phase. (d)

Table 3.3: Specific growth rate and productivity calculated using Equations 3.3 and 3.6

Conditions	μ	Productivity
M9 medium at uncontrolled pH	39.4/day	59.9 Cfu/ml/day
GP medium at uncontrolled pH	39.4/day	47.9 Cfu/ml/day
M9 medium at controlled pH 6.0	43.0/day	13.6 Cfu/ml/day

3.5 Discussion

3.5.1 Bacterial growth curves

The growth curve exhibited all the four distinct growth phases (lag, log, stationary and death phase) of a typical growth curve for batch growth (Fig 3.2). The "lag" period is a phase in which the bacterial cells are adapting to the new medium (environment). Once adapted to the new environment, bacterial cells start growing and dividing, a phase referred to as the log period. As the nutrients start depleting and toxins accumulate (usually in the form of products), cell growth stops and a stationary phase is reached where growth occurs at a constant rate (cell growth = cell death) (Stanbury, 2000). When nutrients are depleted and bacterial growth ceases, the last phase is reached, a period known as the death phase. During this period, most if not all nutrients are converted into products, most of which may inhibit bacterial growth at certain concentrations (Hobson and Feilden, 1982).

Once the growth curve was constructed, the mid-log phase was determined and calculated at four hours after inoculation. At this point, bacteria cells are growing at an exponential rate. This means that cells are most metabolically active. During this phase, the culture is used as a seed culture for bio processing (Stanbury, 2000). The lag and the death phase were quite brief with each lasting only for about 2 hours.

3.5.2 Initial batch experiments to determine fermentation products

Initial batch experiments were carried out as a platform for batch experiments that involved quantification of batch products. The obtained results gave a clue of products produced when varying batch fermentation reagents were available in a system. In C1, containing no inoculum, acetic acid and propionic acid were detected mainly because sweet potato tuber has bacteria associated with it as confirmed previously in chapter 2; however, other products such as ethanol and butyric acid were not detected. In C2, no substrate was added yet acetic acid was detected, this could be attributed to the complex interactions of mixed bacterial populations leading to provision of necessary nutrients for optimal performance (Harrison, 1978). Another reason for this could be that some of the species constituting the used mixed cultures still had some kind of carbon source stored in their cells (Rinnan and Bååth, 2009). As would be expected, there was no liquid or gas product detected in C3 where only the medium was used.

A wider spectrum of products was recorded in batch experiment where substrate and bacteria cultures were used. These observations indicate that a favourable fermentation process requires an adequate amount of both the substrate and the bio-catalyst (bacteria).

3.5.3 Quantification of fermentation products using the M9 minimal growth medium

a) VFAs and ethanol production

Acetic acid, propionic acid and butyric acid were detected. This would be expected as these VFAs have been reported to be products of fermentation (Buyukkamaci and Filibeli, 2004). It has been proven that the medium pH does not only affect fermentation's final product, but also microbial cell density and the rate of fermentation (Vandák et al., 1997; Horiuchi et al., 2002; Niedzielski, 2007). This is mainly because different acid pathways are activated at different pH ranges (Choi and Mathews, 1994; Jiang et al., 2009). As a result, microbial pathways can be altered by changing the medium pH as this will activate different enzymes that are involved in acid or solvent production (Salleh et al., 2008). It was therefore necessary to measure the pH of the medium each time a sample was taken as that would give an indication of which products to expect and this would explain why different acids were produced at different concentrations at different pH levels.

Contradictory findings have been reported on the optimal pH ranges of acids and alcohols (Salleh et al., 2008). Some studies report that acetic, propionic and butyric acids dominate in pH range of 4.5 - 6.0 as opposed to solvents (Ennis and Maddox, 1985; Marchal et al., 1985). On the other hand, other studies report decreased acid production and increased solvent production in that pH range (Nishio et al., 1983; Brum et al., 1989). In this study the recorded pH range was 4.83 - 7.2 and ethanol concentration was highest at pH 6.0, this finding was in agreement with results obtained by Ennis and Maddox (1985) and Marchal et al. (1985).

Of all the three VFAs produced, acetic acid dominated at pH 6.0. These results are in agreement with a study performed by Veny and Hasan (2005), who investigated anaerobic fermentation

process using *Clostridium thermoaceticum* from glucose. They investigated the environmental effects on batch fermentations by varying the pH from 5.0-7.0. Their results showed that acetic acid yield was highest at pH 6.0. However, these results were in contrast with the findings reported by Brum et al. (1989), who reported acetic acid production to be maximal at pH 7.0. Choi and Mathews (1994) also reported that acetic acid production was maximal at pH 7.0.

In this study, propionic acid was produced from as early as day 6; however, its concentration was relatively lower compared to other acids. This could be because propionic acid concentration has been reported to be considerably low at pH range of 5.0 and 7.0 (Horiuchi et al., 2002). Interestingly, ethanol was also produced in moderate amounts with a maximum of 29.48 g/l. This finding was similar to that of Li et al. (2010), who reported no inverse relationship between ethanol and VFAs production. This could be attributed to the complexity of the potato tuber carbohydrate content leading to more side reactions as opposed to studies that use one carbohydrate source such as starch (Li et al., 2010).

There was also considerably high concentration of butyric acid which would be expected as pH range of 5.0-6.0 has been reported to favour butyric acid production by mixed cultures of fermentative bacteria (Vandák et al., 1997; Jiang et al., 2009). The observation that there was considerably lower propionic acid concentration indicates that bacterial routes to propionic acid production were inhibited to some extend at this pH range (Choi and Mathews, 1994).

b) Methane and H_2 production

Reports have shown that metabolic pathways of microorganisms involved in production of methane and H_2 gases are affected mostly by substrate digestion pathway (Wang et al., 2009). As previously mentioned, environmental factors such as pH and media composition also affect

bacterial pathways, hence end products. Thus, fermentation end-products depend on the environmental conditions in which bacteria grow.

In this study, pH was not controlled but was left to spontaneously fluctuate. Eventhough there was an overall low gas concentrations recorded in this study, H_2 was the most dominant gas and the highest concentration was observed at pH 6.0. These results are consistent with research carried out by Li et al. (2010) who reported an increase in H_2 production at a pH higher than 5.0. Acetic acid and butyric acid were the most dominant products in this study and H_2 production is usually correlated with acetic acid and butyric acid as shown in Equations (3.7) and (3.8) below (Liu et al., 2006; Antonopoulou et al., 2008).

$$C_6H_{12}O_6+2H_2O \rightarrow \mathbf{4H_2}+2CH_3COOH(acetate)+2CO_2, \quad (3.7)$$

$$C_6H_{12}O_6 \rightarrow \mathbf{2H_2}+CH_3CH_2CH_2COOH(butyrate)+2CO_2, \quad (3.8)$$

This indicates that high H_2 concentration is associated with high concentrations of acetic and butyric acid as stated by several researchers (Mizuno et al., 2000; Levin et al., 2004; Liu et al., 2006; Antonopoulou et al., 2008; Li et al., 2010). It should also be noted that the ratio of acetic acid to H_2 is 1:4 per mole of glucose, while butyric acid to H_2 is 1:2, indicating that increased H_2 production is associated more with how much acetic acid is present in the system than with butyric acid if both products are present in the system (Mizuno et al., 2000; Levin et al., 2004; Liu et al., 2006).

Many studies have shown that pH also affects gas production especially H_2 production (Levin et al., 2004; Liu et al., 2006; Antonopoulou et al., 2008). However, in this study, there was no huge

change in H_2 production even when pH fluctuated from 5.21 to 6.0. This may indicate that H_2 production may not be sensitive to pH.

The highest pH recorded in this study was 7.21 which was the initial pH on day 0. This could explain such low methane concentration observed in this experiment since the optimum pH for methane production has been shown to be 7.5 which is close to the initial pH recorded but was never reached during the 24 day fermentation period of this study (Liu et al., 2006).

Ethanol was also found in quite high concentrations and since the production of metabolic products accompanied by a negative or zero yields in H_2 results in lower total yields of H_2, this could account for low H_2 gas concentrations observed throughout the study even though it was the dominant gas (Levin et al., 2004). This is generally because reduced end-products such as ethanol contain molecular H_2 (Equation 3.9) that could otherwise be released as gas and therefore minimizes H_2 production to some extent. The fact that alcohols inhibit H_2 liberation to some extend was confirmed by Dabrock et al. 1992, who directed *C. pasteurianum* metabolism towards solvent production by increasing glucose concentrations (Dabrock et al., 1992; Levin et al., 2004).

$$C_6H_{12}O_6 \rightarrow 2CH_3C\mathbf{H_2}OH+2CO_2, \quad \text{(3.9)}$$

3.5.4 Quantification of fermentation products using the GP medium

a) VFAs and ethanol production

This study found that generally, ethanol and butyric acid were the most dominant products while acetic acid and propionic acids were found only in small traces. This could be attributed to the fact that acetic acid production is favoured at a pH less than 5.0 (Jiang et al., 2009). Maximum butyric acid production has been shown to be in the pH range of 5.5 - 6.0. In this study butyric

acid production was favoured at a pH between 6.1 and 6.3. Although these results differ from other published studies, they are consistent with results of Jiang et al. (2009) who reported maximum butyric acid production of 26.2 g/l at this pH range (Choi and Mathews, 1994). Increased concentrations of butyric acid observed in this experiment could also be a result of production of H_2 in the system where the butyric acid works as a H_2 acceptor from NADH (Horiuchi et al., 2002).

It has also been reported that certain concentrations of butyric acid inhibit acetic acid production. Jiang et al. (2009) reported that butyric acid concentrations of up to 10 g/l could inhibit acetic acid production. This could explain the observation that there was a very high concentration of butyric acid (up to 35 g/l) while acetic acid concentration was low.

Glucose fermentation studies have shown that for stoichiometric reasons and for maintaining H_2 and redox balance, acetic acid production is accompanied by propionic acid production with propionic acid pathway inhibited by highly ionized acetic acid at pH range of 5.5 - 7.0 (Choi and Mathews, 1994). This statement implies that at that pH range, opposite production patterns are expected for these acids; however, this was not the case in this instance as concentrations of these two acids were low. One of the possible reasons for this could be that since mixed fermentative cultures were used in this study, bacterial routes to these acids production were inhibited at this pH range (Choi and Mathews, 1994).

The spontaneous decrease in pH that occurred for the first 10 days of fermentation could be a result of acidogenesis at the beginning of the fermentation due to the higher initial pH of the medium which is unsuitable for solventogenesis (Vandák et al., 1997). This means that VFAs

production can rapidly decrease pH and then change the fermentation pathways and end-products.

b) Methane and H_2 production

Reports show that all soluble metabolites obtained in this study - ethanol acetic acid, propionic acid, and butyric acid, could be used as substrates for methane and H_2 production (Hanaki et al., 1994; Buyukkamaci and Filibeli, 2004). If all these substrates are available in a system then, acetic acid will be converted first, followed by ethanol, then butyric acid. Propionic acid is normally used as the last resort to all the other substrates (Ren et al., 2003). Since acetic acid is the most preferred to all the mentioned substrates, all other substrates need to be converted to acetic acid prior to being used for gas production and this happens in this order ethanol > butyric aicd > propionic acid which makes propionic acid an inferior substrate for methane production (Collins and Paskins, 1987; Hanaki et al., 1994; Wang et al., 2006).

In this experiment, relatively low concentrations of H_2 were observed compared to methane. Presence of VFAs in a system either as products or substrates has been reported to have inhibitory effects on substrate degradation (if found as products) or biogas production (if present as substrates) at different concentrations (Wang et al., 2009). VFA concentrations above 4.0 g /l have been found to cause inhibition of glucose degradation, while propionic acid concentration of 5 g/l at pH 7.0 has been reported to decrease methane production (Yeole et al., 1996; Siegert and Banks, 2005). Reports that propionic acid inhibits biogas production have also been confirmed by other researchers (Ren et al., 2003; Wang et al., 2009). This would account for higher methane concentrations observed compared to H_2 since propionic acid was produced at very low concentrations implying that propionic acid concentrations were not high enough to have inhibitory effect on methane production.

This could be further explained by reports that in methane production pathways, high H_2 production increases propionic concentrations in a system (Mosey and Fernandes, 1989; Fynn and yafila, 1990; Wang et al., 2009). This means that the optimal operating conditions for H_2 production commonly produce an inferior substrate for the methanogenic phase (Wang et al., 2009). The reverse could be true suggesting that in this case, relatively low H_2 concentrations could have been a result of low propionic acid concentrations which in return favoured methane production.

Some researchers have however questioned the fact that increased propionic acids leads to increased H_2 production and instead argue that accumulation of propionic acid is not due to increased H_2 production but due to NADH/NAD^+ balance which is required for continuous fermentation implying that more NADH leads to increased production of propionic acid since it produces more NAD^+ therefore bringing about the required NADH/NAD^+ balance (Cohen et al., 1984; Dinopoulou et al., 1988; Harper and Pohland, 1990; Inanc et al., 1996; Ren et al., 1997; Wang et al., 2006). On the other hand, other researchers have associated increased H_2 production to NADH/NAD^+ balance within the bacterial cell instead, stating that more H_2 leads to increased NADH within a cell (Vavilin et al., 1995). Considering all these arguments, it can be deduced that increased H_2 production (hence NADH) favours accumulation of propionic acid (NAD^+) spontaneously for maintaining a proper ratio of NADH/NAD^+.

Anaerobic biogas fermentation is divided into two main phases - acidogenic phase (acidogenesis) which is characterized by production of low alcohols, VFAs (acetic acid, propionic acid and butyric acid) and H_2, and a methanogenic phase (methanogenesis) which produces methane and CO_2 or methane and H_2O depending on the methane production route (Bhatia et al., 1985; Collins and Paskins, 1987; Vavilin et al., 1995; Wang et al., 2006). This Means that short chain

VFAs could be produced with methane and CO_2 (Horiuchi et al., 2002). This would explain the presence of both methane and CO_2 as biogases produced. The finding that there was remarkably high concentration of methane compared to H_2 could be due to the possibility that some of the H_2 was used as substrate for methane production.

The most interesting finding was that when acetic acid concentrations were very low, H_2 concentrations were also lower than those of methane. This further confirms reports that there is a linear relationship between H_2 and acetic acid (Liu et al., 2006; Antonopoulou et al., 2008). Although reports show that molecular H_2 is produced simultaneously with acetic acid and butyric acid production, and consumed during increased production of propionic acid, low H_2 concentrations were recorded even when relatively low propionic acid concentrations were recorded. This suggests that in this case, low H_2 concentrations were a result of low acetic acid concentrations (Horiuchi et al., 2002).

3.5.5 Quantification of fermentation products using the M9 minimal medium at pH 6.0

a) VFAs and ethanol production

Higher bacterial counts were recorded when the M9 minimal growth medium was used compared to when the GP medium was used, indicating that the mixed cultures used in this study had preference for M9 minimal medium than the GP medium. In addition to this, at uncontrolled pH conditions, higher product concentrations were recorded at pH 6.0. Furthermore, it has been reported by several researchers that the optimal pH of the acidification process is about 6.0 (Duarte and Andersen, 1982; Zoetemeyer et al., 1982; Cohen et al., 1984; Horiuchi et al., 2002). As a result, an experiment was carried out under controlled pH 6.0 conditions using the M9 minimal medium.

The main soluble products detected in this experiment were acetic acid, butyric acid and propionic acid while ethanol concentration was rather low. Butyric acid and propionic acid concentration increased to 24.5 g/l and 16.71 g/l respectively, with acetic acid production decreasing from the maximum concentration of 12.81 g/l to a minimum concentration of 10.87 g/l. Since the pH was controlled, the difference in the product distribution over time could be attributed to the change of metabolic pathway in the same bacterial populations.

To confirm the statement above, bacteria were sampled, isolated and sampled every second day as described in chapter 2, to see which species dominated over time. The results revealed that *Klebsiella* species dominated throughout the 24 day period (Table 3.2), confirming that the change in product concentration over time was not due to change in dominant microbial population but change in metabolic pathway within the same bacterial species.

Despite reports that pH 6.0 is the optimum pH for most acids, results obtained in this experiment revealed considerably lower product concentrations when the pH was kept constant at 6.0 compared to uncontrolled pH conditions employed during quantification using both the M9 minimal growth medium and GP medium. Such a discrepancy could be a result of other factors such as the type of medium and mixed cultures used in this study. For example, the medium and growth conditions used in this study allowed for facultative anaerobic growth while most studies used the medium that only allowed for growth of strict anaerobes resulting in anaerobic microbial populations in their system (Horiuchi et al., 2002). The observation that when pH was lowered using added HCl lower concentrations were recorded compared to experiments where pH was allowed to follow its natural course has been reported by other researchers (Vandák et al, 1997).

b) Methane and H_2 production

There was no methane detected in this experiment, only H_2 and CO_2. The main reason for this could be the fact that metabolism of glucose only occurred in acetogenesis stage which only produces H_2 and CO_2 implying either that there were no methane producing bacteria present or a methane pathway was not possible at all for the mixed consortium present under the prevailing conditions. In mixed fermentation processes, the microorganisms may select different pathways while converting sugars, as a response to changes in their environment (pH, sugar concentration). It should be highlighted that the absence or presence of H_2-consuming microorganisms in the microbial consortium also affects the microbial metabolic balance and consequently, the fermentation end-products (Antonopoulou et al., 2008).

It has been reported that high H_2 content leads to increased production of butyric acid because butyric acid acts as a H_2 acceptor resulting in production of NAD^+. Since H_2 was the main gas produced in this experiment, this statement could explain increased butyric acid concentration. It has also been pointed out that H_2 is given off together with acetic acid and butyric acid from glucose (Equations 3.7 and 3.8), while it is used up during the production of propionic acid (Equation 3.10) (Horiuchi et al., 2002; Levin et al., 2004; Antonopoulou et al., 2008). However, results obtained in this study show that as long as there is enough butyric acid and acetic acid in the system, the effect of propionic acid on H_2 is not so profound. This could be attributed to the fact that stoichmetrically, propionic acid production uses up two moles of H_2 per mole of glucose while acetic acid and butyric acid production results in a total of six moles of H_2 per glucose leaving a net total of four moles per glucose produced if all the three products are found in the system Equation 3.11.

If acetic acid, butyric acid and propionic acid are produced in the system then combining Equations (3.7), (3.8) and (3.10) results in Equation 3.11 as shown below.

$C_6H_{12}O_6+\mathbf{2H_2} \rightarrow 2CH_3CH_2COOH(propionate)+2H_2O,$…… (3.10)

$3C_6H_{12}O_6 \rightarrow \mathbf{4H_2}+2CH_3COOH+CH_3CH_2CH_2COOH+2CH_3CH_2COOH+4CO_2,$…… (3.11)

3.5.6 Cell density determination

There are several ways to measure bacterial cell growth. These include methods such as OD measurements using the spectrophotometer and direct count (plate count). The OD method has been reported to have disadvantages such as the inability to differentiate dead from living organisms, variable light scattering by living and dead organisms, discrepant measurements with low and high bacterial concentrations and with aggregating organisms, and interference with by-products of growth. Furthermore, light transmission and scattering by microorganisms change between the logarithmic and stationary phases of growth (Casciato et al., 1975). In addition to these, OD measurements have been reported not to be sensitive enough to the lag phase. On the other hand, plate counts have been reported to differentiate between live and dead cells as only viable cells are included in the count. Direct count method has also been reported to be sensitive to all the four phases of bacterial growth and therefore considered the most effective way for determining viable cells, which is why this method was mostly used to quantify bacteria in this study.

In all the cases, the highest cell density was recorded on a day when there was a huge drop in glucose concentrations, implying that the drop was due to glucose consumption by bacterial

cells. This would suggest that the cell growth energy comes from Embden-Meyerhoff-Parrnas (EMP) of the dicarboxylic pathway, which is a common route for most bacterial metabolic pathway from glucose to propionic and acetic acids (Choi and Mathews, 1994).

There were generally healthy cells of mixed cultures maintained throughout the study evident in overall high bacteria cell counts recorded in all the experiments. This confirmed reports that most bacterial cells grow well in batch cultures under microaerobic conditions than anaerobic environments because microanaerobic conditions result in rapid substrate consumption (Choi and Mathews, 1994; Chen et al., 2003). This therefore means that if some oxygen is present in a system, it acts as an exogenous electron acceptor and this in turn activates the gene expression system for the enzymes in the citric acid cycle providing the energy required for cell growth (Chen et al., 2003). Higher bacterial counts recorded could also imply that the activated pathway leading to production of all these products supplied the maintenance energy resulting in maintenance of healthy cells throughout the fermentation period (Choi and Mathews, 1994).

It should be noted that high product concentrations were recorded in experiments that maintained the highest bacterial counts (experiments where pH was not controlled (Figs 3.7 and 3.10) i.e., as cell populations increased, the amount of product formed increased, suggesting that more cell growth resulted in more substrate consumption which lead to increased product formation (Qureshi et al., 2005). A similar trend was observed in a study done by Amsden and colleagues (2003) on phenol degradation by *P. putida* in a partitioning bio-reactor, who reported a decrease in phenol concentration and a concurrent increase in cell concentration (Amsden et al., 2003).

Reportedly, techniques used for monitoring bacterial metabolic activity include the use of extracellular enzymatic activity measurements, radiolabeled amino acids incorporation and the

direct viable count method (Kogure et al., 1979; Alongi, 1990; Boetius and Lochte, 1994). This confirms that high metabolic activity is associated with higher plate counts and high productivity (Table 3.3). Therefore, higher product concentrations in these experiments were without any doubt due to high cell densities (Shim and Yang, 1999). However, as shown in table 3.3, there was no direct relationship between specific growth rate and productivity. As shown in M9 minimal growth medium at pH 6.0, high specific growth rate did not result in higher productivity. This may result from the fact that specific growth rate was determined in relation to the maximum substrate available in a system and not how much was used up by bacterial cells during batch fermentation.

3.5.7 Glucose consumption/production

As previously stated, sweet potatoes have amylases that covert starch into simple sugars, so do a lot of bacterial species used in this study such as *Bacillus*, *Klebsiella*, *Citrobacter* and *Enterobacter* (Beckord et al., 1945; Brena et al., 1993; Cudney and McPherson , 1993; Kumar and Das, 2000; Sajedi et al., 2005; Xie et al., 2008). This would explain an increase in glucose observed in all the experiments (Figs 3.7, 3.10 and 3.13).

Just like all other enzymes, amylases have their optimum pH and most researchers report that sweet potato amylases have a wide pH range of 4.0-5.0 (Balls et al., 1948; Sawai et al., 2004). An optimum pH of 6.0 has also been reported for sweet potatoes β-amylase (Noda et al., 2001). On the other hand, bacterial amylases work best between pH range of 4.0-6.0 (Kumar and Das, 2000; Sajedi et al., 2005). At pH 6.0, maximum glucose production was achieved (3 g/l) implying that at this pH amylases from the consortium of bacteria and sweet potatoes had higher activity compared to other experiments (uncontrolled pH experiments).

It should also be noted that there was no relationship between glucose production and consumption, indicating that increased starch conversion into glucose units does not necessarily mean increased conversion of glucose conversion into final products.

3.5.8 Dominating microbial populations

Sequencing results obtained from the first two quantification experiments revealed that there was a change in dominating bacterial species (Table 3.2). From observations made in the results, it can be deduced that change in product distribution observed in experiments that were carried out under uncontrolled pH conditions occurred because of change in the dominant microbial populations. This was further confirmed by the time mixed cultures took to change from one dominant species to another (maximum of 8 days). Reportedly, it takes a minimum of 4 days depending on the conditions for dominating microbial species to prevail (Horiuchi et al., 2002). Change in the dominant species was a result of different pH optima for different bacterial populations. Some bacterial species have their optimal pH for butyric acid in the range of 6.0-8.0, acetic acid in the range of 5.0-6.0 and propionic acid lie in the range of 7.0-8.0. Change in metabolic products that is brought about by a shift in a pathway in the same species as observed in the last experiment which involved the use of the M9 medium at the controlled pH 6.0 often happens in one day (Horiuchi et al., 2002).

3.5.9 Comparison of products obtained in this study with literature

Table 3.4: Fermentation products obtained in this study and other studies

			Product g/l						
Substrate	**Microorganism**	**pH**	HEt	HAc	HPr	HBu	H_2	CH_4	**References**
Cane molasses	*Clostridium tyrobutyricum*	6.0	-	2.3	-	40	-	-	Jiang et al., 2009
Glucose	Mixed culture	6.0	0.18	0.71	0.17	3.13	35	8	Horiuchi et al., 2002
Glucose	Mixed culture	6.0	5.9	29.6	12.9	32.4	1	0.037	Fang and Liu, 2002
Glucose	Digested sludge	5.5	8.8	10.7	2.1	20.5	13.1	0	Chunfeng et al., 2009
Sweet potato	Mixed culture	*	26.2	1.2	0.5	18.5	1.2	0.1	This study
Sweet potato	Mixed culture	*	21.6	11.2	7.4	17.6	3	20	This study
Sweet potato	Mixed culture	6.0	7.3	8.2	5.1	2.3	1.5	-	This study

*Fluctuating pH conditions,
HEt= ethanol, HAc= acetic acid, HPr= propionic acid, HBu= butyric acid, H_2= hydrogen and CH_4= methane

Fermentation product concentrations obtained from different substrates using different bacteria are shown above. Varying pH conditions used in this study resulted in overall higher product concentration; however, it should be noted that different bacterial species, conditions (species, pure cultures, mixed culture) and substrates were used in all these studies which could explain any difference in the concentrations of products obtained.

3.6 Conclusion

The pH of the medium does affect the final product distribution and the mixed cultures used in this study preferred fluctuating pH conditions over controlled pH conditions. In addition to this, high glucose production and high specific growth rate do not necessarily result in high productivity; on the other hand, high cell density means high productivity.

Butyric acid and acetic acid increases H_2 production but if both acids are present in the system then acetic acid has more effect. Increased H_2 production favours accumulation of propionic acid and high H_2 content leads to increased production of butyric acid. High concentrations of butyric acid and acetic acid in a system inhibit negative effects of propionic acid on H_2 production.

Chapter 4

Fermentation of sweet potato tuber under continuous operation

4. Introduction

4.1 Continuous culture system

The continuous culture represents an open and steady-state system where a constant supply of nutrients and a steady amount of biomass is maintained in the bio-reactor (Crueger, 1984; Gilbert, 1987). The culture volume and the cell concentration are both kept constant by allowing fresh, sterile medium to enter the culture vessel at the same rate that used medium is removed from the growing culture (Fig 4.1) (Hoskisson and Hobbs, 2005). A continuous culture therefore reaches a stage at which the levels of bacteria, bacterial products, media components, and waste products are constant (Gilbert, 1987; Minihane and Brown, 1986; Mkandawire et al., 2005). Under these conditions, the rate at which new cells are produced in the culture vessel is exactly balanced by the rate at which cells are being lost through the overflow and is related to volume and defined by the flow rate (Hoskisson and Hobbs, 2005).

During continuous operation, the exponential phase is maintained and therefore dominant; this way production is maximized as the most productive phase is maintained (Fraleigh et al., 1989). The principal advantage of continuous culture systems is that the rate of dilution solely controls the rate of microbial growth (Crueger, 1984; Hoskisson and Hobbs, 2005). Continuous system uncouples microbial growth from temporary environmental conditions seen in batch, increasing flexibility which in turn makes the continuous culture system superior to all other known systems both in academic and industrial sectors (Hoskisson and Hobbs, 2005).

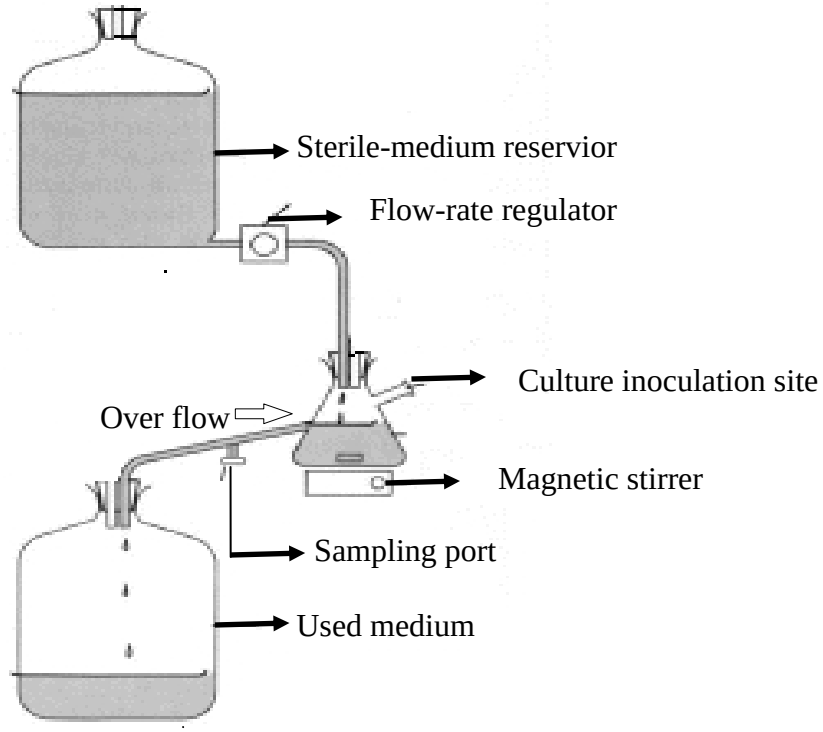


Figure 4.1: General set-up of continuous culture (Adapted from Hoskisson and Hobbs, 2005).

Compared to batch configurations, continuous system is mostly preferred for its ease to control variables or individual strains that determine the rate of reaction for maximized performance (Rani and Rao, 1999). This was further illustrated by Modak and Lim 1992, who studied continuous, repeated fed-batch and repeated batch modes of operation for fermentation processes. Their results revealed that for maximized bio-reactor productivity, continuous operation was the optimum mode of operation. Industrial applications that use the continuous system include butter production which involves continuous fermentation of milk (Rani and Rao, 1999).

Three common types of continuous mode of operation are 1. An auxosatat - a system in which part of the culture is set while other parameters including growth rate vary 2. A turbidostat - where the biomass concentration is predetermined 3. A chemostat - an operation where the

growth rate is kept constant while other constraints are regulated (Fraleigh et al., 1989). The latter is the most popular and has been employed for nearly half a century and still is a potent tool for research in biotechnology.

In theory, culture grown in a chemostat can be controlled at any rate between zero and a maximum value (an intrinsic property of the organism), by changing the dilution rate (Pirt, 1972; Hoskisson and Hobbs, 2005). At low growth rates (low dilution rates), a large amount of the substrate is used for cell maintenance instead of growth (Fraleigh et al., 1989). This is manifested by reduced concentrations of biomass and biomass-related products. As a result, chemostat culture at low dilution is not commercially favoured. The opposite is true in waste treatment processes since low biomass and low residual substrate concentrations are desirable in such processes (Hoskisson and Hobbs, 2005).

Chemostats have a lot of advantages over other configurations and these include:

(i) Growth rate can be regulated by varying substrate concentration. The concept was applied by Tempest and Herbert (1965), in determining the effect of growth rate on respiratory enzymes activity.

(ii) Growth rate can be kept constant while other constraints are varied and controlled (Hoskisson and Hobbs, 2005). This has added little to chemostat technology where the information outputs are relatively simple, but has a huge impact on collection and analysis of the complex data from batch and fed-batch processes and has enabled the processes to be controlled and optimized (Fraleigh et al., 1989).

(iii) Most importantly, chemostats are known for their efficient and quick conversion of substrate to growth-limited products (Pirt, 1972). This has made chemostat necessary for large-scale biomass production and for bioremediation.

Generally advantages of the chemostat are that certain biological parameters assumed to influence the outcomes can be controlled. The operation of the bio-reactor as a chemostat, is economically efficient from the viewpoint of production and is the most frequent means for large-scale production of bio-based chemicals (Pirt, 1972, Mhaskar and Aumi, 2008).

4.2 Biofilm reactors

Biofilm bio-reactors are a group of bio-reactors in which bacterial cells are found in an immobilized form (Van Loosdrecht and Heijnen, 1993). The attached bacterial cells can be attached either on the surface of an inert "carrier" or to one another, forming flocs (Saravanan and Sreekrishnan, 2006).

Biofilm bio-reactors are in general more efficient than other types of reactors because they maintain higher biomass concentration (Qureshi et al., 2005; Zilouei, et al., 2006). Of all biofilm reactors, FBBRs are the most popular (García Encina and Hidalgo, 2005). This is because they offer advantages such as higher substrate break down capacities and higher surface area (Heijnen et al., 1989; Bohlmann and Bohner, 2001; Ochieng et al., 2003; Zilouei et al., 2006). In addition to these, they offer even distribution of the liquid phase bringing about phase homogeneity which allows them to operate efficiently even at high volumes (Saravanane and Murthy, 2000; Ochieng et al., 2003).

4.3 Objectives

Continuous systems are mostly preferred for their ease to control individual strains that determine the rate of reaction for maximized performance (Rani and Rao, 1999). Above all, they are known for their fast conversion of substrate to products making them vital in large scale production (Pirt, 1972). Batch experiments were carried out in smaller scale under different conditions as explained in chapter 3. The aim of this study was consequently to increase product yields as well as to optimize product distribution using batch conditions that resulted in higher product concentrations. A continuous operation was set up in a 7 liter FBBR using mixed consortium.

Mixed cultures have been reported to have improved microbial activity and stability compared to pure cultures (Pirt, 1972). The mixed microflora used in this study was isolated from sweet potato tube as described in chapter 2.

4.4 Materials and Methods

4.4.1 Bio-reactor Description

The FBBR used in this study was constructed of transparent acrylic (perspex) with the following dimensions: 1 m high column with 10 cm internal diameter and a total volume of 7 cm^3. The bio-reactor had an outer water jacket connected to a water bath set at 55 ºC to account for heat loss during operation so that the column temperature was 30 ºC. The temperature was maintained by recirculating heated water from a thermostatic bath through the water jacket. The medium was fed into the reactor through the feeding pump and the effluent recycled through the recycle pump which connected the effluent outlet and the feed inlet. Soluble products were collected via the overflow port and gaseous products were collected from the top of the reactor (Fig 4.2).

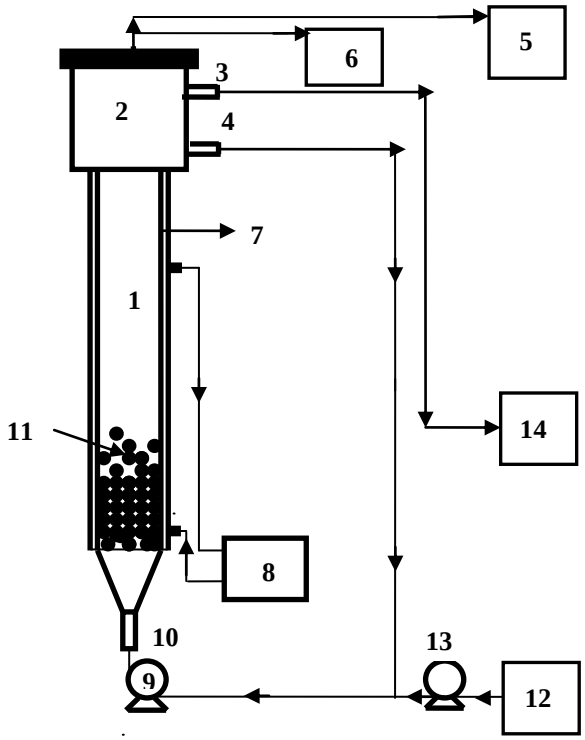


Figure 4.2: Schematic illustration of FBBR. 1- Column, 2- Gas sampling site, 3- Overflow site, 4- FBBR outlet, 5- Gas sample bomb, 6- pH meter probe, 7- Water jacket, 8- Water bath, 9- recycle pump, 10- FBBR inlet, 11- GAC, 12 Reservoir, 13- Feeding pump, 14- Product collection site.

4.4.2 Sterilization of FBBR

Prior to inoculation, the bio-reactor was rinsed with 20% sodium hypo chlorite-containing sanitizer for 1 hour. This was followed by the neutralizing buffer (Difco) for 1 hour and flushed with sterile distilled water as described by Lindsay et al. (2002).

4.4.3 Inoculum preparation

Bacterial consortium isolated from sweet potato tuber were grown in 1 cm^3 NB for four hours at 30 ^oC to allow them to reach their mid-log phase prior to bio-reactor inoculation.

4.4.4 Granular activated charcoal (GAC) as a carrier

GAC was used as a biofilm carrier or attachment medium in FBBR due to its slight electro-positive charge making it more attractive to bacterial cells. Different functional groups and pores on the GAC increase microbial attachment while its rough surface provides excellent shelter and protection for bacterial attachment.

4.4.5 FBBR set-up and operation

The sterilized bio-reactor was first filled with 300 g of GAC. Nutrient broth (1:10) was prepared, sterilized and added to the reactor. The medium was then inoculated with 1 m^3 of the bacterial consortium. The culture was allowed to grow in batch mode by recycling through the reactor for seven days to allow for growth of bacterial cells and the formation of biofilm on GAC particles. Cell growth was indicated by an increased turbidity of the medium. This was also done in order to avoid washout of inactivated biomass. Once sufficient turbidity was established, NB was replaced with a modified sterile M9 minimal medium containing 64 g $Na_2HPO_4.7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5.0 g NH_4Cl, 300 g of cut sweet potato tuber combined with 0.5 ml of 1M $MgSO_4$ and 25 µl of 1M $CaCl_2$/litre.

After a seven day batch operation, the system was then converted to continuous operation by continuously pumping sterile medium into the reaction chamber. To investigate the effect of flow rate on product distribution, the bio-reactor was operated for 42 days with operation at HRT of 6 hours, 3 days and 12 days over a period of 14 days. Data was collected everyday over a 14 day period. The temperature was maintained at 30 ºC by circulating heated water from a thermostatic bath through the column water jackets. pH was not controlled but measured.

4.4.6 Analytical methods

a) Liquid products

The liquid products (alcohols and volatile fatty acids) were analyzed using an Agilent 6820 gas chromatograph (Agilent Technologies, Supelco, 24107, USA) equipped with a thermal conductivity detector (TCD). The operational temperatures of the oven and the detector were 185 °C and 155 °C, respectively. The samples were separated on a fused silica capillary column (30 m × 0.25 mm, 0.25 µm film thickness) using Helium as the carrier gas at a flow rate of 20 ml/0.01min. Soluble products (1 ml) were collected from the bio-reactor, filtered through a 0.4 µl membrane filter and 0.2 µl of the sample injected manually into the GC.

b) Gas products

Gas samples were collected from the top of the reactor and analyzed using a Dani GC (Dani 1000) with pora pack Q column equipped with a TCD detector. The oven temperature was at 200 °C. Argon was used as the carrier gas. Injection port temperature was kept at ambient while the detector temperature was 120 °C. Peak identification of products was based on retention times of reference standards.

4.4.7 Morphology of attached populations by SEM.

GAC granules (1g) were obtained from the reactor and rinsed with sterile distilled water. The granules were first fixed in 1 ml of 3% aqueous glutaraldehyde at room temperature overnight. After fixation, they were rinsed with sterile distilled water once and gradually dehydrated in increasing concentrations of graded ethanol series (20, 30, 40, 50, 60, 70, 80, 90. 95 and 100%) at 10 minute intervals. They were then subjected to critical-point drying at 60 °C for 2 hours. The

particles were then mounted on SEM stubs, attached by silver glue and finally coated with normal gold/palladium for viewing on a Joel® 840 Scanning microscope (Lindsay et al. 2002).

4.4.8 Bacterial cell density determination

Bacterial cell growth was determined so as to reflect on bacterial metabolic activity (Kogure et al., 1979). Bacterial concentrations were determined by preparing serial dilutions of up to 10^{-5}. These were then plated onto Nutrient Agar (NA) plates using standard drop plate method. Plates were incubated at 30 ^{o}C overnight and growth was reported as colony-forming units per ml (cfu/ml).

4.4.9 Determination of continuous culture productivity

Under the continuous culture operation, the balance between cell formation and loss may be explained by Equation 4.1.

$$\frac{dx}{dt} = \textbf{Formation - Loss} \quad \text{(4.1)}$$

This can also be expressed as follows;

$$\frac{dx}{dt} = \mu x - Dx \quad \text{(4.2)}$$

Where

μ = Specific growth rate
X = Cell concentration
D = Dilution rate

Note: Dilution rate (**D**) is the rate of medium addition divided by the reactor volume. Since under steady state conditions, there is no change production rate, then,

$$\frac{dx}{dt} = 0 \quad \text{..... (4.3)}$$

Combining equations 2 and 3, results in Equation 4.4 as follows

$$D = \mu \quad \text{..... (4.4)}$$

Productivity of a continuous culture may be represented as

$$R_{cont} = Dx\left(1 - \frac{t}{T}\right) \quad \text{..... (4.5)}$$

Where

R_{cont} = Culture output in terms of cell concentration per time (Cfu /ml/d)
X = Steady state cell concentration
t = Time period prior steady-state establishment (d)
T = Time period during which steady-state conditions prevail (d)

Note: Hydraulic retention time (HRT) is the volume of the reactor divided by the medium flow rate.

From HRT and D definitions,

$$HRT = \frac{V}{F} \quad \text{and} \quad D = \frac{F}{V}$$

Where

F = medium flow rate (d^{-1})
V= Volume of the bio-reactor (L)

Therefore,

$$D = \frac{1}{HRT} \quad \text{(4.6)}$$

Substituting Equation 4.5 and expressing it in terms of HRT, then

$$R_{cont} = \frac{\left(1 - \frac{t}{T}\right)}{HRT} x \quad \text{(4.7)}$$

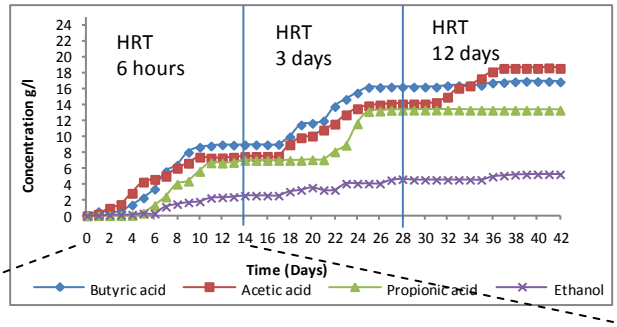
4.5 Results

4.5.1 The Effect of HRT on product distribution

Since HRT is one of the important control parameters affecting fermentation product distribution, concentrations of different products were determined at different HRTs. Production rates were calculated before steady state conditions were reached. Transition from a lower HRT to a higher HRT was done after steady state conditions prevailed (after 14 days). Cumulative concentrations of products at three HRTs over a 42 day period are also shown.

Cumulative concentrations are presented as small graphs while the actual concentrations at different HRT are shown in bigger graphs.

a)



b)

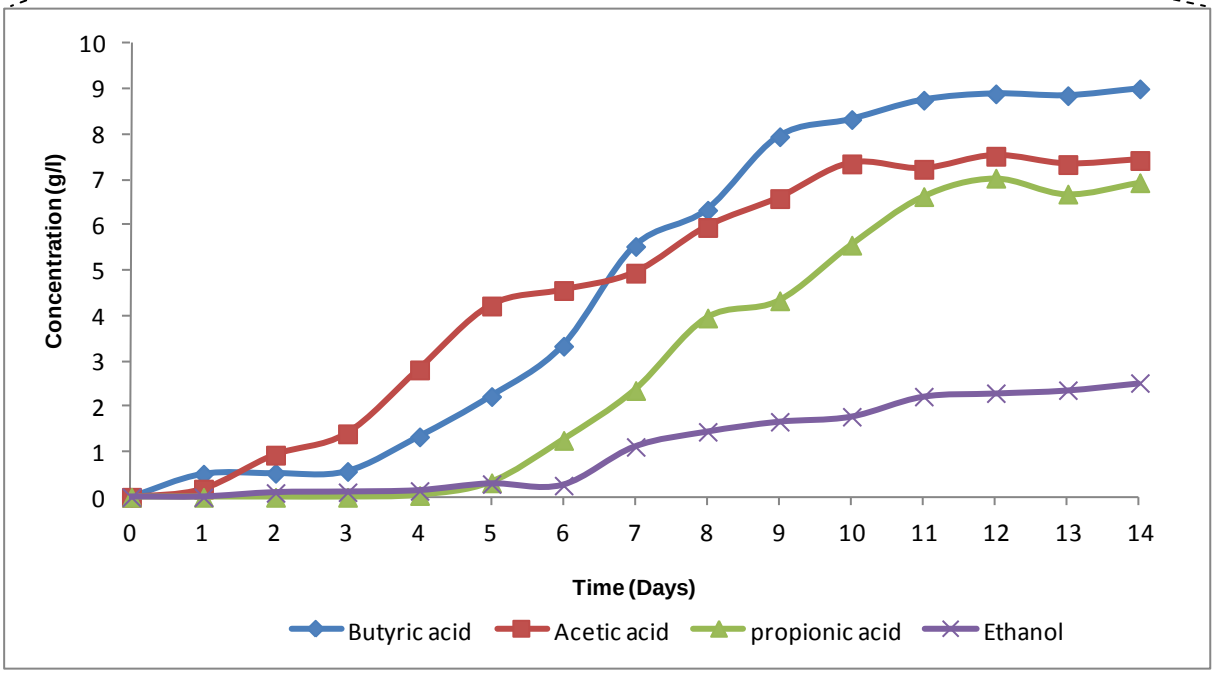


Figure 4.3: Liquids produced during bio-reator operation at HRT of 6 hours. a) Cumulative concentrations of liquid products for all three HRTs over a 42 day period. b) Actual concentrations at HRT of 6 hours for the first 14 days. Aqueous metabolites were analyzed every day and concentrations determined using standard curves (appendix B).

During the continuous operation, liquids products were collected and analyzed. To show product distribution pattern at different HRTs over a 42 day period, cumulative concentrations of products are shown (Fig 4.3 a). Acetic acid and butyric acid were the most dominant metabolites with maximum concentrations of 16.8 g/l and 18.5 g/l respectively. A change in dominating soluble products was seen as butyric acid dominated at HRT of 3 days while acetic acid dominated at the beginning of operation at HRT of 6 hours and for the last four days of operation at HRT of 12 days (Fig 4.3a).

For all three HRTs, propionic acid and ethanol were the least produced with ethanol being the lowest. Concentrations of 13.2 g/l and 5.18 g/l were recorded for propionic acid and ethanol respectively. There was no increase in propionic acid concentration after transition from HRT of 3 days to 12 days.

Closer look at the distribution pattern for the first 14 days shows that acetic acid dominated for the first six days with maximum concentrations of up to 7.5 g/l recorded (Fig 4.3b). Butyric acid gradually increased and dominated from day 7 to day 14 with a concentration increase from 7.5 g/l to 9 g/l. Acetic acid was produced at a rate of 0.8 g/l/d while butyric acid was produced at 0.92 g/l/d.

Propionic acid was produced with a maximum concentration of 7.04 g/l. Its production rate was 0.7 g/l/d. The concentration of ethanol was the least reaching a maximum of only 3 g/l at a rate of 0.22 g/l/d.

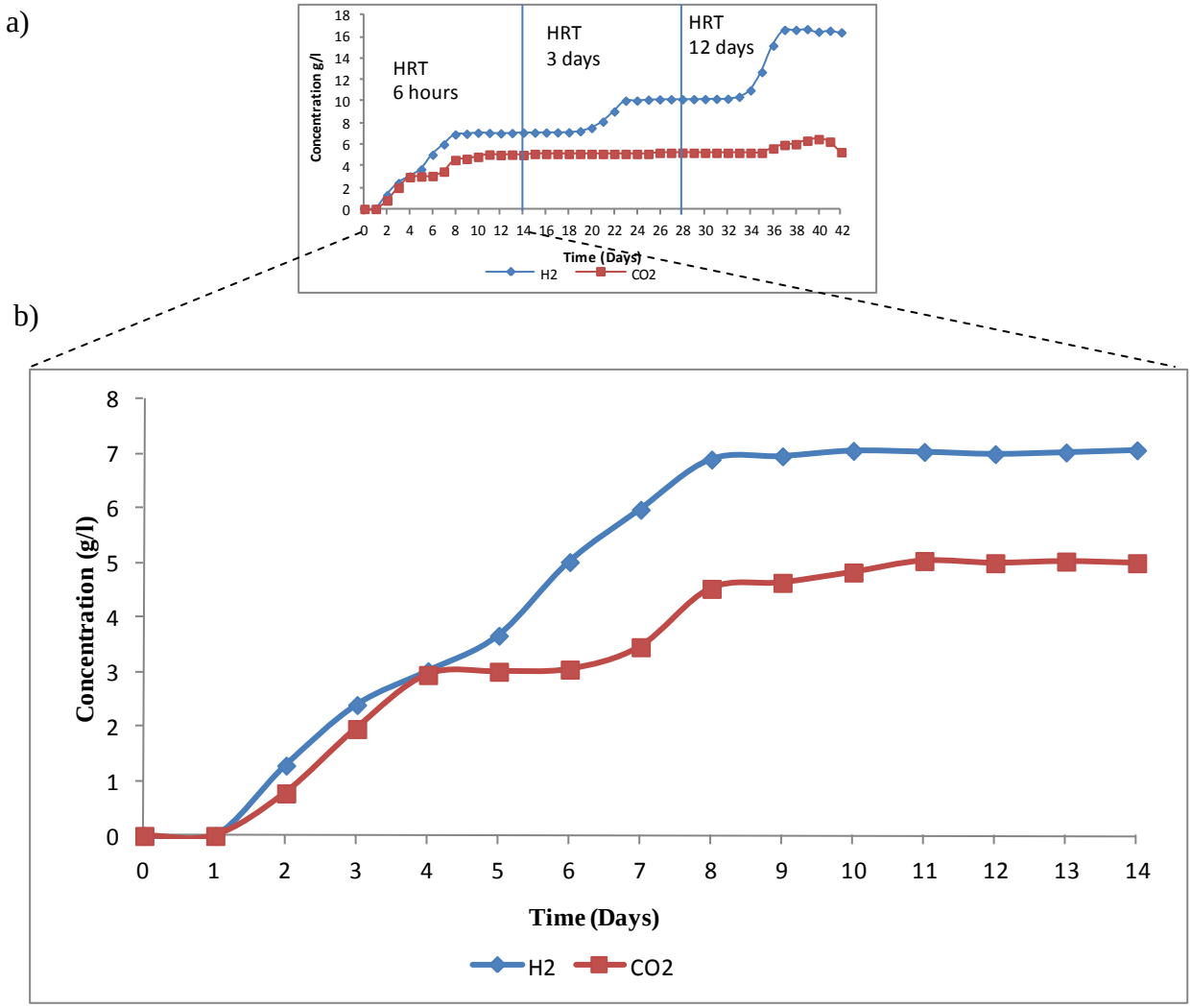
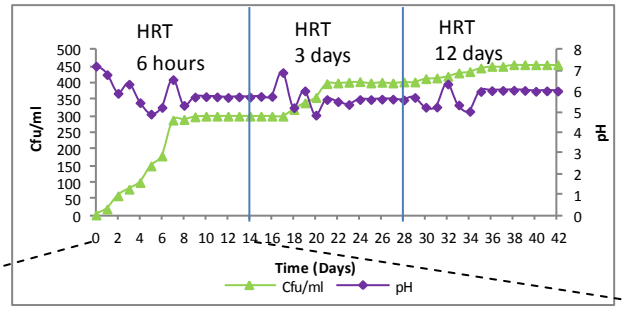


Figure 4.4: Gases produced during bio-reator operation at HRT of 6 hours. a) Cumulative concentrations of detected gas products at three HRTs over a 42 day period. b) Actual gas concentrations at HRT of 6 hours for the first 14 days. Gas analysis was done every day and the concentrations determined using standards curves (Appendix B).

Gaseous products were collected and analyzed. Cumulative concentrations of gases produced during continuous operations are also shown to indicate gas production and distribution over a 42 day at three HRTs (Fig 4.4a). For the first 14 days of operation at HRT of 6 hours, measurable amounts of H_2 and CO_2 were detected from day 2. H_2 dominated with a maximum concentration of 7 g/l at a rate of 1.08 g/l/d while CO_2 was produced at relatively lower rates of only 0.19 g/l/d with a maximum concentration of 5 g/l. H_2 reached a steady state on day 10 while CO_2 reached the steady state on day 11 (Fig 4.3b).

a)



b)

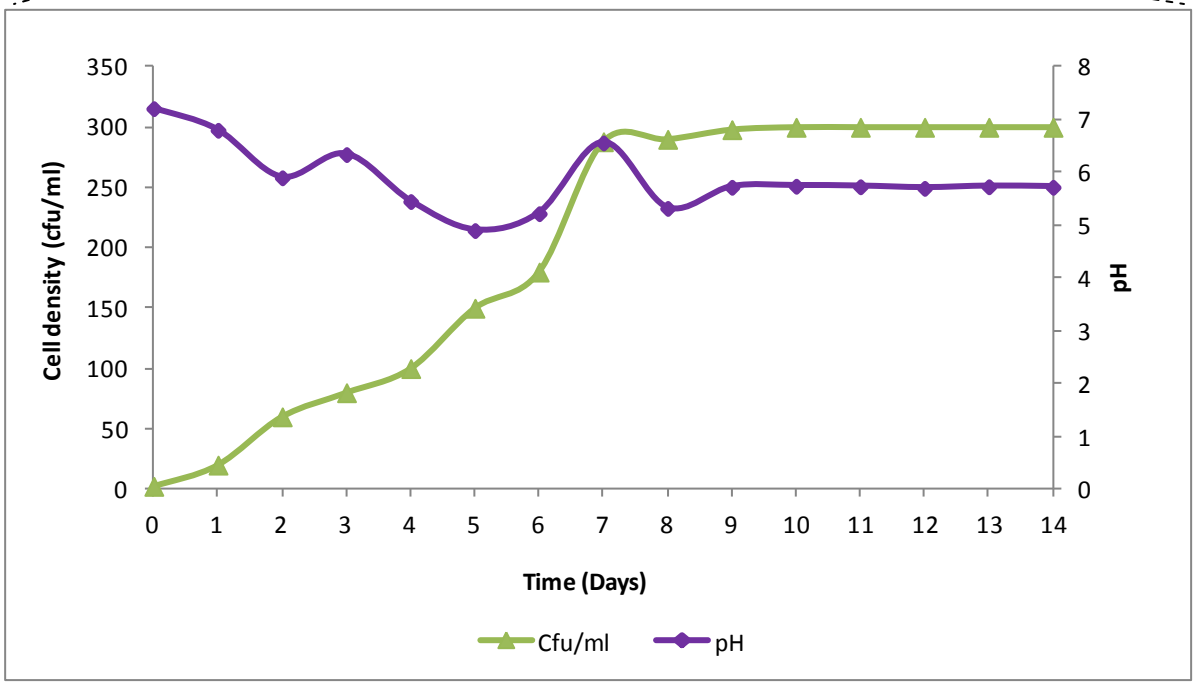


Figure 4.5: Cell density and pH during bio-reator operation at HRT of 6 hours. a) Cumulative cell density and pH at three HRTs over a 42 day period. Actual cell density at HRT of 6 hours for the first 14 days of continuous operation. pH was also recorded during bio-reactor continuous operation.

To indicate how cell density and pH varied at the three different HRTs over 42 days of continuous operation, cumulative cell density and pH were shown in Fig 4.3a. A closer look at operation at HRT of 6 hours for the first 14 days showed that at the beginning of the experiment, pH was 7.21 and at the end, pH decreased to 5.72 (Fig 4.4b). There was an increase in pH on day 7 from 5.22 to 6.56. The lowest pH recorded was 4.9 on day 5. A constant pH of 5.7 was observed from day 9 to day 14. A gradual increase in cell density was observed until day 7 when cell counts of 288 cfu/ml were recorded. Constant concentrations of 300 cfu/ml were obtained from day 9 to day 14.

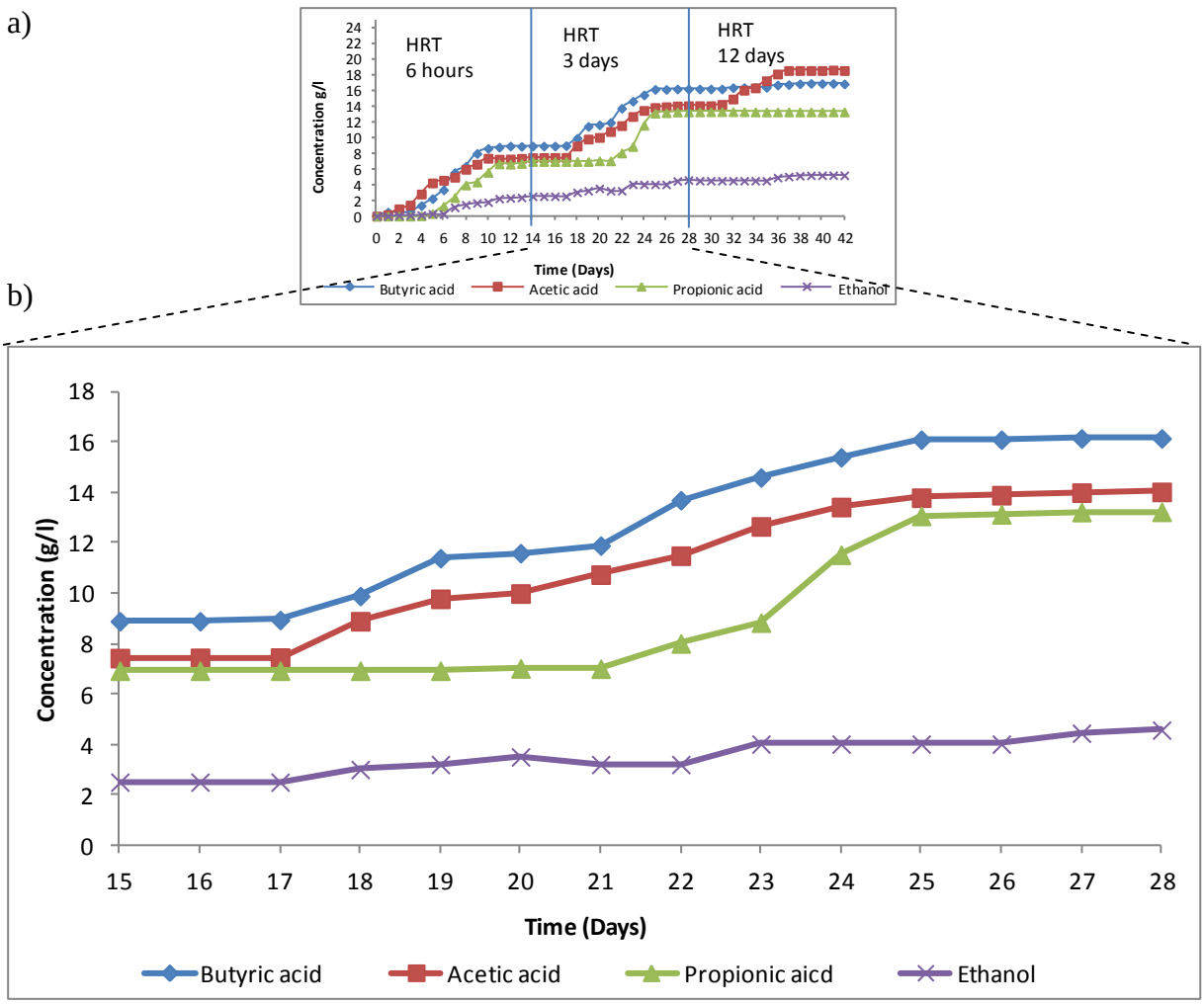


Figure 4.6: Liquids produced during bio-reator operation at HRT of 3 days. a) Cumulative concentrations of detected soluble products at three HRTs over a 42 day period. b) Actual product concentrations during operation of the bio-reactor at HRT of 3 days over a 14 day period after transition from HRT of 6 hours to HRT of 3 days. Liquid analysis was done every day and each detected product quantified.

When HRT was increased from 6 hours to 3 days, it was observed that butyric acid was the most dominant acid with a maximum concentration of 16.2 g/l produced at an average rate of 0.79 g/l/d. A constant concentration of about 16 g/l was measured from day 25 to day 28. There were considerably low ethanol concentrations recorded with maximum amounts of only up to 4.6 g/l at a production rate of 0.45 g/l/d.

Propionic acid increased from 7.04 g/l to 13.20 g/l and production rate increased slightly by 0.04 g/l/d on day 25. Acetic acid production also increased at a rate of 0.72 g/l/d reaching a maximum concentration of 14 g/l day on day 28.

a)

b)

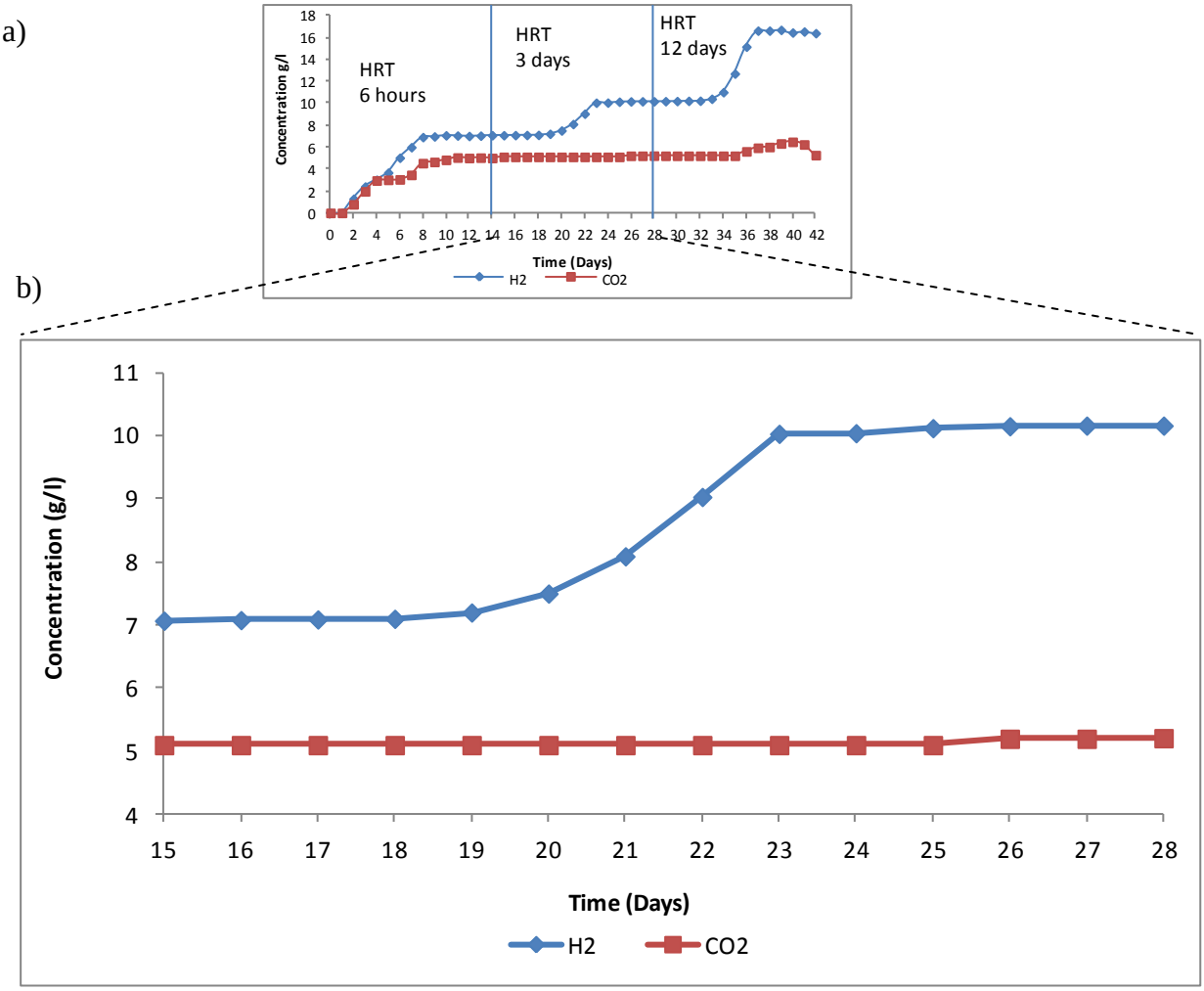
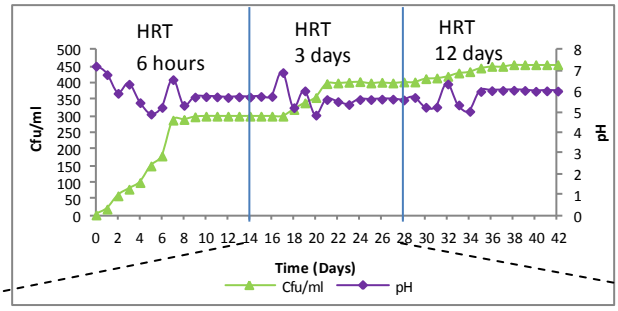


Figure 4.7: Gases produced during bio-reator operation at HRT of 3 days. a) Cumulative concentrations of gases produced at three HRTs over a 42 day period. b) Gas production over a 14 day period after transition from HRT of 6 hours to HRT of 3 days. Gas analysis was done every day and the concentrations determined using standards curves (Appendix B).

When HRT was changed to HRT of 3 days, H_2 increased to 10.1 g/l on day 23 at a rate of 1.01 g/l/d reaching a constant concentration of about 10 g/l. On the other hand, CO_2 production did not change and remained at 5.2 g/l.

a)



b)

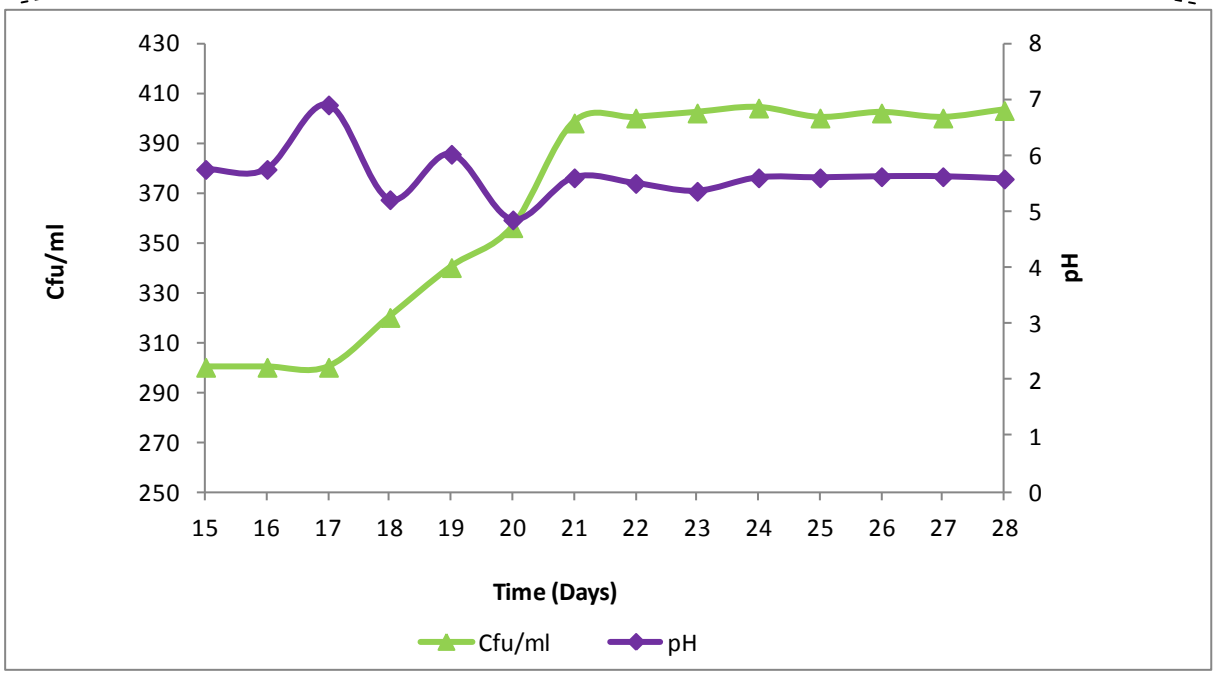


Figure 4.8: Cell density and pH during bio-reator operation at HRT of 3 days. a) Cumulative cell density and pH at three different HRTs over a 42 day period. b) Bacteria cell density and pH during bio-reactor continuous operation at an HRT of 3 days from day 15 to day 28.

During transition from HRT from 6 hours to 3 days, pH level of 5.75 was recorded. There was an increase in pH level on day 16 from 5.75 to 6.90 and another increase from 5.21 to 6.02 on day 19. The lowest pH recorded was 4.85 on day 20. However, pH stayed constant at 5.60 from day 24 to day 28.

No increase in cell density was observed until day 17 with a gradual increase from day 18 to day 21 after which a constant cell density of 400 cfu/ml was recorded.

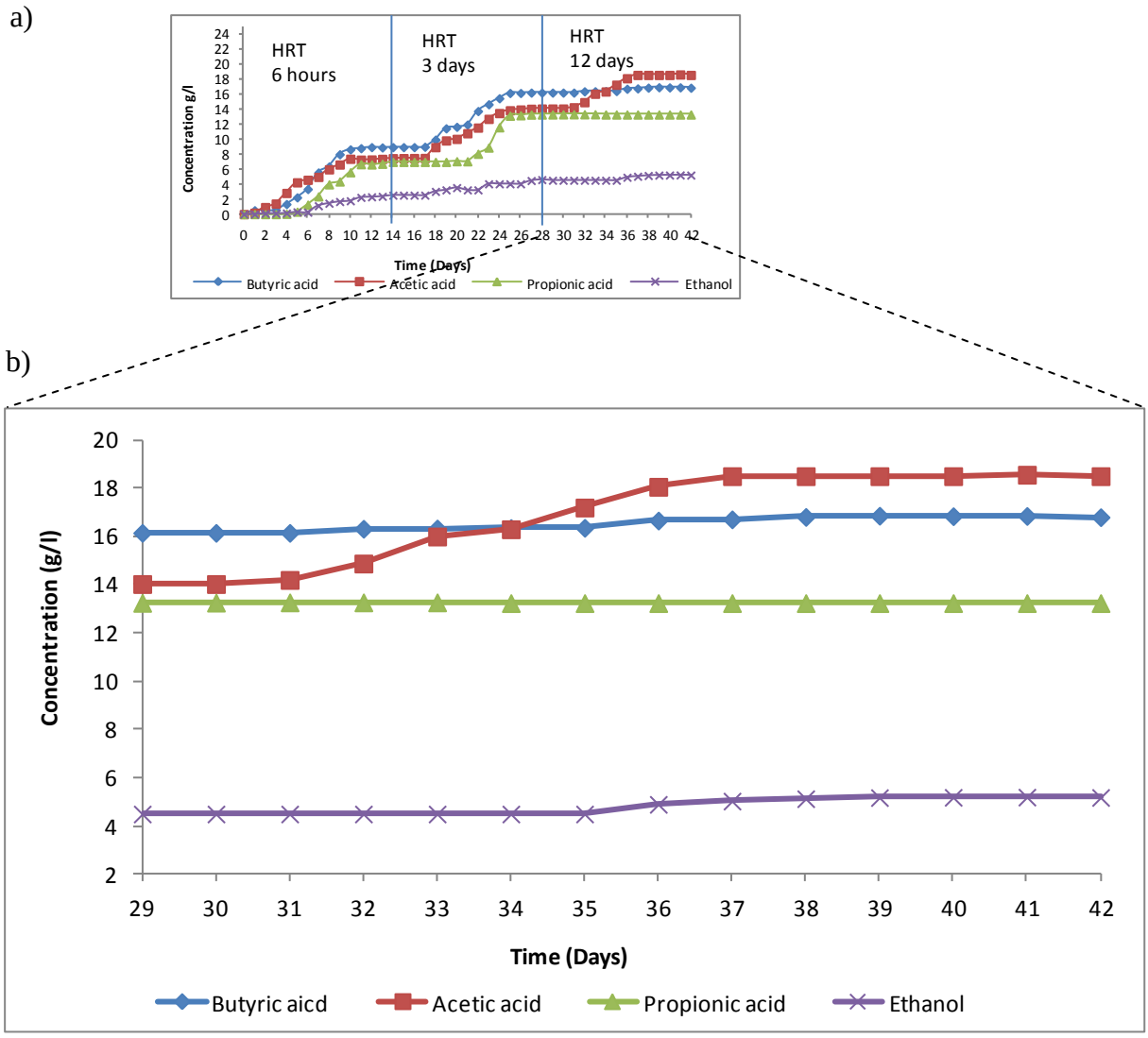


Figure 4.9: Liquids produced during bio-reator operation at HRT of 12 days. a) Cumulative concentrations of detected soluble products at three HRTs over a 42 day period. b) Detected liquid products at an HRT of 12 days over a 14 day period. Liquid products were detected using GC and quantified using standards curves (Appendix B).

Changing the HRT from 3 days to 12 days resulted in acetic acid now dominating. Acetic acid production further increased to 18.5 g/l and the rate also slightly increased by 0.8 g/l/d. Butyric acid only increased by 0.9 g/l and remained constant at 16.9 g/l until day 42. Ethanol was the least produced reaching a maximum of 5.2 g/l - an increace of only 0.6 g/l. Propionic acid however, did not increase but remained constant at 13.2 g/l.

a)

b)

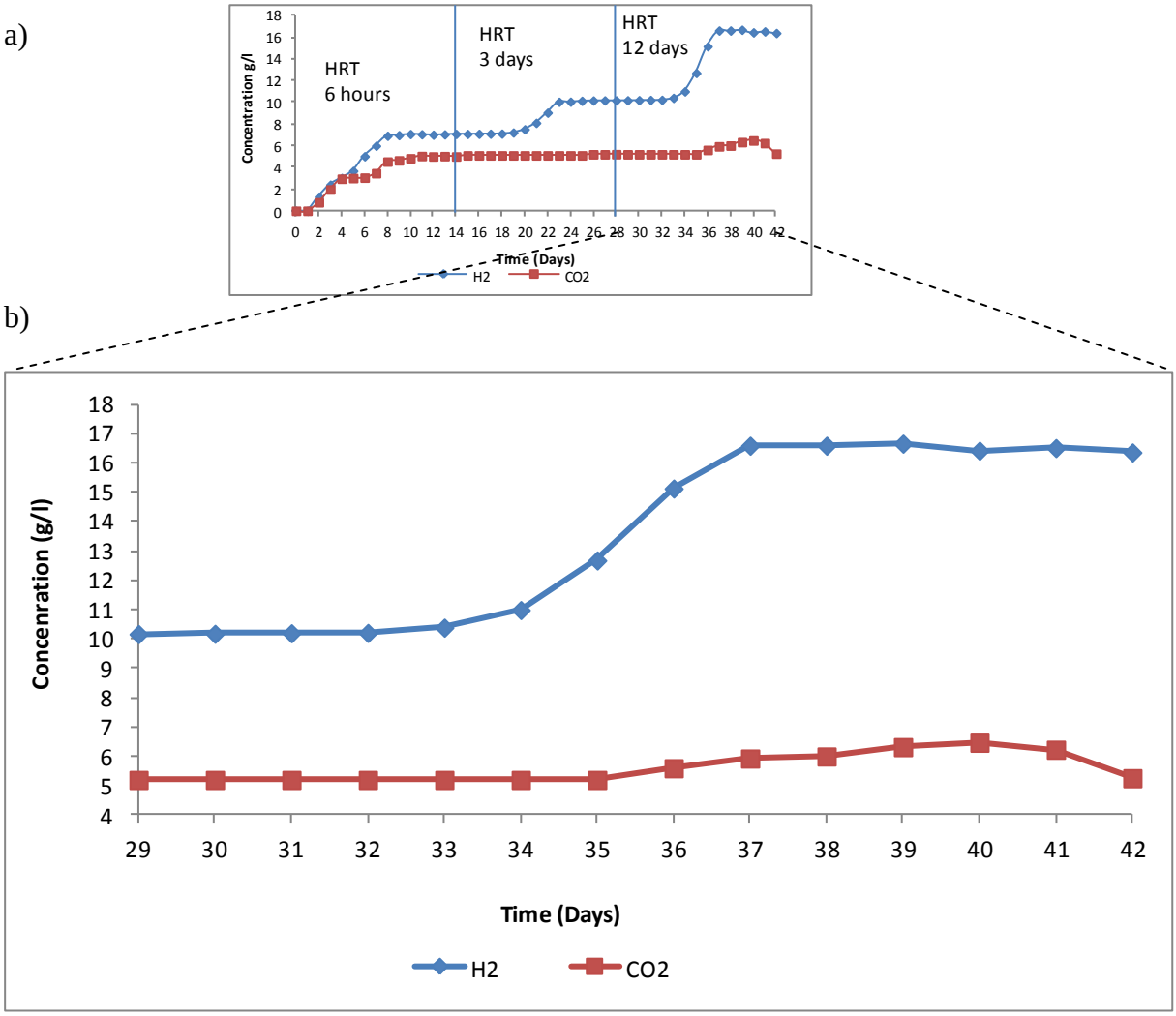


Figure 4.10: Gases produced during bio-reator operation at HRT of 12 days. a) Cumulative concentrations of gases produced at three HRTs over a 42 day period . b) Gas production at HRT of 12 days. Concentration of gases produced over a 14 day period at the HRT of 12 days. Gas analysis was done every day and the concentrations determined using standards curves.

At an HRT of 12 days, H_2 continued to increase reaching a maximum of 16.7 g/l. The rate of production also increased by 0.89 g/l. CO_2 concentration remained low but increased slightly from 5.2 g/l to 6.5 g/l at a production rate of 0.18 g/l/d. A decrease was noted on day 42.

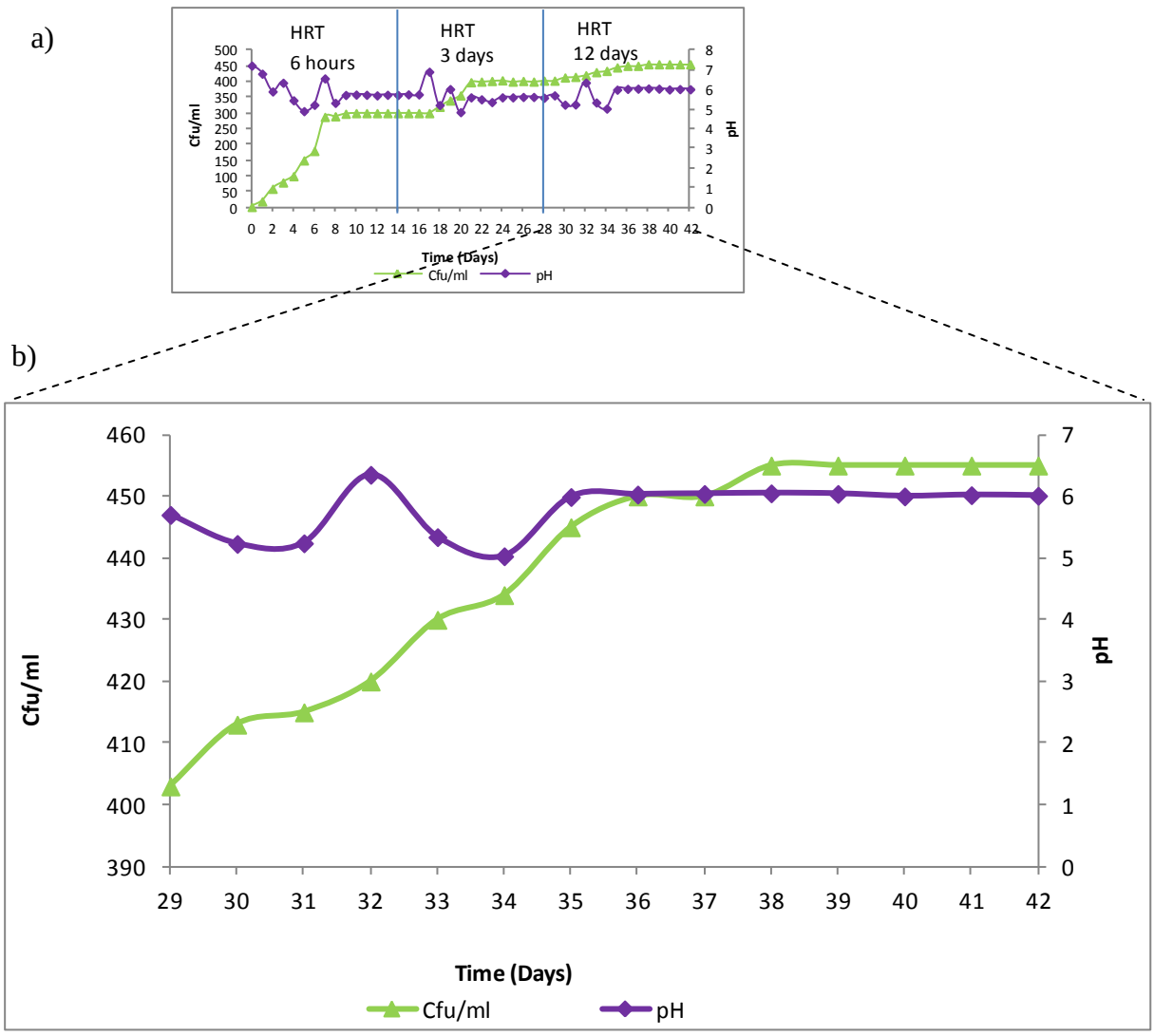


Figure 4.11: Cell density and pH during bio-reator operation at HRT of 12 days. a) Cumulative cell density and pH at three different HRTs over a 42 day period. b) Bacteria cell density and pH variation during continuous operation**.**

During transition of HRT from 3 days to 12 days, pH 5.75 was recorded in the beginning. There was an increase in pH level on day 32 from 5.24 to 6.35. The lowest pH recorded was 5.03 on day 34. There was no change in pH from day 35 to day 42 with pH levels of about 6.0 recorded.

A gradual increase in cell density was observed from day 29 to day 37. A constant cell concentration of 455 cfu/ml was recorded from day 37 to day 42.

Table 4.1: Productivity as calculated using Equation 4.7

HRT (days)	Productivity/R_{cont} (Cfu/ml/d)
0.25 (6 hours)	171
3	19
12	5.4

4.5.2 Scanning electron microscopy (SEM)

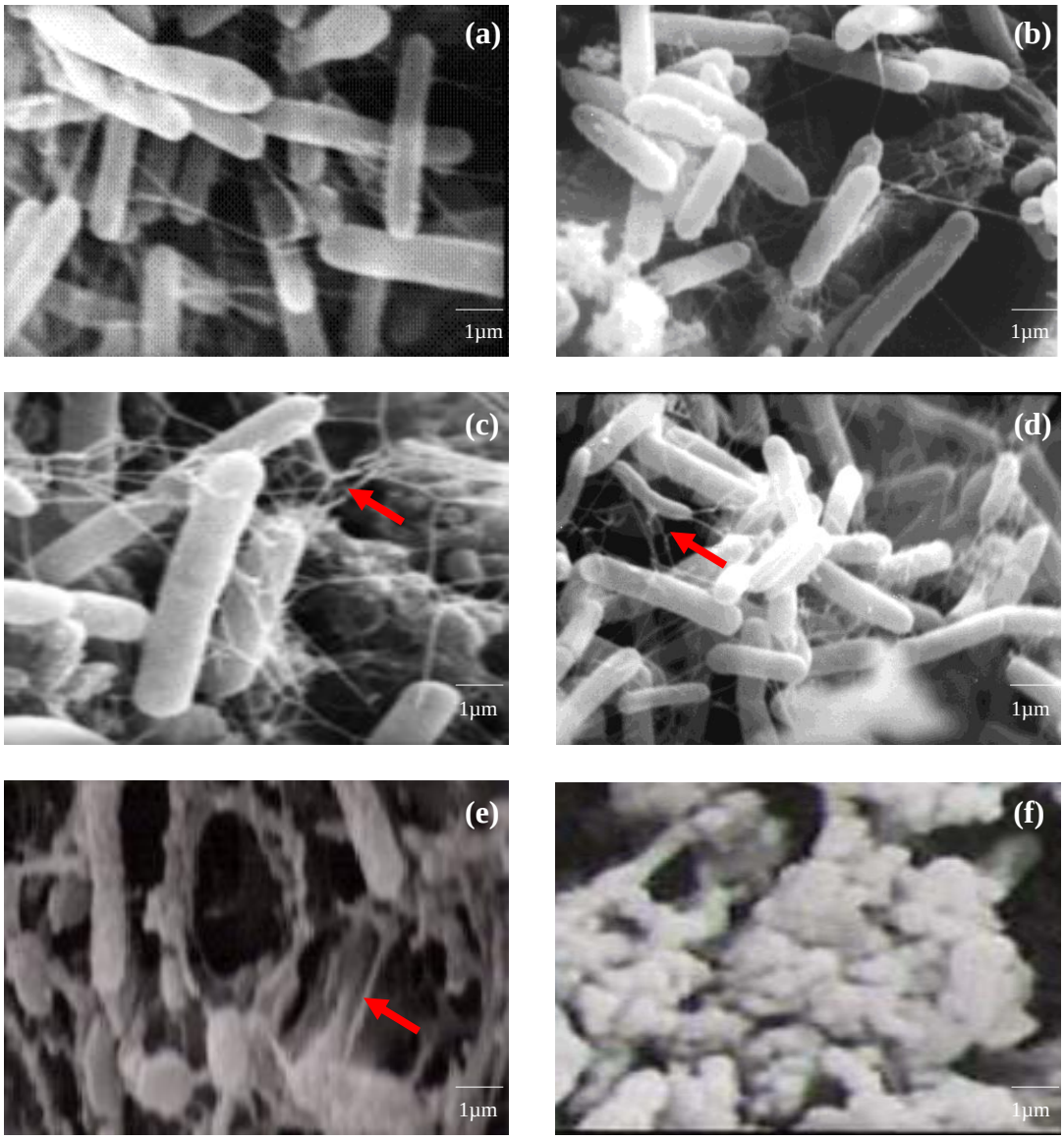


Figure 4.12: SEM micrographs of bacterial cultures used. The micrographs were taken before transition from one HRT to the next. Micrographs (a) and (b) were taken after the bio-reactor was operated at HRT of 6 hours. Micrographs (c) and (d) were taken just after HRT of 3 days and micrographs (e) and (f) were taken after HRT of 12 days.

SEM was used to observe biofilm morphology in laboratory-scale FBBR under continuous system. Micrographs showed that bacterial cells were evenly distributed on the surfaces of GAC and preserved their rod shape features. Most cells exhibited smooth surfaces, while differing shapes and sizes were also observed. All micrographs showed that some cells had clustered on top of each other.

Closer examination of GAC surfaces demonstrated bacterial cells embedded within an extracellular polymeric substance (EPS) (indicated by the red arrow). EPS is a matrix of a mixture of polysaccharides that encloses aggregates of bacterial cells in a biofilm (Bhinu, 2005). Mature biofilms covered in EPS were observed after 42 days of bio-reactor operation (Fig 4.7f).

4.6 Discussion

4.6.1 The effect of HRT on product distribution

Butyric acid and acetic acid were produced the most while propionic acid and ethanol were detected the least for all three HRTs (6 hours, 3 days and 12 days). This was expected since butyric acid and acetic acid are usually the most dominant liquid products in fermentation when bacteria consortium is used (Chen et al., 2001; Fang and Liu, 2002). Several reports indicate that products such as propionic acid are a result of different fermentation pathways that are due to altered electron equivalent distribution (Zhang et al., 2006; Sreethawong, 2010a). The observation that dominant soluble products detected were acetic acid and butyric acid, suggested butyrate-acetate fermentation type in the bio-reactor (Badiei et al., 2011). Microorganisms (species, oxygen demand) and fermentation conditions have also been reported to affect product distribution (Zhang et al., 2006).

High concentrations of H_2 were also recorded for all HRTs with high butyrate and acetate concentration but low propionate concentration. This observation was in agreement with a lot of studies that have reported a linear inverse relationship between propionate and butyrate formation and a linear relationship between acetate, butyrate and H_2 production (Cohen et al. 1995; Mizuno et al., 2000; Levin et al., 2004; Liu et al., 2006; Zhang et al., 2006; Antonopoulou et al., 2008; Mitchell, 2009; Li et al., 2010).

Generally, controversial results have been reported on the effect of HRT on product distribution. Some researchers have suggested that long HRTs favour acetic acid production while others have reported an increase in acetic acid at short HRTs (Dinopoulou et al., 1988; Hwang et al. 2001). In addition to this, some studies have revealed that at very high dilution rates in a shorter

retention time, i.e. near-washout point, microbial activity is maximal hence productivity (Tchobanoglous and Burton 1991; Grady, 1999).

A closer look at the whole system over a 42 day period revealed that running the system at HRT of 6 hours achieved maximum concentrations of 8.9 g/l, 7.4 g/l, 6.9 g/l and 2.5 g/l for butyric acid, acetic acid and propionic acid and ethanol respectively (Fig. 4.3a). However, a transition from HRT of 6 hours to 3 days resulted in production increase of 16.2 g/l, 14 g/l, 13.2 g/l and 4.6 g/l for butyric acid, acetic acid, propionic acid and ethanol respectively. This meant that increasing the HRT in a system resulted in prolonged active state of bacteria cells bringing about increased productivity. Nonetheless, increasing HRT to 12 days resulted in 16.8 g/l, 18.5 g/l, 13.2 g/l and 5.1 g/l for butyric acid, acetic acid, propionic acid and ethanol respectively. No product increase was noted except for acetic acid. These results suggested that at this HRT, only acetic acid production pathway was favoured. These findings are in agreement with those reported by Dinopoulou et al. (1988).

A different trend was observed for gases as there was an increase in H_2 concentration even at a longer HRT of 12 days (Fig 4.4a). These results imply that H_2 producing microorganisms or H_2 producing pathway was favoured even at this HRT. It should be noted that there was however, a slight decrease in CO_2 concentration. It can therefore be deduced that HRT of 12 days was mostly preferred for gas production as it resulted in increased H_2 production and decreased CO_2 production - a trend which is critical in renewable energy concept.

Looking at various HRTs used in this study individually, higher productivity was observed at the lowest HRT of 6 hours (171 cfu/ml/d) while lowest productivity was recorded at the highest

HRT of 12 days (5.4 cfu/ml/d, Table 4.1). This confirmed that lower HRTs are associated with increased microbial activity hence increased productivity (Tchobanoglous and Burton 1991).

Low productivity observed at HRT of 3 and 12 days (19 cfu/ml/d and 5.4 cfu/ml/d respectifully, Table 4.1) could be attributed to unfavourable conditions experienced by bacterial culture as a result of long HRT. These conditions may include starvation and accumulation of other products such as ethanol that may be toxic to bacteria (Mitchell, 2009). This observation suggested that at these retention times, the loading rate is so slow that no growth or metabolism can be supported (Grady, 1999). This could be a result of reduced supply of nutrients to bacterial cells over such long periods (Mitchell, 2009).

It should be noted that when HRT was changed from 3 to 12 days, no increase in propionic acid concentration was detected (Fig 4.9a). This indicated a shift in metabolic pathway which could be a result of either a change in dominating microbial populations or shift in catabolic pathway within the same species (Horiuchi et al., 2002). This observation provided evidence that HRT is an important factor in product distribution as has been reported by several researchers (Zhang 2006; Barros et al., 2010; Xiao et al., 2010; Badiei et al., 2011).

High productivity observed at HRT of 6 hours (Table 4.1) possibly resulted from sufficient nutrient influx resulting in maximum microbial activity at such high flow rates (20 ml/min) (Grady, 1999). In addition to this, at such high flow rates, any inhibitory substances that could be produced were easily washout which would mean increased substrate utilization hence increased product formation (Mitchell, 2009).

Ethanol is known as an unfavourable metabolite for H_2 generation (Badiei et al., 2011). Relatively consistent but low concentrations of ethanol were obtained in this study compared to

VFAs, i.e. ethanol concentration did not vary markedly with varying HRT. The presence of abundant VFAs suggested that H_2 production pathway was favoured (Mohan et al 2007; Sreethawong et al 2010b).

4.6.2 Cell density determination

In this study, cell density and viability was monitored and measured; fairly constant cell densities were recorded for all HRTs studied (Figs 4.5b, 4.8b, 4.11 b) illustrating that the immobilized culture was stable. This was also confirmed by relatively stable pH. Viable bacterial cells were maintained until day 42, suggesting that healthy cells were successfully retained throughout the fermentation period. The maintenance of healthy bacteria cells throughout operation suggested that bacteria growth rate was higher than possible wash out rate (Badiei et al., 2011). This would be expected as bacteria cells used in this study were immobilized resulting in minimized cell wash out hence increased cell density (Qureshi et al., 2005; Badiei et al., 2011).

4.6.3 Scanning electron microscopy (SEM)

One way to distinguish cells in a biofilm from their suspended counterparts is the presence of an EPS matrix (Bhinu, 2005). Biofilms have defined architecture which plays a vital role in microbial behaviour (Donlan, 2002; Guiot et al., 2002). Thus, the study of biofilm architecture is very important. Nonetheless, bacterial cells constituting the biofilm are very small; therefore, microscopes with a high resolution would be used to study biofilm morphology (Guiot et al., 2002). SEM has been successfully used to view biofilm morphology and arrangement (Davey and O'toole, 2000; Wimpenny et al., 2000).

The SEM micrographs (Fig 3.11) showed that particles of GAC were appropriate support materials for bacterial cell immobilization with cells uniformly distributed on the surface. SEM

micrographs also confirmed attachment of cells to GAC surfaces. Most cells had normal short-rod shape which would be expected of most species identified in chapter 2 (*Klebsiella, Enterobacter, Rhodobacter, Bacillus, Citrobacter, Alcaligenes* and *Bordetella*). In addition, the micrographs showed that some cells occurred in pairs or clusters, an indication of potential biolfilm formation (Guiot et al., 2002).

Since cell morphology changes have been associated with cell starvation, normal cell morphology observed in this study implied that cells had been grown under nutrient-rich medium (Jan et al., 2001; Allan et al., 2002). A trace element solution, organic nitrogen source and other nutrients were provided in the medium (Shim and Yang, 2002). The normal rod shaped morphology maintained by most cells can be attributed to the good growth conditions imposed by operating conditions used in this study.

4.7 Conclusion

A mixed culture of bacteria isolated from sweet potato tuber which was used to seed a laboratory-scale fluidized bed bio-reactor, successfully broke down carbohydrates found in sweet potato into bio-based industrial chemicals. This meant that this group of bacteria may be used for fermentation of other biomass.

HRT can be used to select for product distribution as different HRTs favoured formation of different products, e.g. HRT of 12 days favoured production of acetic acid and H_2 while HRT of 3 days favoured production of all soluble products (butyric acid, acetic acid, propionic acid and ethanol). Low HRT of 6 hours favoured formation of all products resulting in high productivity. In addition to this, biofilm consisting of multi-species were observed confirming that species

used in this study were capable of forming biofilms. FBBR technology may be efficient for biomass fermentation of biofuels and bio-based industrial chemicals.

Chapter 5

General discussion and conclusion

5.1 Isolation and identification of bacteria associated with sweet potato tuber

Eight bacteria isolates were confirmed based on their 16S rRNA by DNA PCR amplifications. The amplified PCR fragments were sequenced and compared with the 16S rRNA sequence database via the National Centre for Biotechnology (NCBI) site (http://www.ncbi.nlm.nih gov). The NCBI blast results revealed that the isolates belonged to a wide range of bacterial species which were *Klebsiella, Enterobacter, Rhodobacter, Bacillus, Citrobacter, Alcaligenes* and *Bordetella*. To further classify and determine relationships between species, a phylogenetic tree was constructed. The phylogenetic tree showed that isolates clustered with bacterial species known to break down a wide range biomass material into biofuels such as ethanol and H_2 as well as bio-based chemicals such as acetic acid and butyric acid.

Only populations with the ability to degrade carbohydrates in the sweet potato tuber were used in this study. This could be because bacteria used in this study are found in soil and associated with sweet potato tuber. This was evident in the observation that these bacterial species were capable of using sweet potato tuber as a carbon source. Reportedly, growth of bacterial cells on any surface or material means that the bacterial populations have the ability to utilize the carbon source on that material (Okerentugba and Ezeronye, 2003).

5.2 Fermentation of sweet potato under batch operation

In determining fermentation product spectrum and distribution using the eight isolates obtained from sweet potato tuber, batch experiments were performed. Ethanol, acetic acid, butyric acid, propionic acid, H_2 and methane were identified as sweet potato fermentation products. Different concentrations of these products were obtained under different fermentation conditions such as media type and pH level. This would be expected as previous studies have proven that different bacterial pathways are activated under different conditions.

A relationship between most products was noted in all experiments. For example production of both butyric and acetic acid resulted in increased H_2 production which can be expected as stoichoimetrically, production of these acids liberates H_2 gas. It was also noted that when both acids were present in a system, there was a linear relationship between H_2 production and acetic acid production and not to butyric acid. Another interesting finding was that, although production of propionic acid is known to use up H_2, high concentrations of butyric acid and acetic acid in a system reduce the negative effect of propionic acid production on H_2 production.

Highest cell density was observed with concomitant drop in glucose concentrations, implying that the drop was due to glucose utilization. This would imply that the cell growth energy comes from Embden-Meyerhoff-Parrnas (EMP) of the dicarboxylic pathway, which is a common route for most bacterial metabolic pathway from glucose to propionic and acetic acids (Choi and Mathews, 1994).

5.3 Fermentation of sweet potato tuber under continuous operation

Fermentation of sweet potato tuber using isolates mentioned previously resulted in production of the same products produced in batch experiments but at different concentrations. A laboratory-scale FBBR was operated at different HRTs. Higher productivity was observed at the lowest HRT of 6 hours, an indication that at this retention time, bacterial cells were most active. GAC was used as a carrier for bacteria to allow for bacteria immobilization. Immobilized cell systems were preferred in this study because of findings that immobilized cultures are associated with high density of active cells, high surface area and good operational stability which results in improved reaction rates (Sharanagouda and Karegoudar, 2002; Wang et al., 2004 ; Patil et al., 2006).

SEM was successfully used to view cell morphology. The observed EPS matrix was an indication that biofilms were established in the bio-reactor. Rod shaped cells attached on GAC were also observed which would be anticipated of dominantly rod bacterial species used in this study.

5.4 Significance of the present study

Many small sweet potato farmers successfully harvest lots of crops each year in order to sell. However, most of these farmers are faced with a problem of lot of surplus and do not know how to efficiently use it. It is important to explore and find additional applications of these crops. However, due to ethical issues such as food vs fuel, this has been neglected in research. This study therefore provides insights to how else sweet potato tuber could be used instead of being

disposed as waste. This information could be used in the development of bio-reactors for effective fermentation of carbohydrate rich biomass.

Results obtained in this study show that a robust system maybe employed on farms using naturally occurring bacterial consortium for converting surplus sweet potato into useful biofuels and bio-based industrial chemicals. Bacteria used are usually found in soil and associated with sweet potato tuber. These baceterial are capable of breaking down biomass to valuable alcohols, VFAs and gases. It is very important to use accurate techniques in isolating and quantifying bacterial populations because a better understanding of the microbial diversity and quantification of viable biomass would provide information on mechanics of industrial fermentation unit and eventually aid in achieving higher reactor performance.

The mode of operation is one of the key parameters in the optimization of bio-reactor performance. Sweet potato tuber fermentation was performed under batch and continuous modes. Each of these modes of operation, have advantages and disadvantages with respect to stability and performance. This study also gives details on how different flow rates may affect bacterial cell growth and product distribution, hence, fermentation of biomass.

As mentioned earlier, FBBR operational performance allows for reduced space requirements. This would be particularly advantageous for fermentation industries that very often have space limitations and ferment large amounts of biomass.

5.5 Considerations for future work and limitations

Application of fermentation for production of biofuels and bio-based chemicals is a well known concept. These products are normally produced as very dilute mixtures since production is normally quite low. The important step would therefore be separation of these mixtures in dilute aqueous solution to pure chemicals. This way, individual chemicals could be obtained and used for their respective industrial applications. Even though there have been reports that separating chemicals from each other and large amount of water can be more costly to even compete with production of the same chemicals from fossil fuel sources, fossil fuel depletion and ever increasing prices of petrochemical based products has given scientists no choice but to see renewable energy through since it is the only hope. As a result, a study based on more economical way of separating fermentation products from one another and water would be very important.

Activated charcoal was used as a support material for bacterial cells. However, no flocks were formed. This shows that attachment of bacterial cells to biofilm carriers is a very complex process. It has been reported that attachment of bacterial cells to support materials is affected by several factors such as substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and various properties of the cell surface. Since good adhesion of bacterial cells to its support material is a critical factor in determining bacterial efficiency in fermentation process, a study on these factors would be very important. It would therefore be necessary to carry out experiments investigating the effects of these variables on attachment of different support material. This way, the variables could be controlled and conditions optimized in such a way that the optimum attachment is achieved.

A limitation in this study was that only SEM was used to visualize bacterial morphology. Although this technique provides information on bacterial adhesion, other alternatives for studying hydrated microbial cells and cell viability, such as confocal scanning laser microscopy (CSLM) could have been used as well.

Chapter 6

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Appendix A

16S rRNA sequences of isolates from sweet potato tuber

Isolate 1 (JF920410)

```
  1 gctctcgggt gacgagtggc ggacgggtga gtaatgtctg ggaaactgcc tgggggacct
 61 tcgggcctct tgccatcaga tgtgcccaga tgggattagc tagtaggtgg ggtaacggct
121 cacctaggcg acgatcccta gctggtctga gaggatgacc agccacactg gaactgagac
181 acggtccaga ctcctacggg aggcagcagt ggggaatatt gcacaatggg cgcaagcctg
241 atgcagccat gccgcgtgta tgaagaaggc
```

Isolate 2 (JF920411)

```
  1 gggtgacgag tggcggacgg gtgagtaatg tctgggaaac tgcctgatgg aggataacta
 61 ctggaaacgg tagctaatac cgcataacgt cgcaagacca aagaggggga ccttcgggcc
121 tcttgccatc agatgtgccc agatgggatt agctagtagg tggggtaacc tcacctaggc
181 gacgatccct agctggtctg agaggatgac cagccacact ggaactgaga cacggtccag
241 actcctacgg gaggcagcag tggggaatat tgcacaatcc ccgcaagcct gatgcagcca
301 tgccgcgtgt atgaagaacc ccttcgggtt gtaaagtact ttcagcgggg aggaaggtgw
361 tgaggttaat aacctcrkca attgacgtta cccgcagaag aagcaccggc taactccgtg
421 ccagcagccg cggtaatacg gagggtgcaa gcgttaatc
```

Isolate dy4 (JF920417)

```
  1 tggcggacgg gtgagtaatg tctgggaaac tgcccgatgg agggggataa ctactggaaa
 61 cggtagctaa taccgcataa tgtcgcaaga ccaaagaggg ggaccttcgg gcctcttgcc
121 atcggatgtg cccagatggg attagctagt tggtgaggta acggctcacc aaggcgacga
181 tccctagctg gtctgagagg atgaccagcc acactggaac tgagacacgg tccagactcc
241 tacgggaggc agcagtgggg aatattgcac aatgggcgca agcctgatgc agccatgccg
301 cgtgtatgaa gaaggccttc gggttgtaaa gtactttcag cggggaggaa ggggttaagg
```

Isolate 2B (JF920415)

```
  1 tgctctcttg gcggcgagtg gcggacgggt gagtaatata tcggaacgtg cccagtagcg
 61 ggggataact actcgaaaga gtggctaata ccgcatacgc cctacggggg aaaggggggg
121 atcgcaagac ctctcactat tggagcggcc gatatcggat tagctagttg gtggggtaaa
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241 agacacggcc cagactccta cgggaggcag cagtggggaa ttttggacaa tgggggaaac
301 cctgatccag ccatcccgcg tgtatgatga aggccttcgg gttgtaaagt acttttggca
361 gagaagaaaa g
```

Isolate 1A (JF920412)

```
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 61 gggataacta ctcgaaagag tggctaatac cgcatacgcc ctacggggga aaggggggga
121 tcgcaagacc tctcactatt ggagcggccg atatcggatt agctagttgg tggggtaaag
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241 gacacggccc agactcctac gggaggcagc agtggggaat tttggacaat gggggaaacc
301 ctgatccagc catcccgcgt gtatgatgaa ggccttcggg ttgtaaagta cttttggcag
```

Isolate 2A (JF920414)

```
  1 gcccttcact ctgggataag cactggaaac ggtgtctaat actggatatg cacaatggcc
 61 gcatggtctg ttgtgggaaa gatttatcgg tgaaggatgg gctcgcggcc tatcagcttg
121 ttggtgaggt agtggctcac caaggcgacg acgggtagcc ggcctgagag ggtgaccggc
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301 acctctttta gtagggaaga agcgaaagtg acggtacctg cagaaaaagc accggctaac
361 tacg
```

Isolate 1B (JF920413)

```
  1 gctctcttgg cggcgagtgg cggacgggtg agtaatatat cggaacgtgc ccagtagcgg
 61 gggataacta ctcgaaagag tggctaatac cgcatacgcc ctacggggga akgggggga
121 tcgcaagacc tctcactatt ggagcggccg atatcggatt agctagttgg tggggtaaag
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241 gacacggccc agactcctac gggaggcagc agtggggaat tttggacaat gggggaaacc
301 ctgatccagc catcccgcgt gtatgatgaa ggccttcggg ttgtaaagta cttttggcag
361 araagaaaag gtacctccta atacgaggta ctg
```

Isolate 2C (JF920416)

```
  1 ggcggacggg tgagtaatat atcggaacgt gcccagtagc gggggataac tactcgaaag
 61 agtggctaat accgcatacg ccctacgggg gaargggggg gatcgcaaga cctctcacta
121 tkggagcggc cgatatcgga ttagctagtk ggkggggtaa aggctcacca aggcaacgat
181 ccgtagctgg tttgaragga craccagcca cactgggact garacacggc ccaractcct
241 acgggaggca gcagtgggga wttttggaca atgggggaaa ccctgatcca sccwtcccgc
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```
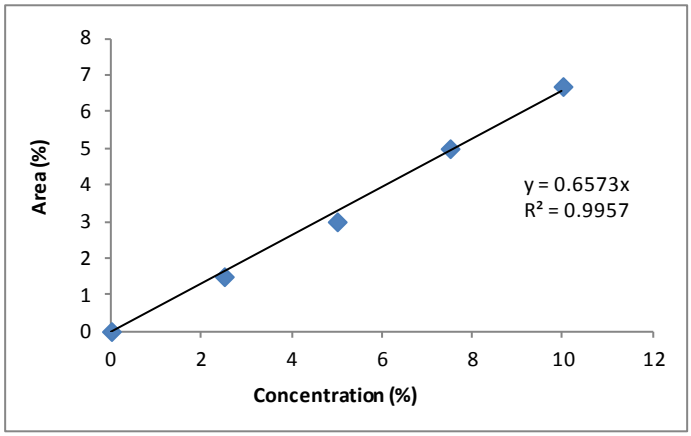
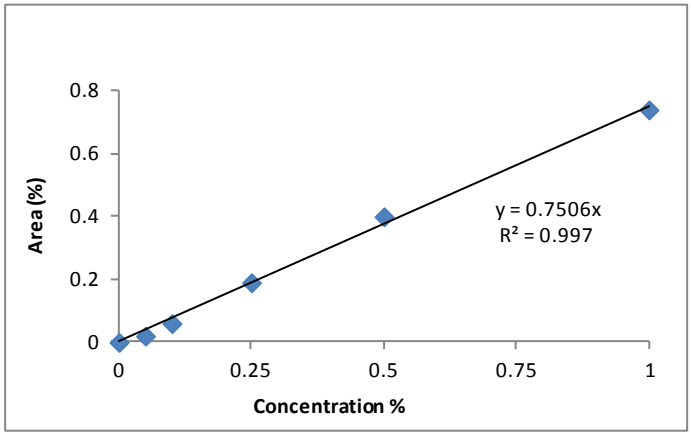

Appendix B

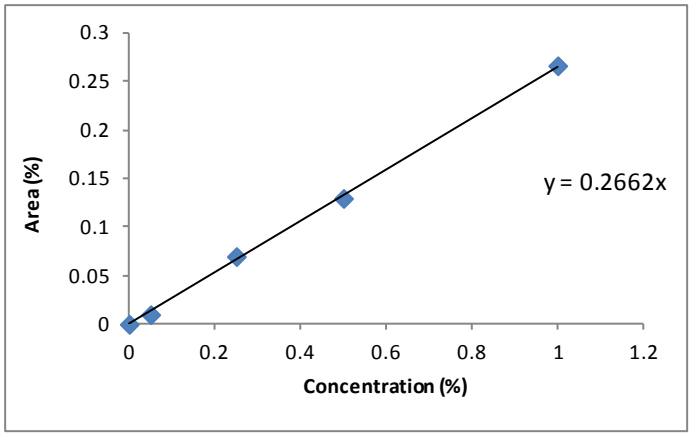
Calibration curves of liquid standards used to determine concentrations in GC



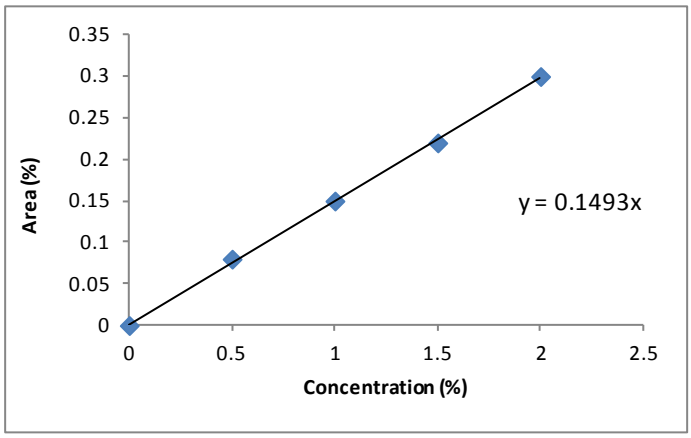
Ethanol starndard curve



Butyric aicd standard curve

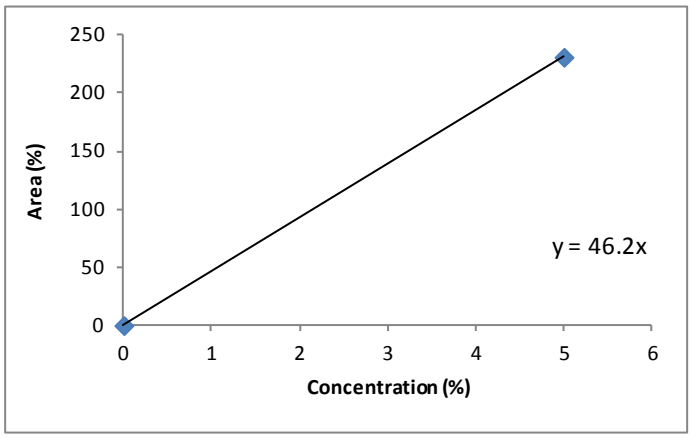


Propionic acid starndard curve

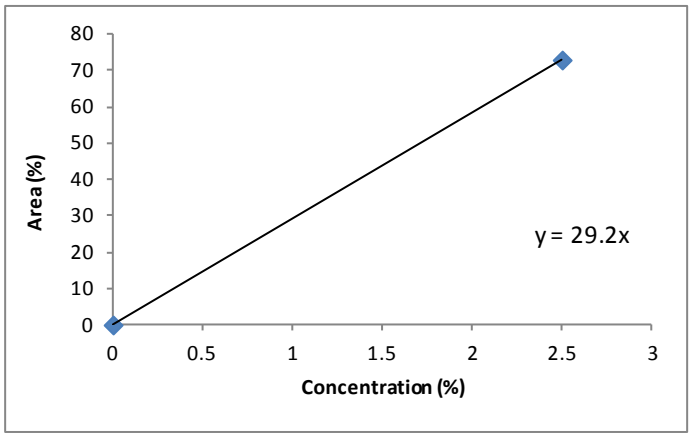


Acetic acid standard curve

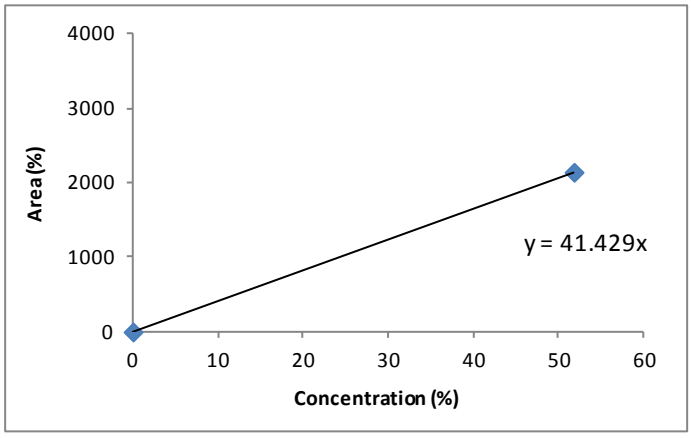
Calibration curves of gas standards used to determine concentrations in GC



Hydrogen standard curve



Methane standard curve



Carbon dioxide standard curve