Investigation of brewery waste grains and microbial fuel cells as value-additive technologies improving solvent production yields in *Clostridium acetobutylicum* (ATCC 824) fermentation

A thesis submitted in the fulfilment of the requirement for the degree of

MASTER OF SCIENCE

in BIOTECHNOLOGY

RHODES UNIVERSITY

by

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February 2023

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Abstract

The production of the solvent compounds acetone, ethanol and butanol through fermentation of organic feedstocks using *Clostridia* species could be a promising route for biofuel production. However, the cost of raw materials, low yields and the complexity of anaerobic fermentation continue to hinder this means of generating these compounds. The research presented in this Thesis investigated low-cost interventions that could decrease the costs of production and to direct the synthesis of fuel compounds using microbial fuel cells. Low-cost anaerobic chambers were designed and constructed for the propagation and manipulation of *Clostridium acetobutylicum*, selected as a low-risk microbial catalyst. Fermentation was monitored using *in situ* pH measurements and a combination of turbidity measurements, nutrient assays (especially total carbohydrates) and HPLC-RI detection as a means of monitoring the consumption of nutrients (glucose), production of precursor compounds (butyric acid) and the formation of solvent molecules (acetone/ethanol and butanol) during fermentation by this organism. Brewer's spent grains were tested as a sustainable and low-cost feedstock for solvent production, comparing the effects of sterilising before fermentation, or allowing resident microflora to remain during *Clostridium*-catalysed solvent production. Sterilised spent grains significantly improved the production of solvent molecules (e.g. 12.97 ± 0.38 g/L of butanol yielded, compared to 0.40 ± 0.33 g/L for defined media sampled during the solventogenic phase); compared to these, the use of non-sterilised brewer's grain decreased both the reproducibility and yields of fermentation (8.66 ± 1.6 g/L of butanol). Microbial fuel cells were studied as a possible means of altering electron transfer to/from electrode-attached *Clostridia* to control the metabolic shift in bacteria from acidogenesis to solventogenesis. The base line MFC (11.00 ± 4.69 g/L) fermentation experiment did produce higher acetone/ethanol than the baseline batch experiment MB (5.47 ± 4.48 g/L), indicating an improvement to solvent production in *C. acetobutylicum* (ATCC 824) in a MFC fermentation. In this study, MFC-1 demonstrated remarkable superiority over MB in terms of butyric acid production, yielding significantly higher concentrations while also improving acetone and ethanol production. However, the enhanced butyric acid production did not correspond to significantly increased butanol yields when compared to batch fermentation of chemically defined media. These findings highlight the potential of MFC-1 as an efficient approach for enhancing the fermentative production of valuable compounds, with a particular focus on butyric acid and acetone/ethanol.

Acknowledgements

There are many people who come and go in and out of your life. I have been fortunate to have many people who have contributed to this dialogue. A professor once said to me, research is like a conversation and a conversation can last a lifetime. I dedicate this thesis to my late forefathers, my grandfather, Ben and father, Andre, who imparted the ability to see the beauty in the simple things and respect all people and their differences. My conversation with them was cut short too early but I can be grateful they are liberated from the suffering of the living.

To the imperviously strong women in my family; Mom, without your dedication to my education and your relentless work ethic I would not be writing this, you are an incredible woman and I have yet to meet a woman as strong as you. To Nana Anna for her perseverance in always showing love, joy and compassion, I am so proud of you. To Jade for doing my homework with me when there was no one else. To my partner Sam who has been there every step of the way, without whom I would not be writing this.

To friends Chris and Stef Uys for always considering my wellbeing. The hours spent with your family have been some of the best times of my life over the last decade. To Mark and Sasha Dawe for their love and support in the final hours upon the Sashalon. Thank you to a friend I made during this project, Mr. van Rooyen for machining the anaerobic chambers.

Profesors Andre' Calitz and Margeret Calitz for their professional support.

Dr S. Abboo for the introductory training on the HPLC. To Marvin and Shirley for their assistance in the Electron microscopy unit.

Thank you to (Sarchi) NRF and co-supervisor Prof. Limson for Funding the research and providing financial support.

Special thanks to Prof. Earl Prinsloo for all the support and understanding. The world will be a better place with more scientists like you.

To my Supervisor Dr Ronen Fogel. If I said that your patience and grace for me is abundant. It would be an understatement. Your inspiration within biotechnology and perseverance with me during this project is honourable. Over such a long haul with so many obstructions and hurdles I cannot express my gratitude for having you as a supervisor. Thank you.

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List of Abbreviations

ABE	Acetone Butanol Ethanol
ATCC	American Type Culture Collection
BSG	Brewers Spent Grain
CNM	Clostridial Nutrient Media
DAFF	Department of Agriculture, Forestry's and Fisheries
DNA	Deoxyribonucleic acid
EDTA	Ethylenedinitrilo-tetra-acetic acid disodium salt dihydrate
FePc	Unsubstituted iron (II) phthalocyanine
×*g*	relative centrifugal force (multiples of gravitational force equivalents)
HPLC	High performance liquid chromatography
M	Molar concentration
MB	Media B
MEC	Microbial electrolysis cell
MFC	Microbial Fuel cell
Nox	Nitrous Oxides
NSBSG	Non-sterile brewers spent grain.
PEM	Proton exchange membrane
pH	Potential Hydrogen
redox	Reduction/oxidation
RI	Refractive index / refractive index detector
SBSG	Sterile Brewers spent grain.
SEM	Scanning Electron Microscopy
***spp*.**	Species
UV	Ultraviolet radiation
V	Volts

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Chapter 1: Introduction

1.1 Background

1.1.1 Fossil fuel demand

The use of non-renewable sources of fuels further intensifies environmentally-damaging effects such as extreme weather, raising sea levels, food shortages and the collapse of natural ecosystems (Gomez, Steele-King and McQueen-Mason, 2008). Excessive pollutants such as CO_2 and NO_x from combustion of fossil fuels plague the security of future natural resources. Although a grave concern worldwide, a major driving force for further research is the rising costs of energy for transport and industry (Melendez et al., 2022; Rip Jeon et al., 2023; Nigam and Singh, 2011).

The main issue that is being faced today is the reduction of CO_2 emissions, linked to anthropogenic activities such as burning fossil fuels. The global demand for fuel-based energy cannot be met with conventional oil fuels alone but can be supplemented with biomass-based fuels (Nigam and Singh, 2011).

Biomass-based fuel sources provide alternative energy solutions, which surpass our global demand for energy (Ibrahim et al., 2017a). Terrestrial plants produce more energy than the rate at which fossil fuel derived energy is consumed, globally. The introduction of fuel supplements derived from fermentation processes provides an environmentally conscious solution to the growing economic demand for energy (Jeswani, Chilvers and Azapagic, 2020).

There are shortcomings and added costs in development of customised facilities and infrastructure to produce biofuels. Findings from further investigation suggest that certain types or processing methods of biofuels have a greater environmental and economic impact, compared to non-renewable fuel sources (Jeswani et al., 2020).

To meet the global demand for fuel production by bacterial fermentation as opposed to yeast bio-ethanol production or biodiesel trans-esterification, bacterial fermentation is technologically still a long way from being a true contender (Green, 2011; Walker, 2009). With the implementation of current technologies such as metabolic and genetic engineering and machine learning tools for bioconversion, these advancements are positive prospects for efficient biofuel production models

in the future (Joshi and Mishra, 2022; Melendez et al., 2022; Rip Jeon et al., 2023). Greater research on biofuels is required to provide solutions to global energy demands and mitigate growing environmental challenges (Lee, 2012). As a result of the increasing demand for energy feasibility of petrochemical products is becoming economically unviable, further incentivising the need for biofuels (Van Der Merwe et al., 2013).

1.1.2 Organic biomass and feedstock

The term biomass refers to matter derived from a biological source contains chemical energy. Biomass is a renewable energy source that refers to organic matter, such as plants, cells and waste products that can be used to generate heat, electricity and biofuels. Examples of biomass include wood, crops (such as corn and sugarcane), agricultural and forestry waste (such as straw, sawdust, and manure) and organic municipal waste (sewerage and food waste). Treatments are necessary for lignocellulosic biomass to liberate fermentable sugars for biofuel production (Devi et al., 2016).

At its simplest, the combustion of biomass as a source of energy is considered a renewable resource and its use can reduce greenhouse gas emissions, compared to the use of fossil fuels. However, the sustainability of biomass energy depends on the specific type of biomass and the methods used for its production and utilization (Gomez et al., 2008; Pothiraj, Kanmani and Balaji, 2006).

The energy stored in biomass can be used to feed bacteria to be converted into biofuels through processes such as fermentation (Daniell, Köpke and Simpson, 2012). The mixture of compounds comprising biomass are subjected to biochemical changes by exposure to enzymes and other organic products produced by the catalysing organism (Kheyrandish et al., 2015). The resulting products from the breakdown of the feedstock are typically increased biomass of the catalysing cell and the formation of metabolic products, which include specific chemical compounds that are desired from the intended industrial fermentation (Hassan et al., 2015; Walker, 2009).

1.1.3 Overview of fermentation

Fermentation is a metabolic process in which microorganisms, such as bacteria, yeast, or fungi, convert organic compounds, such as sugars or carbohydrates, into simpler compounds, such as

alcohol or lactic acid, in the absence of oxygen (White, Yohannan and Walker, 2008). This process is used in various industries, including food and beverage production, biofuel production, and pharmaceuticals. All living cells have energy-generating metabolic pathways that convert chemical energy stored in organic compounds into usable energy in the form of ATP (adenosine triphosphate). This process can be categorised as predominately occurring either through respiration processes or fermentative processes (Albert, 2008).

For most industrial organisms, biochemical fermentation occurs under anaerobic conditions, where a carbon source such as glucose and an active cellular organism such as yeast, are present (Albert, 2008). Depending on the organism used in the fermentation, understanding the effects of the presence of oxygen on fermentation can strongly influence the yields and array of metabolites produced. In the presence of oxygen, the most effective form of cellular respiration can take place, aerobic respiration. ATP is produced efficiently under aerobic respiration by the complete oxidisation of the carbon source (e.g. glucose or starch) and the regeneration of NAD+ through the use of molecular oxygen as an electron acceptor. In the absence of oxygen (or other suitable electron acceptors for anaerobic respiration), the organism regenerates NAD+ through the oxidation reaction of NADH via fermentation so that glycolysis can be induced by sufficient NAD+ in the presence of glucose as a carbon source (Ding et al., 2018; Albert, 2008).

During respiration, the pyruvate produced from glycolysis undergoes further oxidation by oxidative phosphorylation, ultimately converted to carbon dioxide and water end products by the electron transport chain. In contrast, fermentation emphasises ATP production via the glycolytic pathway and does not require the electron transport chain. While both respiration and fermentation utilize NAD+, respiration produces more ATP per glucose molecule compared to fermentation. This is because respiration can extract more energy from glucose by fully oxidizing it to carbon dioxide and water, whereas fermentation only partially oxidizes glucose to produce a limited amount of ATP, releasing organic compounds as end products (Huberts, Niebel, and Heinemann, 2012).

In the context of fuel production, fermentation centres on the conversion of sugars or other organic compounds into short-chained aliphatic compounds, such as butanol, through the action of microorganisms. The majority of industrial fermentations utilise microorganisms as the biocatalyst rather than enzymes in other bioprocessessuch as ABE fermentation (Qureshi et al., 2010; Xue et

al., 2013). Microbial fermentations can be one of many solutions to address the current fuel crisis (Moon et al., 2016a; Nigam and Singh, 2011). The specific type of biofuel produced depends on the type of microorganism used and the type of feedstock and the conditions of the fermentation process. For example, yeast can be used to ferment sugars into ethanol, while some bacteria can produce biohydrogen or biobutanol through the fermentation of organic matter (Luo et al., 2015; White, Yohannan and Walker, 2008).

In general, the focus of fermentation for the production of biofuels is to provide a renewable and sustainable source of energy that can replace traditional fossil fuels. By harnessing the power of microorganisms to convert organic matter into fuel, the goal is to reduce carbon emissions and promote a more sustainable future of energy consumption. The sustainability of fermented products is directly correlated to the substrate utilized in fermentation.

1.1.4 Fermentation for biofuel production

In the context of the feedstocks that are used to produce the various types, biofuels can be classified into first-, second- and third-generation biofuels. Through thorough research and development, the categorisation of biofuels has provided a better understanding of the manufacturing process (Awogbemi et al., 2021). Conventional biofuels are often related to first-generation or developed biofuels as their productions are less complex and generally rely on the use of a food source as feedstock for the bioprocess. The drive towards researching and developing advanced biofuels are the result of environmental concerns, as there is competition for natural resources such as land, water and energy between fuel and food production when producing first-generation biofuels. This crisis could be addressed through innovative methods, including second-generation biofuels (derived from cellulosic biomass), third-generation biofuels (algae biomass) or fourth-generation biofuels and includes genetic modification and nanotechnology applications (Walker, 2009).

For a viable solution to the growing demand for energy, a mixture of technologies and fuel sources will be imperative. Higher alcohols such as butanol from bacterial fermentations has gained increased attention over other alcohol biofuels, or more specifically ethanol, as butanol offers a higher calorific energy value, low volatility, low corrosiveness and hygroscopicity (Ebrahimian, Denayer, and Karimi 2022; Moon et al. 2016a; W. Lee 2012).

The higher calorific energy value of the biofuel product is a result of increased hydrogen- carbon and hydroxyl group covalent bonds which are broken down when the energy is released during combustion. Butanol is a four-carbon alcohol and ethanol is a two-carbon alcohol. The increased length of the hydrocarbon carbon chain changes the calorific value of the two separate alcohols. Higher alcohols, referring to alcohols with greater number of carbon than methanol ethanol for example, have a greater thermodynamic efficiency (Veza, Said,and Latiff, 2019)

Currently, bioethanol is blended with fossil fuels in some countries as a supplement. Although the mandate for reduced greenhouse emissions has been proved on a global stage by blending bio-ethanol with petroleum fuels, the incentive for increased biofuel production is limited as a result of socio-economic influences in developing countries especially due to the lack of resources and government support (Mvelase, Ferrer and Mustapha, 2023). Microbial bioprocessing by algae, fungi and bacteria requires further research as possible alternatives to produce organic solvents and biofuel supplements to existing petroleum based fuels (Mujtaba et al. 2023).

1.1.5 Bio-ethanol fermentation by yeasts

Yeast-based fermentation is carried out by a variety of yeast species, such as *Saccharomyces cerevisiae* (White et al., 2008). This type of fermentation is commonly used to produce alcoholic beverages, such as beer, wine and distilled spirits.

Yeast-based fermentation is widely used in the production of bioethanol, a biofuel produced by the fermentation of sugars derived from crops (Luo et al., 2015). The production process typically involves the hydrolysis of carbohydrates into glucose, followed by the fermentation of glucose into bioethanol by yeast. The bioethanol is then distilled to concentrate and purify it, removing impurities such as proteins and lipids. Despite the significant advancements that have been made in bioethanol production using modern yeast fermentation systems, the bioethanol industry continues to face challenges, such as competition with food production for a source of fermentable carbohydrates, as well as concerns about the environmental impact of bioethanol production. Nevertheless, bioethanol remains a critical component of the biofuel industry and efforts are ongoing to further improve the efficiency and sustainability of bioethanol production using modern yeast systems (Bianco et al., 2020; Shin, Zeikus and Jain, 2002).

Modern yeast systems have significantly improved the efficiency, with yields of 23 to 81% of bioethanol production (Bianco et al., 2020). These systems utilize highly efficient yeast strains that have been specifically selected and genetically modified for their ability to ferment sugars into bioethanol in large-scale bioreactors. The implementation and development of modern yeast systems over the last two decades has enabled the bioethanol industry to increase production while reducing costs and improving the sustainability of bioethanol production (Bianco et al., 2020; White et al., 2008).

Biochemically, ethanol alcohol fermentation similar to butanol biosynthesis, is a reduction reaction of acetaldehyde which consumes NADH to produce a hydroxyl group forming the alcohol product and regenerating NAD^+ (Arimura, 2000). The major difference between yeast and bacterial fermentation is the direct conversion of pyruvate to acetaldehyde for subsequent ethanol production that would include additional metabolic steps for bacterial fermentation. Acetaldehyde is therefore, used as an electron acceptor in the absence of oxygen (Albert, 2008), with the produced ethanol subsequently released by the cell. Figure 1.1 provides an illustration of the breakdown of glucose to form ethanol through yeast fermentation.

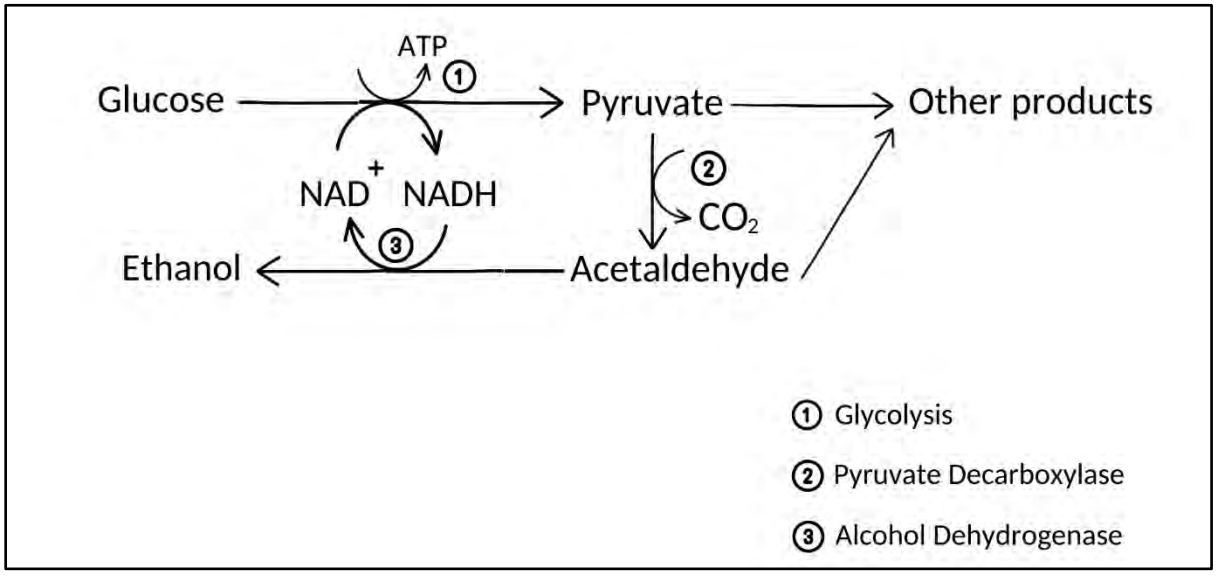

Figure 1.1: Overview of the metabolic intermediates during ethanol biosynthesis by the fermentation of glucose by yeast.
The regeneration of NAD+ from NADH by Alcohol dehydrogenase ③ to form ethanol in the presence of glucose for glycolysis ① to produce pyruvate and subsequent enzymatic conversion of pyruvate-by-pyruvate decarboxylase② to release CO2 and acetaldehyde. Adapted from Arimura (2000)

The main difference between yeast and bacterial fermentation is the end-products produced and the specific enzymatic pathways used. Yeast and bacterial fermentation of glucose differ in the enzymes involved and the products produced. In both processes, the NAD+ is needed for glycolysis to proceed and produce some ATP. However, yeast fermentation requires the enzymatic step of Pyruvate decarboxylase, which commits the pyruvate formed by glycolysis to be fermented as opposed to undergoing respiration. This results in the production of ethanol and carbon dioxide as end-products. The anaerobic fermentation of butanol by *Clostridium spp.*, central to the work reported in this thesis, shares many similarities to yeast alcohol fermentation (Luo et al., 2015) and is discussed in greater detail below.

1.1.6 *Clostridium* fermentation of ABE compounds

Fermentations producing combinations of acetone, butanol and ethanol (ABE fermentation) has historically played a significant role in human development in the last century using *Clostridium acetobutylicum*, classified as BioSafety Level 1 organism (i.e. mostly considered to be safe to manipulate and to culture). Advancement of molecular and biotechnological techniques has provided a platform where butanol derived from *Clostridia* fermentation, can be used in conjunction with conventional petrol or as a substitute (Lapuerta, Ballesteros and Barba, 2017; Wu et al., 2015).

A major downfall of bio-based butanol is the low fermentation yield. Butanol by existing bacteria and the energy-expensive downstream processing separation methods required to purify this fuel. The increased yield and abundance of raw materials have the potential to align the process with a sustainable practice, made possible using current knowledge and technologies, which can increase the ABE concentration from fermentation to 26.64 g/L; more specifically, yields of butanol up to 17.3 g/L have been reported (Kheyrandish et al., 2015; Qureshi et al., 2010; Su et al., 2020), typically in the range between13 and 15 g/L of butanol under optimum conditions (Chen et al., 2013).

The growth cycle of *C. acetobutylicum* commences with the exponential growth phase, vegetative cells digest glucose during the acetogenic metabolic phase leading to the assimilation of organic

acids like butyrate. Subsequently, a pivotal metabolic shift in the growth cycle occurs, marked by a decrease in pH, which signals the initiation of sporulation. This phase is characterized by the activation of solventogenic metabolism and the development of endospores and concludes in cell lysis (Al-Hinai, Jones and Papoutsakis, 2015; Barber et al., 1979; Long, Jones and, Woods 1983).

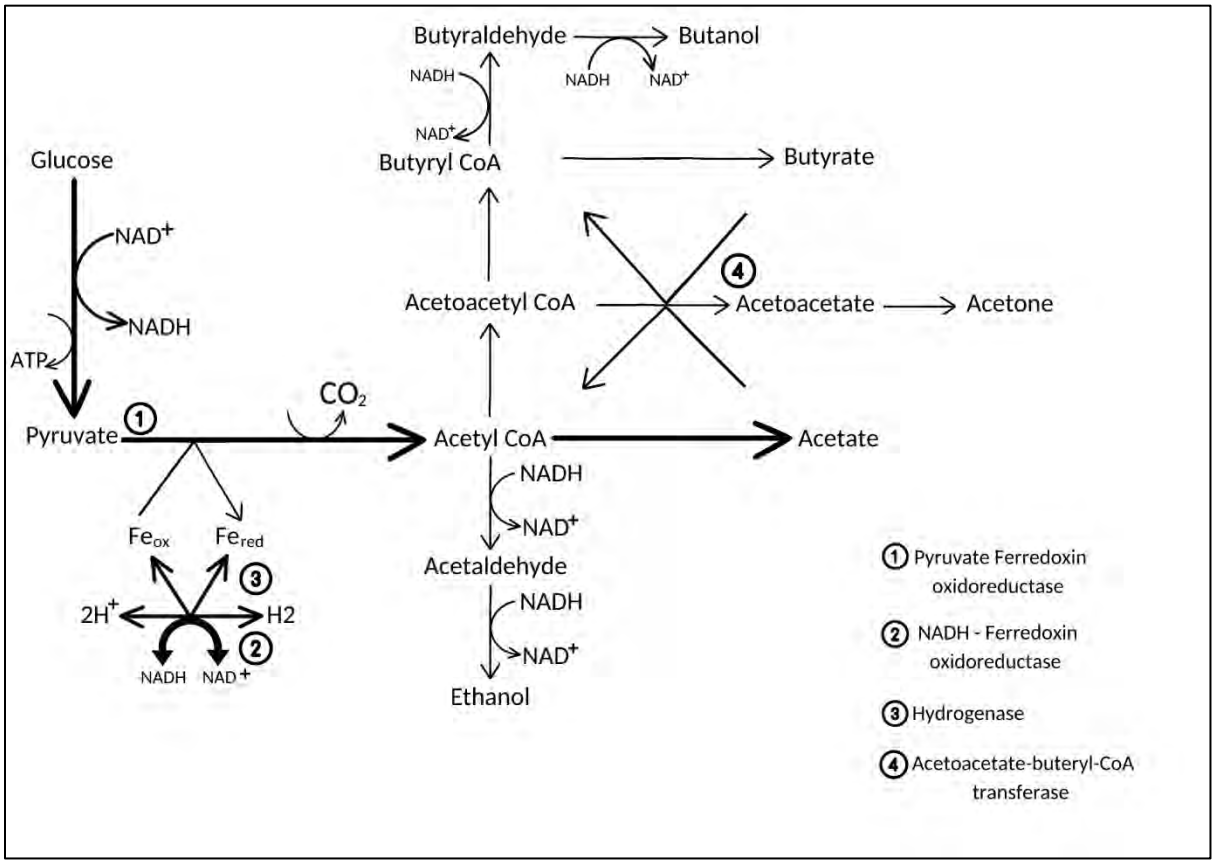

Figure 1.2: Summary of metabolic pathways for fermentation of glucose by *Clostridium acetobutylicum* resulting in the production of, indicating enzymatic reactions of precursors. During the acidogenic phase, *C. acetobutylicum* converts pyruvate to acetate and butyrate. In the solventogenic phase, the same pathway is utilized, but the electron flow and carbon flux are re-routed to produce solvents such as acetone, butanol, and ethanol, with the conversion of pyruvate to acetyl-CoA, acetoacetyl-CoA and butyryl-CoA by the enzymes numbered 1-4. Thiolase is not mentioned for the reaction of Acetyl CoA to Acetoacetyl CoA. The regeneration of NAD+ is catalised from the synthesis of alcohols from driving the reactions numbered 2 (Ferredoxin oxidoreductase) and 3 (Hydrogenase) interacting with the redox reaction of ferredoxin and associated hydrogenase (3) activity. Adapted from (Choi and Sang, 2016; Putsakis, 2008).

Both *C. acetobutylicum* ABE fermentation and yeast bioethanol fermentation pathways require the regeneration of NAD+ to continue the production of their respective products. The biosynthetic secretory pathway of *C. acetobutylicum* involves the production of acids and solvents, including ethanol, while yeast bioethanol fermentation produces ethanol. The enzymes and pathway of electron flow in both pathways are different but serve the same purpose of NAD+ regeneration. The induction of butanol synthesis relies on the accumulation of NADH and the metabolic precursor, butyrate, which is oxidised to regenerate NAD+, forming butanol (Nigam and Singh, 2011).

C. acetobutylicum employs various electron acceptors including downstream acidic metabolites (butyric acid) and Ferrous ions (Fe2+) being prominent for the regeneration of NAD+. These ions efficiently facilitate anaerobic respiration, sustaining metabolic activities and energy production in the absence of oxygen. This involves biochemical reactions, primarily reducing ferrous ions to ferric ions (Fe3+). The bacterium's electron transport chain functions independently of molecular oxygen indicating the obligate anaerobic respiration of *C. acetobutylicum,* utilizing enzymes (hydrogenase and ferredoxin oxidoreductase) and electron carriers to transfer electrons from NADH and $FADH_2$ to ferrous ions. This generates a proton motive force across the cell membrane, driving ATP synthesis via the generation of a proton gradient or chemiosmotic coupling (Grupe and Gottschalk 1992).

During solventogenesis, the organic acids produced during acidogenesis are fermented to alcohols with the simultaneous reduction of protons to H_2 (Plaza et al., 2017). Thiolase enzymatic activity has been found to be a redox dependent switch for bi-phasic metabolism switching between acidogenesis to solventogenesis in *C. acetobutylicum* (Kim et al., 2015; Li et al., 2014a).

The production of acetone, butanol and ethanol by *Clostridium* spp. (the ABE process) has been widely researched. Currently, the consensus is that these produce low yields at relatively high costs (Magalhães et al., 2018). The yield of solvent production is strongly influenced by inhibitory organic acids and metabolites produced by *Clostridium* (Magalhães et al., 2018). Similar to the production of bioethanol by yeasts, the economic feasibility of production of ABE by *Clostridia spp.* Via fermentation is limited by considerations of the initial and treatment costs of raw substrate (Moon et al. ,2016b). For example, the energy costs of heating substrate for sterilization or additional enzymes for further hydrolysis increase costs of production over and above the cost of

raw materials without treatment. The competition between food stocks and sustainable feedstocks for biofuel production is a recurring battle that may be mitigated in the context of a circular economy and waste valorisation (Mujtaba et al., 2023). The saccharolytic expression of solventogenic *Clostridia* incentivises research into the diverse possibilities of sustainable substrates (Ibrahim et al., 2017b).

1.1.7 Application of ABE fermentation to non-food feedstocks and the case for brewers' spent grain.

Conventional methods of acetone, butanol and ethanol (ABE) production via fermentation of molasses by *Clostridium* spp. Produce these fuel compounds at low yields at a high cost, making it cost-ineffective compared to petroleum-based fuel synthesis (Zheng et al., 2009).

In the past, biofuels have faced criticism for utilizing food sources as the substrate for fermentation (Singh and Singh, 2011). Waste streams that are ideal for the application of a sustainable low-cost feedstock for biofuel fermentation processes can be identified from waste products sourced from other industrial fermentation operations, such as brewers' spent grain (BSG), from beer or spirits production (Plaza et al., 2017). Biofuels production are the result from a source of microbial fermentation, as discussed in Sections 1.1.4 to 1.1.7. Feedstocks that have high levels of available reducing sugars and starches require very little by means of pre-treatment to poise the feedstock as a fermentable substrate (Robertson et al., 2010).

Lignocellulosic feedstock often necessitates pre-treatment procedures, including enzymatic hydrolysis, to liberate fermentable carbohydrates. The choice of pre-treatment or hydrolysis method hinges significantly on the specific composition of the lignocellulosic feedstock or the capabilities of the pretreatment facility in question. The selection of the most efficient strategy may be constrained by socio-economic considerations (Ravindran et al., 2018).

Acquisition of raw materials can be achieved through financial means or via service procurement. For numerous industries, the management and disposal of waste materials involve compensation for waste removal services, which in turn impacts the cost structure of the raw material. In contrast, the cost associated with raw materials derived from waste streams is contingent upon the commercial demand for their application, such as their utility as animal feed or competition with

other waste remediation services. The cost and effort to produce a fermentable feedstock from cellulosic sources is still not economically viable due to the cost of inputs such as energy and enzymes coupled with the inefficiency of treatment strategies (Ravindran et al., 2018).

Unlike yeasts, *Clostridium* species can metabolise a much wider variety of potential compounds to serve as feedstocks for ABE fermentation. The metabolism of starch for example, requires amylase enzymes to carry out saccharification to make the glucose readily available for fermentation. A report by Parchami, Ferreira,and Taherzadeh (2021) revealed that the primary constituent of Brewer's Spent Grain (BSG) are polysaccharides: starch (20.88 ± 0.10 % dry weight), cellulose (17.52 ± 1.43 %) and hemicellulose (25.31 ± 2.89 %). Through successful hydrothermal pretreatments, a large portion of this starch (82.62 ± 2.81 %) was effectively liberated, resulting in a notable soluble starch content in aqueous extracts. As described by Kheyrandish et al., (2015), *C. acetobutylicum* is capable of directly fermenting starch to form ABE products. Similarly, due to the noted cellulolytic ability of *C. acetobutylicum*, various sources of lignocellulosic substrates applied ABE fermentation (Lee, Forsberg and Gibbins, 1985; Kheyrandish et al., 2015; Magalhães et al., 2018). Investigating the potential of brewing by-products provides a potential for creation of value-added technologies, combining the conversion of lignocellulosic wastes from breweries with microbial synthesis of fuels by ABE fermentation (Plaza et al., 2017).

The practice of beer brewing is a waste-intensive process; organic-rich byproducts such as carbohydrate-rich brewer's spent grain (BSG) and protein-rich yeast biomass and hazes are traditional by-products of beer brewing. In a move towards more sustainable manufacturing processes, large commercial breweries have invested in waste-repurposing and recycling technologies. Small-scale breweries, however, lack comparable infrastructure and resources to install and operate similar processes.

1.1.8 Microbial fuel cells

Microbial Fuel Cell (MFCs) are a type of bio-electrochemical system that use bacteria to convert organic matter into electricity. MFCs are a promising technology for generating clean and renewable energy from waste materials (Davis and Higson, 2007). MFCs are based on the

observation that exoelectrogenic bacteria in the presence of electrodes are capable of exchanging electrons in a microbial fuel cell or electrolysis cell configuration (Logan, 2009) .

Microbes with efficient electron transfer abilities will have accelerated respiratory rates, which can lead to faster degradation of the recalcitrant organic matter, which in turn can result in higher power outputs from the MFC. In MFCs, the term "bioanode" refers to the anode where oxidising biological processes take place and where electrons enter the fuel cell. At the anode, exoelectrogenic bacteria catabolise organic matter (often, wastewaters or other forms of organic waste), transferring electrons from this process to the electrode (Read et al. 2010). The bioanode can be composed of various materials, including graphite, carbon cloth, and carbon paper. The term cathode refers to the electrode where the electrons from the bioanode are collected and transferred to a terminal electron acceptor. Potential differences between the anodic and cathodic electron-transfer reactions drive the flow of current between the electrode and dictate the overall energy obtainable from these systems (Davis and Higson 2007; Read et al. 2010).

Figure 1.3 provides a summary of the general processes in MFCs, using glucose as a feedstock.

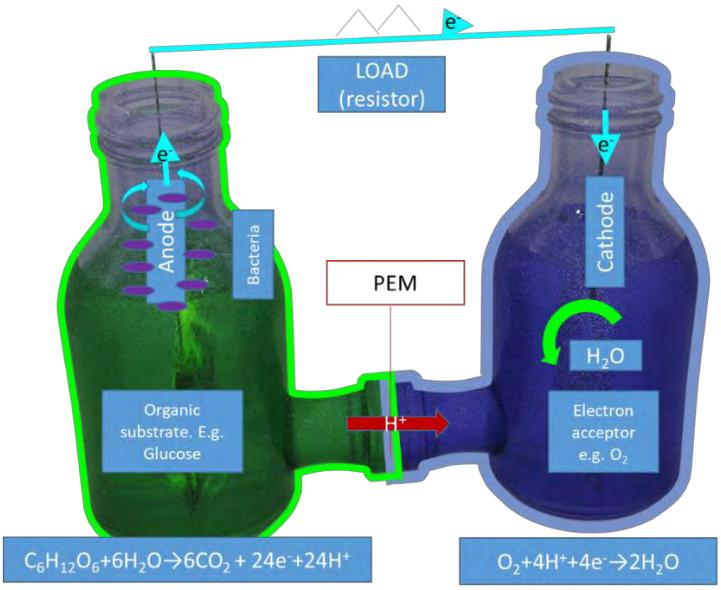

Figure 1.3: Schematic of a microbial fuel cell (MFC) for glucose respiration by bacteria in the anodes showing the electron path to the cathode to bond with oxygen to form water.
The schematic of the fuel cell above shows the reactions and the chemical equation that occur in both the anode and the cathode. The indication of bacteria transferring electrons to the anode also indicates the path of the electrons to the cathode where H_2O is produced from the available electron in the presence of oxygen.

As described in Figure 1.3, the function of the bacteria attached to the anode in the MFC is to degrade organic matter, producing electrons and protons in the process via respiration. Respiratory electrons can be gathered by the anode and used to generate electricity, while the protons diffuse to the cathode through a proton-exchange membrane to create a flow of electrical current. The cathode provides the reaction with an electron sink where the action of exchange of electrons occurs from the cathode to the catholyte.

With the understanding from section 1.1.6, that *C. acetobutylicum* utilises the ferredoxin oxidoreductase enzyme pathway and metabolic products as electron acceptors for anaerobic respiration. Investigating the anaerobically-respiring *Clostridium* during fermentation within microbial fuel cells can provide the ability to tune their metabolism between acidogenesis and fermentation, by providing the bacteria with an exogenous electron acceptor in the form of the anode electrode of the fuel cell. The anode acts as an electron acceptor driving the regeneration of NAD+ from NADH+, to induce ATP production by glycolysis in the presence of glucose or the oxidation of precursor metabolites such as butyrate as illustrated in Figure 1.2. During *Clostridial* ABE fermentation, the organic metabolites are produced at different metabolic phases of the fermentation, which experience bottlenecking that affects the metabolic fluxes in the bio-butanol synthesis pathway (Buehler and Mesbah, 2016a).

The availability of precursors such as glucose or butyrate and the inducer NADH+ and NAD+ are required to catalyse the reaction as they indicate the fermentation conditions which dictate the electron flux. The exoelectrogenic ability of *C. acetobutylicum* in a MFC can alleviate the bottlenecking by acting as an electron acceptor to prevent the accumulation of enzymatic inducers, which redirect the metabolism of carbon to metabolites other than desired products (Finch et al. 2011; Kracke et al., 2016).

The proposed aim of this research is to investigate whether this form of technology produces a method of engineering the metabolism of attached *Clostridium* cells to improve ABE yield. Utilizing the exoelectrogenic ability of *Clostridium* and incorporating electrochemical intervention into the *Clostridial* fermentation of butanol could result in greater control over the process and decrease the cost of production.

Bioanodic MFCs generate organic compounds using microorganisms in close proximity to electrodes. This is achieved through the ability of electrodes to act as an acceptor for electrons generated during catabolism/respiration (Zheng et al., 2009). While the absence of exogenous electron acceptors in the environment is known to function as a regulatory 'switch' to promote fermentative metabolism (Zheng et al., 2009) and the ability of *Clostridium* to simultaneously generate power in an MFC and produce ABE has been reported (Finch et al., 2011), this leaves the so-far novel question as to whether one can switch between metabolic phases of *Clostridium* mediated ABE by directly manipulating the ability of an electrode to accept respiratory electrons.

The use of MFCs can alter fermentation kinetics, as different bacterial species have different metabolic processes and growth patterns, which rely on the transfer of electrons to the anode. Metabolism of *Clostridium acetobutylicum* have been shown to be associated with power generation in MFCs. Previous research incorporating *Clostridium s*pp. Within fuel cells showed some validation of the hypothesis: peak voltage of the fuel cell correlated to the onset of acidogenesis and solventogenesis, respectively (Finch et al., 2011).

It is proposed that by altering the connections of MFCs' anodes their counterpart cathode electrodes, it may be possible to manipulate the metabolic state of the attached bacteria (Choi and Sang, 2016). By the precise manipulation of the connection state during *Clostridium* fermentation of feedstock, this technology may be beneficial in the maximisation and control of ABE fermentation. For similar reasons, an investigation whether the cellulolytic activity of attached clostridial cells may be improved by this manipulation is also justified.

1.2 Problem statement

The global imperative to mitigate carbon emissions has increased demand for liquid biofuels. Biobutanol production through ABE fermentation, historically never practiced on a large scale, is now subject to re-evaluation in light of modern technological and biotechnological advancements. The efficiency of ABE fermentation hinges on multifaceted environmental factors, necessitating the application of intricate operating processes to improve its sustainability. An array of analytical methods, including HPLC and spectrophotometry, can be explored to compare efficiency in solvent production during *C. acetobutylicum* fermentations under controlled conditions. Initially,

it is imperative to establish an appropriate facility for anaerobic culture of microorganisms, alongside robust protocols for media preparation, inoculation, and operation. Quality assurance measures are vital to guarantee culture maintenance, culture viability for product formation and successful fermentation outcomes.

Furthermore, food wastes (especially, lignocellulosic-rich biomass) has emerged as a promising, sustainable, substrate for biofuel production in microbial fermentations, as it avoids competition with food stocks and is abundant. However, the expense associated with pre-treatments to enhance product yields pose a challenge to using these substrates. Due to its composition, Brewer's Spent Grain (BSG) stands as an available waste stream with the potential to serve as a low-cost feedstock for biofuel production, contingent upon appropriate pre-treatment selection. The feasibility of utilizing such feedstocks must be rigorously evaluated to propagate, maintain, and effectively harness low-cost technologies, particularly *C. acetobutylicum*, for proficient ABE fermentation.

Finally, the incorporation of Microbial Fuel Cell (MFC) technology utilizing *Clostridium* could potentially enhance butanol production and yield valuable by-products. This augmentation may lead to the optimization of *Clostridium* fermentation in industrial settings, allowing for the operation of *Clostridium* fermentation adjacent to existing production plants that utilize organic materials, such as brewery waste (BSG). Such integration holds the promise of reducing energy consumption and maximizing the output of *Clostridium* fermentation for industrial biofuel production.

1.3 Aims and Objectives

The study aims to determine the plausibility of BSG being used as a value additive feedstock for ABE fermentation and the use of electro fermentations in the form of microbial fuel cells for increased solvent production.

The Hypothesis explored in the studies presented in the subsequent Chapters does not take into consideration facility and economic viability. Nor does it attempt to develop an applicable protocol for breweries to practice. Other carbohydrate sources could be explored to measure a wider range of complex carbohydrates and reducing sugars. *C. acetobutylicum* has been studied for production

of amylases and cellulases and more research is needed on catabolism of lignocellulosic and cellulosic carbohydrates in bacterial solvent-producing fermentations.

Objective:

- Develop a chemically defined media for ABE fermentation to characterise concurrent solvent production and sporulation.
- Track specific parameters of the ABE fermentations using different techniques to best analyse the kinetics of the fermentation using different media.
- Select protocols which identify and analyse the fermentation with focus cell density and carbon metabolism focusing on carbohydrates, acidic metabolites and solvent synthesis.
- Refine HPLC protocol for the detection of carbon source, intermediate metabolites and desired ABE solvent products.
- Utilisation of microscopy to identify mature *C. acetobutylicum* cells.
- Comparison of fermentation kinetics of *C. acetobutylicum* for chemically defined media and different pretreatments of BSG.
- Fermentation kinetics of *C. acetobutylicum* in a microbial fuel cell configuration
- Increase ABE efficiency and sustainability by use of *C. acetobutylicum* in both electro fermentation and its ability to convert lignocellulosic carbohydrates such as brewers spent grain to value added products.

Chapter 2: Developing the bioprocess of *Clostridium acetobutylicum* (ATCC 824) ® ABE fermentation.

2.1 Introduction

One of the major challenges surrounding the use of *Clostridium acetobutylicum* as a microbial catalyst is the cost of maintaining and culturing this species: due largely to the obligate anaerobic growth requirements (O'Brien and Morris, 1971). Strict anaerobic culturing techniques, in turn, require specialised equipment and reagents to conduct culture maintenance and solvent production studies using this organism. Sterile technique by flame sterilisation, for example, is not possible in an anoxic environment (de Lemos Chernicharo, 2015; Edwards et al., 2013).

To all microbial industrial applications, mutation and strain continuity are a threat to the efficiency and consistency of solvent production, requiring additional culture maintenance considerations. This is further complicated in the case of *C. acetobutylicum*, due to the multiple growth phases it exhibits during culture, along with its tendency for spontaneous autolysis (Barber at al., 1979; Liu et al., 2015).

When cultured from endospores, *C. acetobutylicum* undergoes a vegetative mitotic growth stage simultaneously producing organic acids referred to as acidogenesis. Solventogenesis which follows acidogenesis in the biphasic metabolism of *C. acetobutylicum,* involves the production of solvents, such as acetone and butanol, during the second phase of fermentation in response to environmental stress and accumulation of metabolic intermediates (Papoutsakis, 2015).

Sporogenesis and cell autolysis are signalled by the production of bacteriocins towards the end of vegetative growth, at the peak of acid assimilation. After solventogenesis, the subsequent spore production and autolysis of a significant number of cells occurs, due to the onset of limitations of carbon sources during culture (Barber et al., 1979). Autolysis prevents the harvesting of vegetative bacterial cells and their long-term storage during routine culture, as commonly occurs for other bacterial strains. Co-occurring endospore development in the culture and solvent production is therefore essential to maintain viable solvent-producing cultures over the long term (Al-Hinai, Jones, and Papoutsakis, 2015 ;Long, Jones and Woods ,1983);

The bioproduction of ABE is also affected by product inhibition. Historically, yields of butanol in traditional fermentation systems are very low, as solvent toxicity occurs at concentrations between 2 % to 3 % (i.e. 20 to 30 g/L) for acetone and butanol (Putsakis, 2008). Typically, solvent yields are even further below this toxicity threshold, for example the 13-15 g/l of butanol reported elsewhere (Chen et al., 2013) and are affected by other culture conditions e.g., nutrient deficiencies, or when using mutant strains produced during storage and maintenance (Edwards et al., 2013; Long et al., 1983). To ensure adequate solvent production, both maintenance of a solventogenic strain and sufficient nutrients in culture media is required, to support solventogenesis and to ensure that sporulation occurs efficiently at the end of the fermentation.

The detection of ABE solvents is indicative of the fermentation of *C. acetobutylicum* and the ability of the culture to produce solvents from metabolic precursors and substrates (Vohra et al., 2015). The method used in this research for tracking ABE fermentation kinetics of carbohydrate feedstock, metabolic precursor and solvent product analysis along with the viability of solventogenic bacteria is the chromatographic detection of the solvent product by HPLC. Other highly-sensitive alternatives for the detection and quantification of ABE fermentation products include Gas Chromatography (GC) (Tsuey et al., 2006). The Refractive Index detector (RI) HPLC method is relevant for the analysis of feedstocks and metabolites quantified between different ABE fermentation methods (Finch et al., 2011; Mussatto, Dragone and Roberto, 2006; Plaza et al., 2017) used in this research.

2.2 Aims

The aim of the research in this Chapter is to effectively propagate *C.acetobutlyicum* from a commercial spore sample and to compare various reported chemically-defined media for suitability as a basic preparation for further solvent production studies outlined in Chapters 3 and 4, and for culture maintenance.

To achieve this, a low-cost anaerobic chamber and accompanying equipment had to be designed, manufactured and tested. Producing a chamber that is sealable to minimise oxygen entry and that remains operator-friendly for the aims of this project presented multiple challenges.

2.3 Methodology

2.3.1 Reagents and apparatus

Unless otherwise stated, all reagents were of technical grade (i.e., ≥ 95%) or higher. Ammonium acetate, ethylenediaminetetraacetic disodium salt dehydrate (EDTA), hydrochloric acid (32 % $^{w}/_{v}$), methylene blue (≥82% $^{w}/_{w}$), nitric acid (55% $^{w}/_{v}$), orthophosphoric acid (85 % $^{w}/_{v}$), sulfuric acid (98% $^{w}/_{v}$), and thiamine were sourced from Merck, as were the following inorganic salts: KCl, $MgSO_4 \bullet 7H_2O$, $MnSO_4 \bullet H_2O$, NaH_2PO_4, Na_2HPO_4 and NaOH.

Biotin, D-(+)-glucose, glycerol, para-aminobenzoic acid and starch (from potato) were all sourced from Sigma, as were the following inorganic salts: $CaCO_3$, $FeSO_4$, K_2HPO_4, KH_2PO_4, and NaCl. Anhydrous calcium sulphate ($CaSO_4$) was sourced from Thomas Scientific (United States of America).

5.0 Baseline Nitrogen (80% $^{w}/_{v}$) was sourced from Afrox® South Africa. This was desiccated and de-aerated inline using a glass column packed with oxygen scavenging Drierite® desiccant (Thomas Scientific).

Iodophor – Iodine contact Steriliser (brewmart) was used for sterilisation of the reference electrode for the MECs.

C. acetobutylicum (ATCC 824) was sourced directly from American Type Culture Collection.

The predominant growth media used in this study was based on Sigma Aldrich's Clostridial Nutrient media (CNM). This was composed of: 10 g/L meat extract, 5 g/L peptone, 3.5 g/L yeast extract, 1 g/L starch, 5 g/L sodium chloride, 3 g/L sodium acetate, 0.5 g/L L-Cysteine hydrochloride and 0.5 g/L agar. This was prepared as per the manufacturer's instructions.

HPLC measurements were conducted using a Shimadzu High Performance Liquid Chromatography Unit (Japan), using Refractive Index Detection, operated using Lab Solution software. The column used was a Phenomenex Rezex ROA-Organic Acid Column H+ (8%) 300×7.8mm (l×d) containing 8 µm-sized particles. Spectrophotometry was conducted using a FLUOstar® Omega Microplate reader, using polypropylene-based 96-well microtiter plates (Lasec, South Africa). Scanning electron microscopy was conducted using a TESCAN Vega TS 5136LM microscope.

2.3.2 Formulation of P2 micronutrient supplement

P2 micronutrient supplement (described by Kheyrandish, 2015) was prepared as a solution in 100 ml batches and stored at 4 °C. Each batch of P2 was prepared with 100 ml H_2O and was composed of: 2g/l $CaCO_3$, 50g/l KH_2PO_4, 50g/l K_2HPO_4, 220 g/L ammonium acetate, 0.1 g/L para-aminobenzoic acid, 0.1 g/L thiamine, 1 mg/L biotin, 20 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L $MnSO_4 \bullet H_2O$,1 g/L $FeSO_4$ and 1g/L of NaCl. The pH of P2 was adjusted to 6.2 using 5M NaOH solution and was filter sterilised under aseptic technique using a 0.22 µm cellulose acetate syringe filter before storage. P2 was prepared fresh in small batches and stored in the fridge for no longer than a month in the prepared solution. P2 was added to media at a rate of 0.1% after autoclaving of the media, once the media had cooled to approximately 60 °C. The addition of P2 to the media was only performed for experiments where solventogenesis was induced, or for the maintainance of a pure culture where endospore formation was necessary. P2 was not added to propagation media when the culture was grown either from spores or from cryogenically-preserved glycerol stocks.

2.3.3 Other media amendments

Four compositions of chemically-defined media, Sporulation medias SA, SB, SC, and SD were evaluated, by supplementing CNM with varying carbohydrates and adding P2 amendment (Kheyrandish et al., 2015). Carbohydrates were added before autoclaving of the media and P2 was added after the media had cooled.

Four amendments, affecting the carbohydrate concentration, were applied to CNM supplemented using P2 micronutrient solution. CNM contains 5 g/L glucose and 1 g/L starch in stock form. Sporulation media A (SA) included 45 g/L of glucose, Sporulation media B (SB) included an amended 20 g/L of glucose, media C (SC) included 5 g/L of glucose and 20 g/L starch and Sporulation media D (SD) included 20 g/L of glucose and 20 g/L starch. Filter-sterilised P2 was added to all media after autoclaving. The cultures were subsequently incubated for 120 hours at 37 °C. Progress of fermentation was tracked by measurements of pH, OD_{600} and HPLC method.

Differing amounts of glucose and starch concentrations were compared for the effect on solventogenesis and viable endospore formation:

- SA: 45 g/L of glucose added.
- SB: 20 g/L of glucose.
- SC: 5 g/L of glucose and 20 g/L starch.
- SD: 20 g/L of glucose and 20 g/L starch.

Sporulation Media were formulated from Al-Hinai et al. (2015); Kheyrandish et al. (2015) and Long et al. (1983). Media were additionally supplemented with 5 mM methylene blue (Yhong-he, 2016) for fermentation experiments conducted for longer than 48 hours. Methylene blue was not added to media for cultures grown from spore or from cryogenic glycerol stocks.

2.3.4 Design and development of the anaerobic culturing chamber

For anaerobe culture of *C. acetobutylicum,* a custom-designed anaerobic glove box was developed that is purged with nitrogen to create an anoxic environment. Adaptations were made to a MAC Afric sand blasting cabinet GCABSB-001.

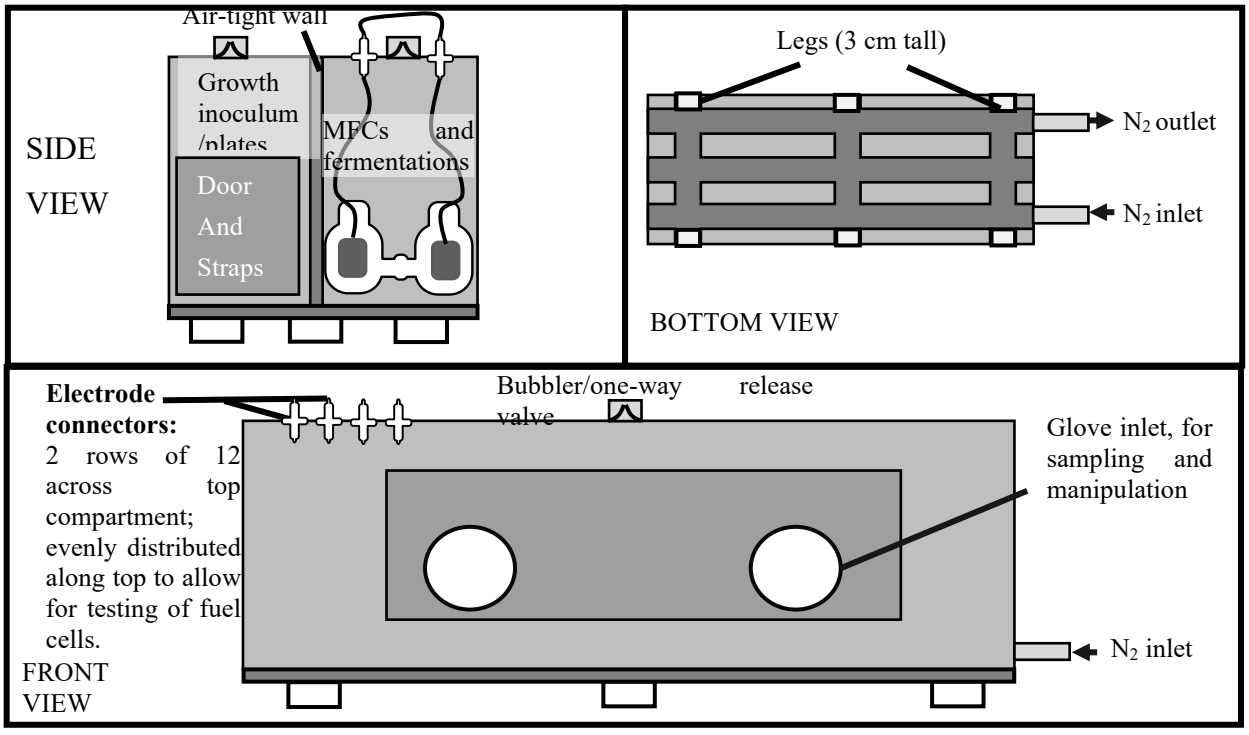

Figure 2.1: Schematic of designed anaerobic fermentation chamber based on sandblasting chamber.

Modifications to the sandblasting chamber were as follows: animal inspection gloves were fitted inside the original sandblasting gloves and held in place with duct tape; gloves were used to access the chamber interior. Glove access points were essential in order to maintain anaerobic conditions when culturing or sampling anaerobic fermentation cultures or to electrically connect the bacteria with internal electrodes (Figure 2.2 D) for electrolysis or exoelectrogenesis discussed in Chapter 4. The total height of the anaerobic fuel cell chamber was increased for ease-of-operation in a congested space. An additional 50 cm was added to the height of the chamber using 3 mm-thick steel plates. The lid of the chamber was modified to allow ten MFCs to be electrically connected to isolated external circuits and to allow potential measurements to take place outside the chamber. The terminals were constructed from crocodile clips and banana clips which allowed for easy connection to prepared individual MFCs and measurements to be taken externally without opening the anaerobic chamber. The final MFC-compatible anaerobic fermentation chamber was constructed and is presented in Figure 2.2, below. MFC-compatible anaerobic fermentation chamber was constructed and is presented in Figure 2.2, below.

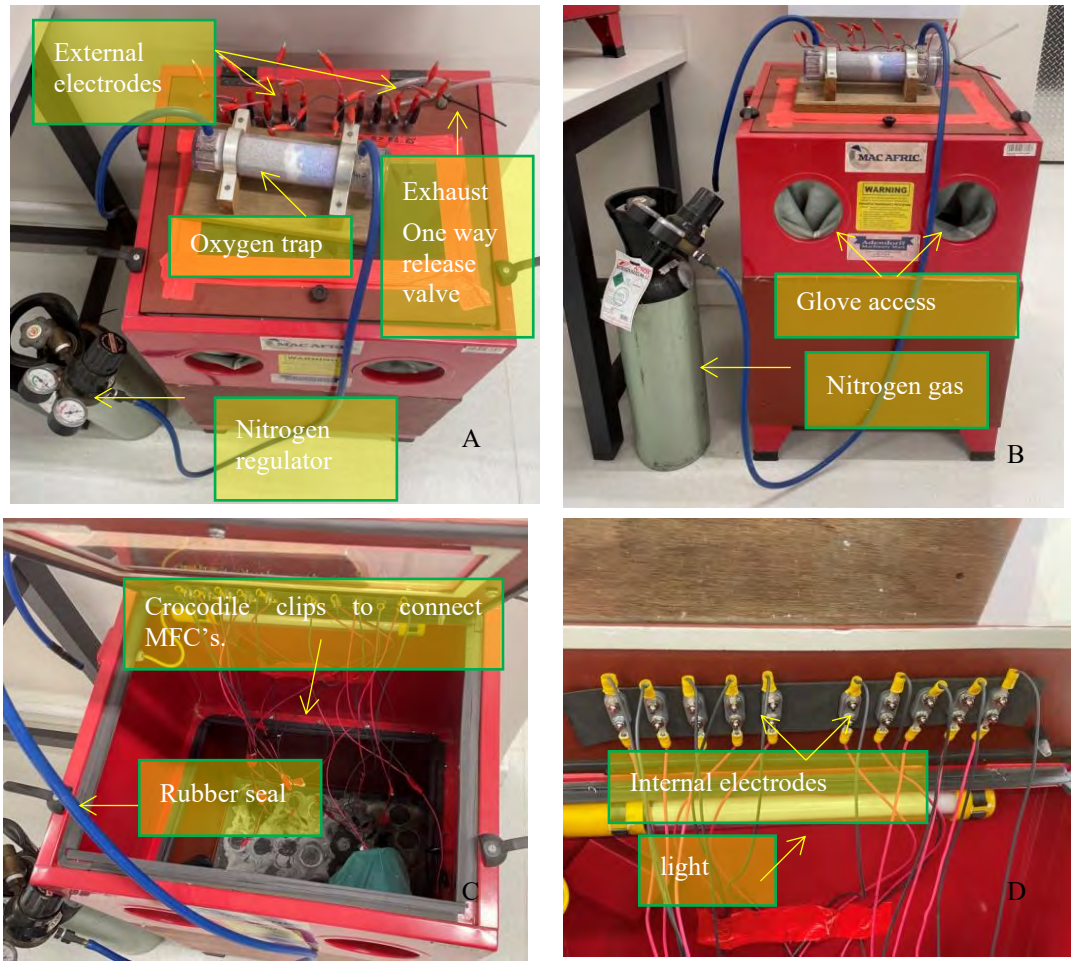

Figure 2.2: Photographs of the interior and exterior of the anaerobic fermentation chamber constructed for this research.
Anaerobic fermentation and anaerobic MFC chamber External view; Top (A) showing oxygen filter, external electrodes and viewing port / Front (B) Gas bottle, regulator, and glove inserts. Internal view; Base to lid (C) showing MFC electrode connections with crocodile clips, Lid (D) showing internal electrode connections.

A second sandblasting chamber was converted to create an inoculation chamber in which the conditions for fermentation (media formulation, etc.) were prepared and the culture was manipulated. This was performed in sealed containers that would be transferred to the anaerobic fermentation chamber in Figures 2.1 and 2.2. This inoculation chamber was constructed to be similar in dimension and features to the anaerobic fermentation chamber in Figure 2.1 but lacked internal compartments in order to maximise the working volume and lacked the height extension created for the MFC. The completed inoculation chamber is presented in Figure 2.3, below.

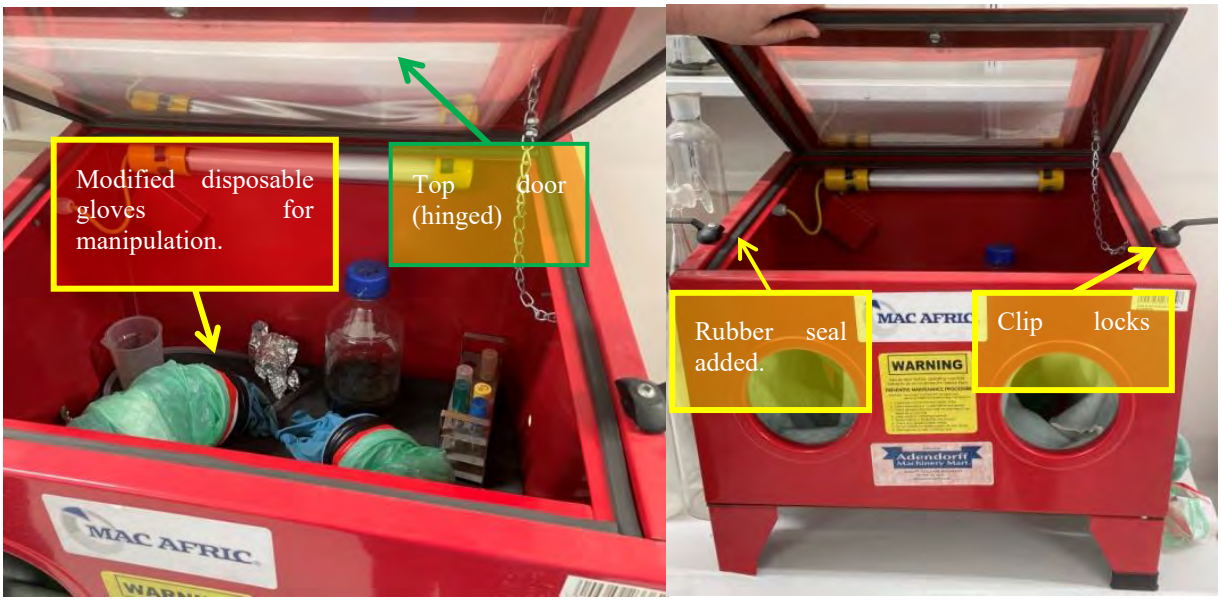

Figure 2.3: Photographs of the interior inoculation chamber constructed for this research.
The main characteristics of the anaerobic chamber are annotated within the diagram.

2.3.5 Inoculation chamber preparation and anaerobic culture manipulation.

Many of the procedures were adapted from the culturing and maintenance of other obligate anaerobes also belonging to the genus *Clostridium,* such as *Clostridium difficile* (Edwards et al. ,2013). The anaerobic inoculation chamber was prepared by sterilising the chamber with 70% ethanol solution and sealing the chamber with the clip locks and rubber seals to ensure an anoxic and aseptic environment was maintained. All media and tools and apparatus were autoclaved or sterilised with ethanol before being placed into the inoculation chamber. When using more delicate equipment (such as the pH probe), autoclaved water was used to rinse the probe, reducing the bacterial load on the probe. Before flasks containing media were opened, oxygen exposure was

mitigated by flushing the chamber with nitrogen for between 10 and 15 minutes. Inoculation took place under induced positive pressure using nitrogen gas.

Oxygen was removed by two methods depending on the quantity of media. Large volumes of media (i.e., $\geq$ 500 ml) were vacuum degassed for between 30 and 60 minutes, while smaller volumes were purged by bubbling nitrogen gas through them for between 10 and 15 minutes. The lids of culture bottles were loosely fitted to allow gas to escape under positive pressure, while preventing the opening of the media containing bottle from being exposed to atmospheric contaminants. When de-aerating with nitrogen gas, a sterile glass pipette was used to deliver the gas to the media and the opening was covered with tinfoil. The glass pipette and the mouth of the bottle were flame sterilised. Purged media were then placed within the anaerobic culturing chamber.

In order to release degassed media from vacuum under anaerobic conditions, the vacuum chamber was connected to a positive pressure feed Baseline Nitrogen line and slowly brought back to atmospheric pressures. Bottles were then sealed and loaded into the anaerobic inoculation chamber.

2.3.6 Propagation of *C. acetobutylicum* from spores.

The lyophilised spores of *C. acetobutylicum* were received on the 19th of October 2018. Propagation was carried out in accordance with manufacturer instructions.

The spores were suspended in 0.5 ml-1 ml of liquid CNM and inoculated into two separate culture flasks A and B containing 10 ml CNM each. These are labelled A1 and B1 for primary culture from commercial spores. Inoculated CNM was incubated at 37 °C under anaerobic conditions for 48-72 hours. Growth was measured by turbidity (OD_{600nm}) measurements; at the end of incubation, samples were removed for morphological analysis using Gram staining as detailed in (Smith and Hussey, 2005). Glycerol stocks were prepared after 48 hours, cryopreserved at -80℃ and thawed on a rack to room temperature when needed.

2.3.7 General *C. acetobutylicum* culturing conditions

The inoculum volume was set to not exceed 10 % of the total media volume. A 1 ml glycerol stock would inoculate 10 ml of CNM; this was cultured between 12 and 24 hours under static anaerobic conditions before being used to as a 10% seed culture of total media to be inoculated, as described above for larger volumes of bulk media for experimentation.

To ensure the appropriate pH range (between 6.2 and 6.8), the medium was titrated accordingly. Subsequently, sterilization was achieved through autoclaving, followed by de-aeration for 30-60 minutes or purging with nitrogen (N_2) for 15 minutes prior to inoculation. During the initial experiments to establish baseline data for the growth of C. acetobutylicum, samples were collected at 12-hour intervals for optical density at 600 nm (OD600) measurements and for in situ pH monitoring to assess growth kinetics.

2.3.8 Total carbohydrate analysis by phenol sulphuric acid assay

Using a 96-well plate spectrophotometer, measuring absorbance at 485 nm phenol sulphuric acid assay was conducted to determine the total carbohydrates of the fermentation experiments (duBois et al., 1956). Stock solutions were prepared using glucose as a standard (0.1 to 1 mg/ml).

30 µl of sample or standard was added to a well and combined with undiluted sulphuric (150 µl); the yellow browning reaction was allowed to occur (10 minutes) before the addition of 5 % phenol solution, diluted with MiliQ water. The mixture was then allowed to incubate for a further 10 minutes before the absorbance was recorded. In order to best represent the total carbohydrates from the data acquired the presented data for total carbohydrates was normalised with respect to the negative uninoculated control. The uninoculated control did not get inoculated at t=0 hours and best represents the initial total carbohydrate content of the media.

2.3.9 HPLC method development and application to ABE production

Existing HPLC protocols for ABE fermentation were selected, which could simultaneously determine both the carbohydrate composition of the media and the solvents produced in the ABE fermentation (Tsuey et al., 2006; Vohra et al., 2015). The protocol for carbohydrate and solvent

chromatography was stipulated by the column manufacturer with certain parameters adapted for this study.

HPLC Sample vials were cleaned via soaking in 5% nitric acid solution for a few hours; subsequently rinsed with sterile miliQ water and allowed to dry before use. Only 20 samples were loaded and sampled in a batch to avoid baseline drift between samples. Between batches of analyses, the system was cleaned with successive rinses of sterile water and of pure methanol. Following a minimum of a few hours' exposure to ethanol, the mobile phase used for the HPLC protocol above was used to purge the lines before processing more samples.

Samples were prepared by centrifuging 1-1.5 mL of the solution at 12,000 x g for 10 minutes and sterilizing it using a 0.22 µm filter. The resulting solution was then de-aerated for 30 minutes. The auto-sampler was auto-purged and the line was purged for 15-30 minutes before zeroing and waiting for one hour. The prepared samples were loaded into the HPLC with the correct column and method, and either a single or batch run was selected. The Run time was determined by the method and number of samples, the mobile phase was constantly monitored to ensure that it did not run dry. The resolution of the separation was increased by adjusting the flow rate and oven temperature parameters. The refractive index HPLC was used with the Phenomenex Rezex ROA organic Acid Column H+ (8%) 300×7.8mm 8 µm column at an oven temperature of 80°C, and a degassed mobile phase of 5 mM (0.005 N) sulphuric acid at a flow rate of 0.4 mL/min. Finally, 10 µl of the prepared sample was injected and the run was carried out for 60 minutes for ABE fermentation products to be analysed.

2.3.10 Preparation of samples for scanning electron microscopy.

Broth media containing cultured *C. acetobutylicum* cells were examined via Scanning Electron microscopy. A broth sample was processed for electron microscopy analysis using the following method. A 1-1.5 mL aliquot of the sample was centrifuged at 4000×*g* for 4 minutes to form a pellet, which was then rinsed in 0.1 M phosphate-buffered solution. The sample was subsequently incubated in 2.5 % glutaraldehyde (dissolved in 0.1 M phosphate buffer, pH 7.4) overnight. Following this, the sample was fixed using critical point desiccation. To dehydrate the samples, the sample was flooded with different concentrations of ethanol by exposing samples with

successively higher ethanol concentrations (30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % and 95 %) for 5 minutes each. The sample was then rinsed twice with absolute ethanol for 10 minutes each and allowed to dry for 30 minutes to 2 hours at 60°C. Finally, the sample was gold-coated for electron microscopy imaging (Relucenti et al., 2021). Microscopy for morphological evidence of sporulation and presence of exopolysaccharides.

2.4 Results and discussion

In the following sub-section, the successful propagation, storage and culturing of *C. acetobutylicum* in chemically defined media will be discussed with focus on the characterization methods which identify trends in carbon sequestration, utilization and ABE metabolite biosynthesis.

2.4.1 Propagation, microscopy and storage of *C. acetobutylicum* cultures

For the propagation of *C. acetobutylicum*, the suspended spores in CNM were inoculated into two vials (A and B), to prepare reserve cryogenic stocks from the first generation of the commercial spores. The graph below (Figure 2.4) is indicative of cell growth in CNM at the time that the glycerol stocks were prepared.

The graph below (Figure 2.4) is indicative of cell growth in CNM at the time that the glycerol stocks were prepared.

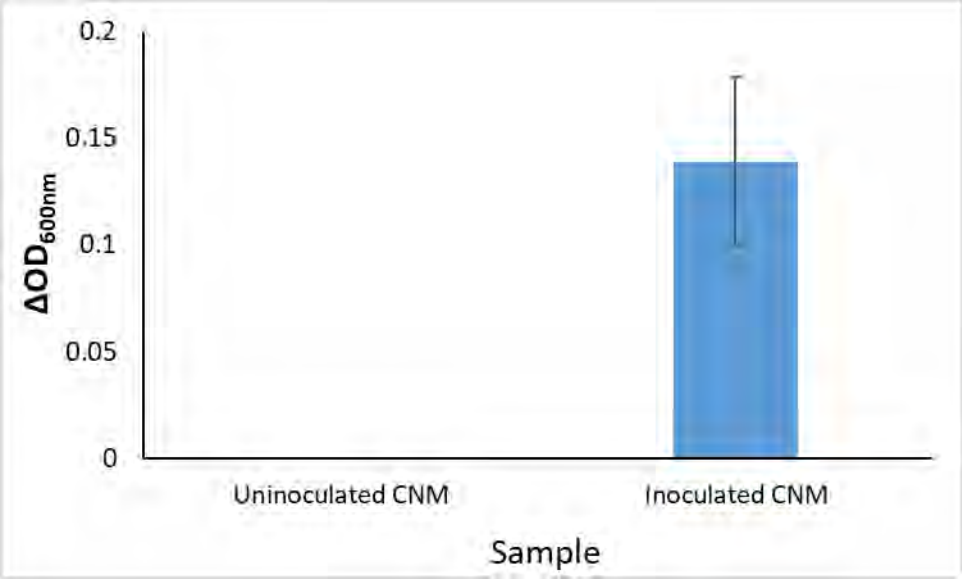

Figure 2.4: Change in turbidity for CNM inoculated with *C. acetobutylicum* after 48 hours of incubation, compared to uninoculated CNM, using the initial commercial spores provided.

The graph above (Figure 2.4) indicates the growth of *C. acetobutylicum* in CNM from commercial spore stock. This OD_{600} measurement was a quality control check the rapid analysis of successful bacterial growth. The indication of a higher OD_{600} reading ensures that subsequent fermentations are inoculated with a viable culture that is compared to an uninoculated control.

To determine the phase of fermentation with the comparison of different methods for viability and efficiency. Below is an image of *C. acetobutylicum* grams' stain by light microscopy. The image was taken for the effort of determining the purity of the culture and the morphological characteristics that are associated with endospore formation.

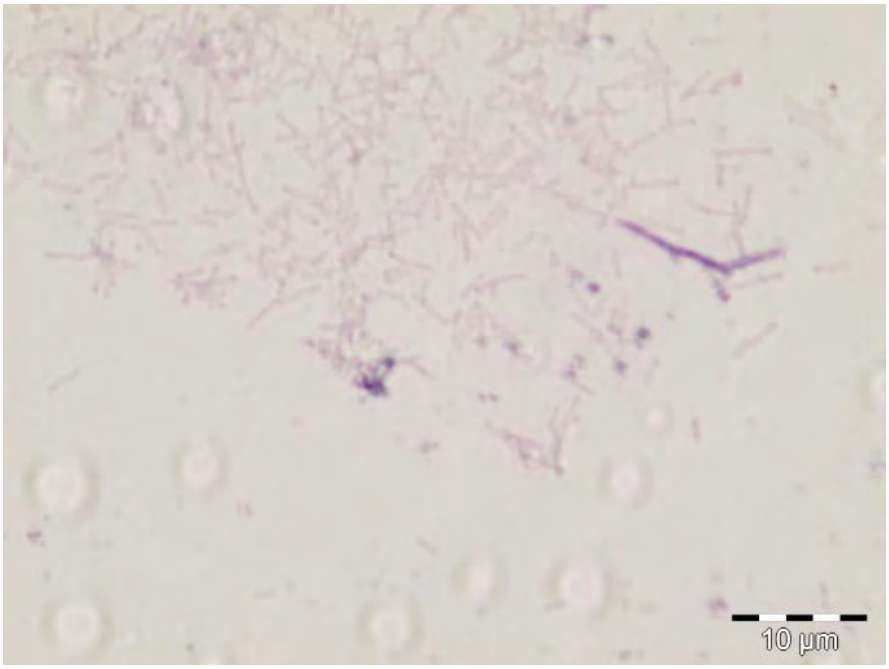

Figure 2.5: Example of gram-stained sample of cultured *C. acetobutyricum* under 500× magnification (oil immersion) light microscopy.

Viewed by optical microscopy (Figure 2.5), the cell shapes appear to be bacilli in shape, but have variable lengths, and exhibit some degree of aggregation, which may indicate the production of exopolysaccharides used in the formation of biofilm. As illustrated in Figure 2.5, most Gram stains taken throughout the study depicted both Gram-negative and Gram-positive bacilli cells. While *C. acetobutylicum* is classified as Gram-positive bacilli cells (Dürre, 2005), Gram-variability is a known feature of *Clostridium* cells (Biebl, 1999). Immature cells in vegetative phase are more easily identified as Gram-positive bacillus; conversely, due to cell wall damage caused by solvent exposure and autolysis, endospore-forming cells in the solventogenic phase do not retain crystal violet and often present as Gram-negative cells (Smith and Hussey, 2005).

Gram-variability is a limitation of the characterisation of whether the culture is axenic using optical microscopy means alone. For this reason, SEM (Figure 2.6) was performed. To confirm the initial culture contained *Clostridia* cells, scanning electron microscopy (SEM) was used for greater accuracy for the morphological characterization of *C. acetobutylicum* using a limited number of samples during the research. Examples of these can be seen in Figure 2.6 below.

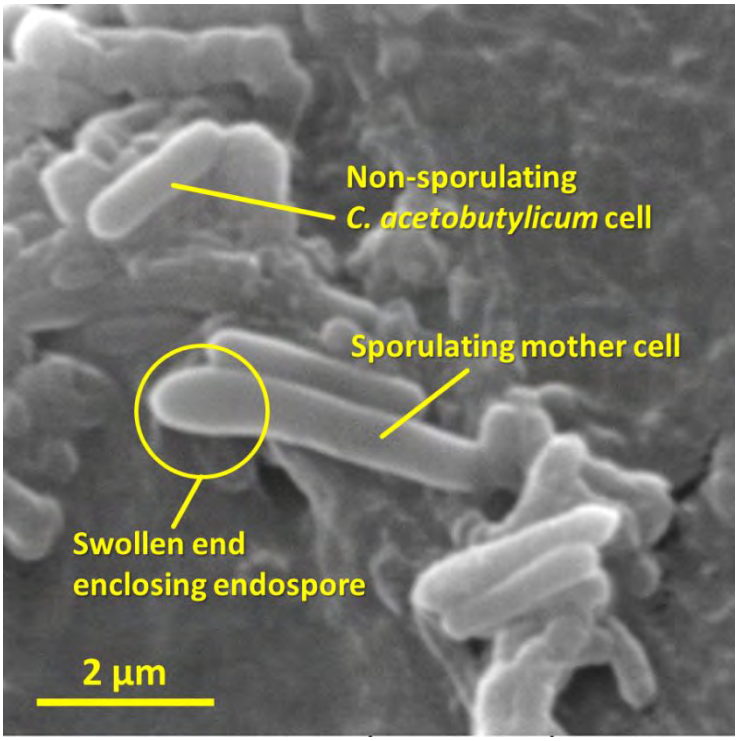

Figure 2.6: SEM image of *C. acetobutylicum* cells obtained from batch culture.
The characteristic endospore formation in a mother cell and the typical bacillus shape of vegetative (non-sporulating) cells are annotated. As Figure 2.6 shows, the morphology of *Clostridium acetobutylicum* was identified by the swollen mother cells that take on a golf club – like shape that can be seen in the Figure 2.6 (Durre, 2005).

The ambiguity of optical microscopy results when attempting to evaluate the purity of subsequent cultures was deemed unreliable for *Clostridium spp*. It was subsequently used as a rapid test to enumerate cells to confirm cell densities but was not considered a viable option for determining if a pure culture was being maintained, or for determining maturity of *Clostridium* cells. For the remainder of the experiments, Gram staining was only used intermittently between experiments to ensure the culture was devoid of obvious contamination from other microorganisms i.e. that predominantly rod-shaped cells were observed at the start of fermentation. Due to the Gram-variability noted in Figure 2.5, the rod-shaped bacillus morphology was used to determine the subsequent purity of the cultures used in this research when using optical microscopy, rather than cell colour.

2.4.2 Development of chemically defined media

The aim of this experiment was to measure growth characteristics of *C. acetobutylicum* cultured in CNM with changes to carbohydrate constituents, to determine the optimal media for solvent production, culture maintenance and storage. As solventogenesis and sporulation phases proceed concurrently, the inability to distinguish the products of each process, without corresponding physiological markers or product detection, suggests a potential loss of functionality in both pathways (Al-Hinai et al., 2015; Long et al., 1983).

To compare the influence of media composition on subsequent propagation of *C. acetobutylicum* cultures, the dependence of the rate and extent of cell turbidity (measured using OD_{600}) during culture with the different media was compared. Figure 2.7 below, presents background subtracted OD600 measurements for the study described below.

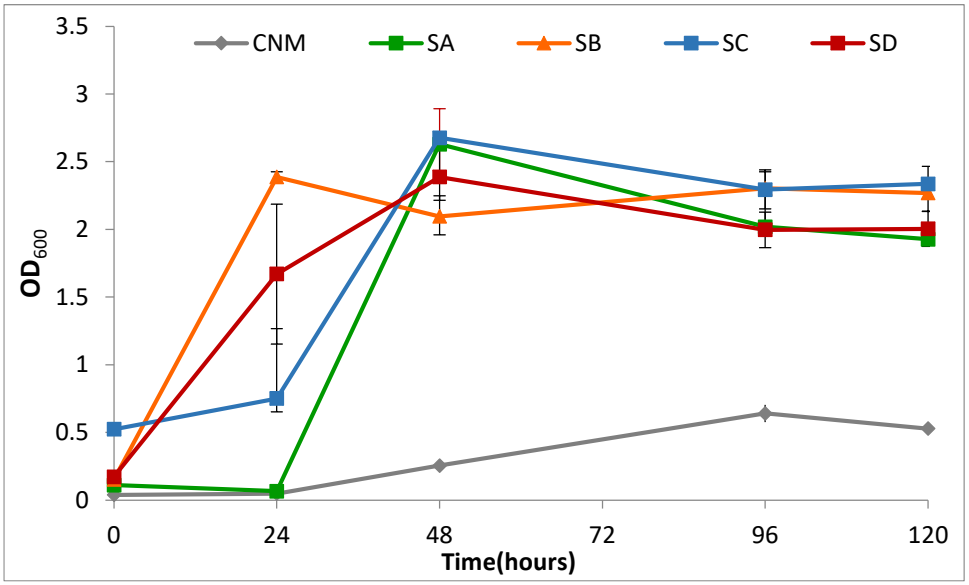

Figure 2.7: Influence of supplementation of CNM with P2 micronutrient solution and carbohydrates on subsequent growth kinetics of inoculated C. *acetobutylicum*, measured using turbidity of suspended cells.

In Figure 2.7 the increase in cell density within the first 24 hours of fermentation indicates general trend signifying successful growth. A slight decrease in cell density is seen after 24 to 48 hours of fermentation of inoculated media. This is likely to be caused by solvent toxicity and the production of bacteriocin after the assimilation of acids to form solvents in the solventogenic phase. This has

been one of the major limiting characteristics of ABE fermentation and successful culture maintenance for the intention of efficient ABE production. Although cell density does decrease this is also significant to identify the viability of solvent producing cells characteristic of *C. acetobutylicum* fermentation (Barber et al., 1979; Long et al., 1983). This further incentivised the need for the measurement for multiple parameters to best identify trends in fermentation kinetics under different physiological condition (different substrates) for *C. acetobutylicum* ABE fermentation. For this reason, pH measurements were also conducted in parallel with turbidity measurements.

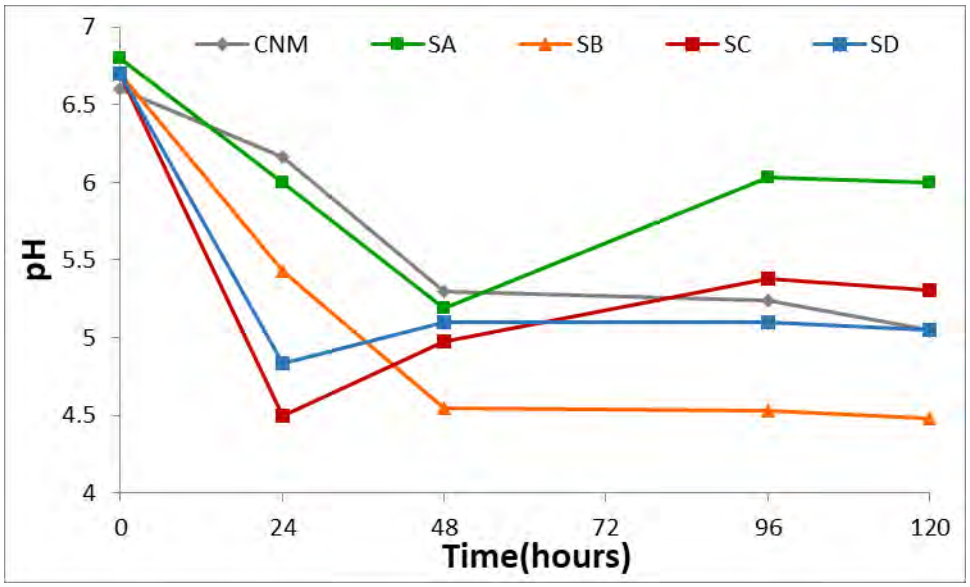

Figure 2.8: Influence of supplementation of CNM with P2 micronutrient solution and carbohydrates on culture pH of *C. acetobutylicum* cells.

Illustrated by Figure 2.8 above, pH rapidly dropped after inoculation of the *C. acetobutylicum*, reaching a low, for all, media between 24 and 48 hours, followed by a subsequent increase. According to-Li et al. (2012) a minimum pH of around 4.5 is essential for Butanol production in *C. acetobutylicum*, as this is the traditional method in determining the metabolic phase shift from acidogenesis to solventogenesis. Successful production of acids was identified by observing a minimum pH and stabilization or slight increase in pH as solvents were produced. From Figure

2.8 the shift from acidogenic phase to solventogenic phase occurs as the pH reaches a minimum between 48-72 hours. This indicates that solventogenesis will commence from 48-72 hours.

Sporulation media D was adapted from literature as the original spore maintenance media for *C. acetobutylicum* included a source of starch (Long et al., 1983). However, CNM does contain starch. Chemically defined media B was the most economically viable for glycerol stocks and analysis of the metabolic phase shift of *C. acetobutylicum.* HPLC data from the above study was processed on a laboratory computer/Software controlling the HPLC that was stolen, preventing re-analysis for 8 months at time of writing. The experiment did not directly answer the main hypothesis/research question – the objective was simply to determine a chemical media which successfully resulted in solvent production by the fermentation of *C. acetobutylicum.* The data were noted manually by the researcher and indicated a chemically defined Media was selected as a viable candidate for subsequent experiments. However, after 120 hours all media variations SA, SB, SC, and SD were positive for butanol production using studies similar to those reported below, in Section 2.4.6. From the above studies, Sporulation media B was identified as ideal for culture maintenance and spore production. Sporulation media B was selected as the chemically defined media for characterization of fermentation studies in later Chapters and will be referred to as Media B (MB).

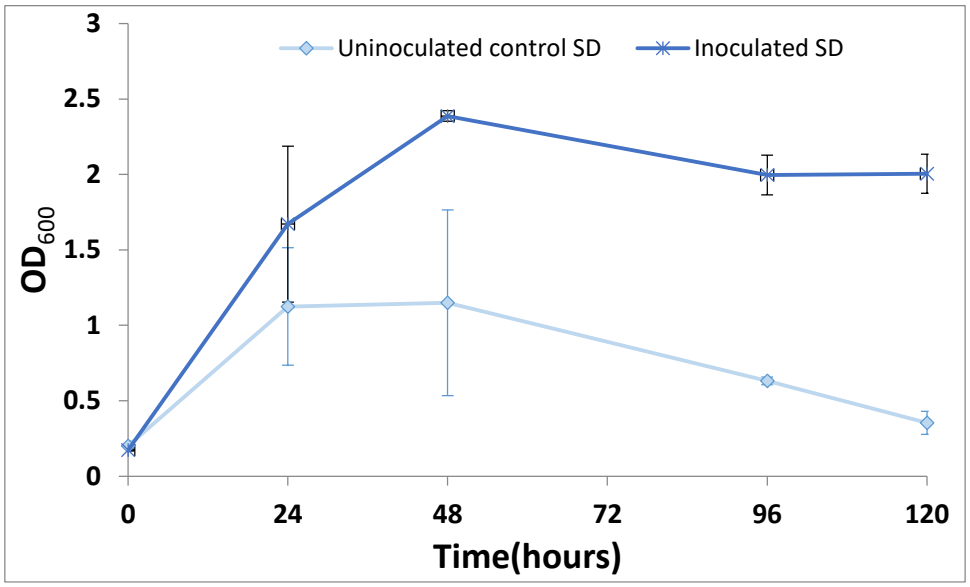

Figure 2.9: *C. acetobutylicum* cultured in Sporulation media D showing turbidity for inoculated replicates vs. contamination in uninoculated control.

The restrictive operating conditions inside the anaerobic chambers mitigated the use of traditional aseptic techniques. The endospores produced by *C. acetobutylicum* are dormant until a favourable environment such as the one that was prepared in the anaerobic culturing chamber or specifically the uninoculated control presents in Figure 2.9 as an opportunity to initiate a vegetative growth cycle, increasing cell density. The source of contamination may have been equipment or on the internal surfaces of the anaerobic chamber. The inoculated media SD indicates a higher OD compared to the uninoculated control. These results indicate the careful preparation before inoculation and operation during fermentation as essential to reduce the impact of cross contamination from sampling tools and probes.

Based on and Time vs pH/OD_{600} in Figures 2.7 and 2.8, Media SB (i.e. CNM amended with P2 and 20g glucose) presented as the optimal media for sporulation and solventogenesis. Media SB indicated the most rapid increase in OD_{600} in the first 24 hours of fermentation, utilising glucose as the main carbon source similar to the media used in previous studies incorporating *C. acetobutylicum* fermentations (Finch et al., 2010). As seen in Figure 2.8 The uncontrolled pH drop during times 0 to 48 hours and subsequent stabalisation from 48 to 120 hours at around pH of 4.5 for media B successfully indicated similar results to that found previously (Al-Shorgani et al., 2018). Consequently, Figure 2.8 MB was selected as a control media for other studies and is referred to as “Media B” (MB).

Methylene Blue was an addition to the media that occurred after the study in Figures 2.8. Preliminary studies during section of the research showed it to interfere with spectrophotometric analysis in a time-, media- (especially, L-cysteine) and cell-density-dependant manner, affecting subsequent turbidity measurement (Fernández-Pérez, Valdés-Solís and Marbán, 2019). All subsequent fermentations therefore included 5 mM of methylene blue for the indication of oxygen and the ability to act as an electron mediator. Furthermore *Clostridium kluyveri* in the presence of mediators (methylene blue) has been studied for its ability to receive electrons from electrodes with no ability to directly interact with the electrode. A flow of electrons was recorded with no connection to metabolism in the presence of methylene blue (Koch et al., 2017). The understanding of the affect methylene blue has on metabolism will be imperative to the interpretation of finding presented in later chapters.

Media B is likely to induce acetone production from the presence of methylene blue (Kim and Kim, 1988a) Furthermore, methylene blue does have then redox potential (+10mV) to affect glucose metabolism, blocking the degradation of pyruvate and interacting with hydrogen-generating biochemical sites (Katagiri et al., 1960). The biochemical reduction of methylene blue in the glucose fermentation by *C. acetobutylicum* may indicate that the accumulation of hydrogen in the form of NADH was not favoured as opposed to transferring hydrogen to methylene blue (Demuez et al., 2007; S. Kim et al., 2015; Matta-el-Amouri et al., 1985). Without the generation and accumulation of NADH, the complexes that drive the metabolic flux towards butyrate and butanol synthesis are disturbed and not induced (Jiang et al., 2009). *C. acetobutylicum* thiolase enzymes which selectively catalyses complexes Aceto-CoA towards Acetoacetyl-CoA butanol production is possibly responsible for the redox dependant switch controlling metabolic phase change from acidogenesis to solventogenesis.

The Thiolase complexes formed in *C. acetobutylicum* interact with thiols as cellular reductants and is possibly the interaction of methylene blue blocks the redox-dependant regulatory switch to solventogenesis (Katagiri et al., 1960; Kim et al. 2015). Methylene blue in the context of this research was an additional amendment for the electron mediating abilities within a microbial fuel cell. The development of the chemically defined media for the fermentation carried out by *C. acetobutylicum* was carried out in an equal manner for static fermentation and MFC fermentation where the associated effects of Methylene blue were not intended within batch fermentations that

were not in MFC configuration. The synthesis of ABE solvents for chemically defined media containing methylene blue or devoid of methylene blue is directly linked to the availability of the HPLC facility or lack thereof as described in the following section.

2.4.3 HPLC analysis of *C. acetobutylicum* fermentations

For the quantification of fermentation product yield, HPLC methods need to identify the correlating substrate, metabolic precursors, and solvents. Here, the HPLC method is explored for the determination of the successive metabolites produced from the carbohydrate substrate during fermentation. HPLC chromatogram of ABE fermentation metabolite retention times agree with literature (Vohra, 2015). The Figure below is an indication of the most important compounds incorporated as standard concentrations that will be compared for the fermentation of glucose by *C. acetobutylicum* and product formulation in ABE fermentation.

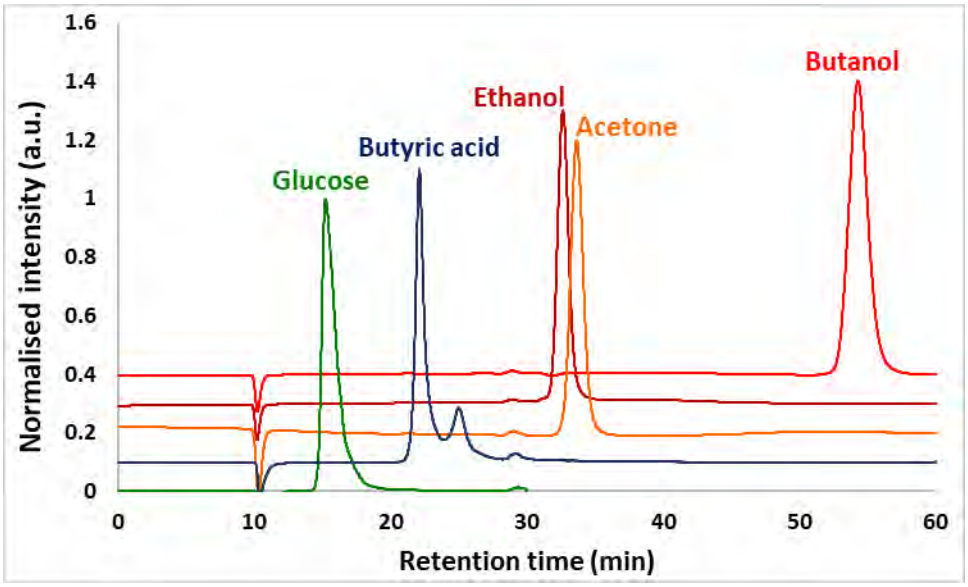

Figure 2.10: Normalised HPLC chromatograms comparing retention times and normalised peak profile of tested ABE fermentation standards.
The refractive index HPLC was used with the Phenomenex Rezex ROA organic Acid Column H+ (8%) 300x7.8mm 8 µm column at an oven temperature of 80°C, and mobile phase at a flow rate of 0.4mL/min. Peak responses of separate injections of standards are annotated above: glucose (~ 15min), butyric acid (~22 min), ethanol (~ 31min), acetone (~32min), and butanol (~ 55min).

Figure 2.10 shows normalised chromatograms which compare the separation between the standards of the compounds of interest in this research. For ease-of-comparison of the peaks, all presented chromatograms in this research have been normalised so that the distance from the baseline to the maximum peak heights are ~ 1 unit. Different signal intensities - i.e. peak heights and peak areas – obtained from chromatograms were evident between the standards (See Appendices: Appendix A-E) for the obtained standard curves) but are not presented in these chromatograms.

After an initial void volume response at ~ 10 minutes post-injection (evident as a dip in the signal at that point), the HPLC method successfully separated the solvent precursor molecules (glucose and butyric acid) and butanol from the other standards – evident as the separation of the peaks from one-another in Figure 2.10. However, the peaks for acetone and ethanol do not show good separation, evident as the overlapping of the overlaid peaks for these two standards in Figure 2.10. The lack of separation between ethanol and acetone makes it difficult to differentiate exact quantities of these two compounds.

Table 2.1: Comparison of retention times obtained for the standards with those reported in literature.

Retention time (minutes)		
Compound (Marker)	**Experimental**	**Reported**
Glucose (nutrient consumption)	15.085	15.506
Butyric acid (38ontent38atio)	22.113	27.470
Acetone (solventogenesis)	33.783	28.045
Ethanol (solventogenesis)	32.891	29.685
Butanol (solventogenesis)	54.958	45.017

Reported values adapted from Vohra et al. (2015)

Table 2.1 indicates the retention times of standards of compounds of interest (glucose, butyric acids, acetone, ethanol and butanol) that were achieved in comparison to similar HPLC operating conditions and procedures in literature. Operating conditions were closely matched for the

generation of comparable retention times from literature presented in table 2.1. Successful separation for individual quantifiable detection of glucose (15.085 minutes), butyric acid (22.113 minutes) and butanol (54.958 minutes) were achieved in this study. The retention time of butanol in this study was retained in the column for a longer time (45.017 minutes) than Vohra et al. (2015) reported. Where butyric acid was separated more efficiently in the results above than reported. However, the method applied in this study did not adequately separate ethanol and acetone. Interestingly acetone is detected following a shorter retention time than ethanol in which the opposite was recorded in literature (Vohra et al., 2015).

The main difference was the hardware used, referring specifically to the column and the hardware (pump, detector etc), which were from different manufacturers. The Phenomenex column used in this study like that of the reported study is comprised of a 8 % resin cross-linked Hydrogen ionic form with a size of 300×7.8 mm with the difference in a particle size of 8µm as opposed to the 9µm particle size for the Aminex HPX-87H column used in the literature, which may explain the longer retention time of larger molecules such as butanol from the smaller particle size of the column used in this study (Vohra et al., 2015).

The other causative variation in retention times is the reaction of the compounds present in the sample in response to the environmental conditions created by the HPLC. Diffusion of compounds in the mobile phase changes when exposed to different oven temperatures of 80 ℃ which dynamically relates to pressure or back pressure of the system, affecting elution of the compounds through the column (Vohra et al., 2015). The methods used in literature are guidelines and changes can be made to protocols for the desired outcome.

Albeit, for subsequent fermentation experiment analysis, it is imperative that the best separation of all compounds of interest is clear for quantification. While a lower flow rate could have further improved resolution between acetone and ethanol, this would have significantly increased the total run time of analyses for all the samples processed. As mentioned in literature, methods such as flow rate and oven temperature can be changed for desired outcomes. However, for large amounts of samples that take over an hour each to complete analysis, this may not be viable in some situations.

It is possible in commercial applications where all compounds will be of interest throughout the entire duration of the fermentation and can be effectively quantified in half the time than the

method used in this study (Vohra et al., 2015). Standard curves varying the concentrations of the above standards are illustrated in the appendices (Appendix: B, D and E). These show that, using independent measurements, the standards could be accurately quantified from 30 g/L to 0.01 g/L in a linear fashion, once converted to log-log format.

The obtained HPLC protocol was then tested for compatibility with complex media samples, illustrated in Figure 2.11, below.

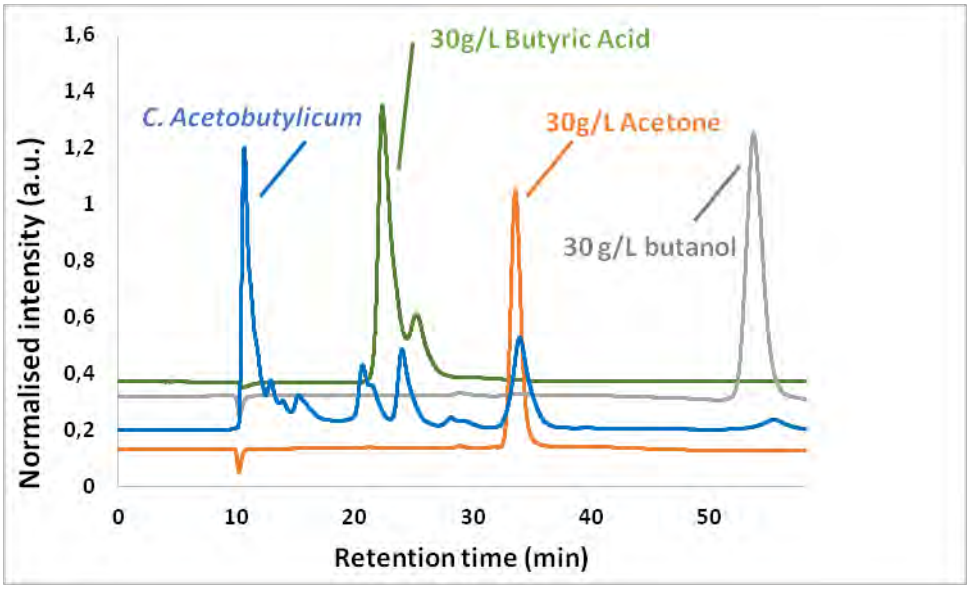

Figure 2.11: Normalised HPLC chromatograms demonstrating ABE production by *C. acetobutylicum* fermentation.
C. acetobutylicum was cultured on chemically defined media B for 48hrs before sampling. Peak detection of metabolic products and corresponding standard peaks of; Acetone (32min), Butyric acid (22min), and Butanol (55min) are supplied for comparison.

From Figure 2.11 were the HPLC chromatogram of standards are compared to *C. acetobutylicum* fermentation on chemically defined media. From literature it is known that the other compounds that are detected by the HPLC are present, however the comparison to select standards analysed using the same methods for HPLC protocol such as flow rate the compounds of interest can be singled out. For the protocol used in this study and the Chapters that follow, acetone and ethanol were not adequately separated for individual quantification and the identification of both peaks with very close retention times distorts the concentration of either acetone or ethanol. For this reason, the concentrations of the solvents, acetone and ethanol when quantified by HPLC for ABE

fermentation products should be referred as the quantification of a single compound. Butanol, glucose and butyric acid indicated successful separation for individual quantification, acetone ethanol will be referred to collectively.

2.5 Conclusions

The experiments reported on this Chapter describe the kinetics of *C. acetobutylicum* fermentation in chemically defined media. *C. acetobutylicum* is characterised for metabolite production and sporulation using pH measurements, light microscopy, OD_{600}, SEM and HPLC. Sporulation media B was chosen for the rapid fermentation time and metabolite production. Further experimentation for the rest of this thesis will use sporulation media B and refer to the media as media B (MB).

The experiments were conducted in an anaerobic chamber, which limited the use of flame sterilisation for aseptic technique. Sterilisation by traditional aseptic techniques is not possible during operation and the addition of a UV sterilisation system would have been an ideal solution to overcome this. Sampling in a small, enclosed area was found to be the most debilitating design flaw. The flasks and fermenters inside the chamber, had to be kept within a certain scale, taking the headroom for sampling with a pipette into consideration. For a simple design, maintaining an airtight environment is the main objective.

Exposure of the entire contents of the chamber to the atmosphere for successive sampling during ABE fermentation may be a higher initial investment that can possibly prove to be economical in the long run. Repetitively purging a large space for sampling, rather than maintaining a consistently anaerobic environment requires increased use of costly consumables to combat the threat of oxygen contamination. ABE fermentation is a complex operation, however it can be simplified with the correct hardware and facilities. With the intention of manipulating different variables within fermentation kinetics, fermentation operation needs to be efficient and reduce the impact of uncontrollable variables because of the system design.

The chemically defined media B was an adaption for the later experimentation of batch and MFC fermentation experiments that utilised a similar carbohydrate composition to that used in previous *C. acetobutylicum* MFC studies (Finch, 2010). *C. acetobutylicum* does not require amendments to CNM for vegetative growth, moreover amendments are necessary when shifting metabolism to

solventogenesis and for endospore production. The addition of P2 for micronutrient supplementation aided *C. acetobutylicum* in its fermentative ability to produce solvents and spores (Kheyrandish et al., 2015). SEM analysis was used to determine morphological characteristics that indicate endospore formation. Swelling in the mother cell identified in Figure 2.6 is evidence that *C. acetobutylicum* will produce spores in chemically defined media in batch fermentation.

HPLC analysis confirmed that the key fermentation parameters (glucose, butyric acid, acetone, butanol, and ethanol content) could be detected and (apart from ethanol and acetone) were separable in chromatographic analysis. Increasing column oven temperature and decreasing flow rate in HPLC analysis for ABE fermentation from the conditions reported in literature produced a chromatogram with a greater separation resolution of peaks for the standards (Vohra et al., 2015).

This is especially important when fermenting complex carbohydrates and to quantify metabolic products with similar molecular sizes. For example, glucose and butyric acid or acetone and ethanol peaks have very close retention times for the HPLC method used in this study, decreasing the flow rate from 0.4 ml/min and oven temperature rom 80℃ would have resulted in the merging/amalgamation of peak area/heights (Vohra et al., 2015 & Tsuey et al., 2006). The HPLC method used for the analysis of ABE fermentation by *C. acetobutylicum* described in this Chapter will be used in the following Chapters. It is important to remember that successful separation of acetone and ethanol was not achieved, and concentrations of either solvent will be referred to collectively as acetone/ethanol.

Degassing Media in the vacuum chamber was the most economically viable option as it did not require as much nitrogen gas. Therefore, larger volumes of media purging with nitrogen for 15 minutes was not an option.

This Chapter identified methods to measure the fermentation kinetics of *C. acetobutylicum* fermentation, using chemically defined media for the objective of culture maintenance and quantification of metabolic products and precursors.

Chapter 3: Comparing substrates for the characterization of brewers spent grain wastes as a feedstock for enhanced *C. acetobutylicum* batch fermentations.

3.1 Introduction

During brewing, the largest contributor to the total percentage of waste is produced in the form of brewer's spent grain (BSG) (Mussatto et al., 2006). This is residual grain biomass after the mashing stage of grains (converting starches to simplified carbohydrates for subsequent brewing). This was the focus as a potential waste stream for value additive technologies.

BSG is a high-value waste product and provides a low-cost/high-volume feed source to livestock in agriculture. Removing this commodity from downstream processing channels such as BSG animal feed stocks, reiterates the debate of the use of food or feed for biofuel production. (Aliyu & Bala, 2011; Mussatto, 2014). Lignocellulosic materials such as BSG have been explored for the production of bio-butanol (Ibrahim et al., 2017).

As mentioned in Section 1.1.7 of Chapter 1, BSG is considered a viable candidate as a lignocellulosic feedstock and has been investigated as a low-cost feedstock for ABE fermentation (Mussatto et al., 2013). Due to the removal of most of the easily-fermentable carbohydrates and soluble proteins, BSG is considered not a directly-usable food source for humans, but is however a feedstock rich in cellulose (Aliyu & Bala, 2011; Mussatto, 2014). Of the brewing wastes, BSG is the most abundant carbon source and requires the least processing for ABE fermentation, compared to other brewing waste streams (Aliyu & Bala, 2011; Kerby & Vriesekoop, 2017; Plaza et al., 2017; White et al., 2008; Xiros & Christakopoulos, 2012).

BSG contains proteins and polysaccharides (starch, cellulose, and hemi-cellulose) that can serve as nutrients to catalysing microorganisms (Parchami, Ferreira, and Taherzadeh, 2021; Xiros and Christakopoulos, 2012). For this study, the starch component of the BSG polysaccharides will be of focus and the liberation strategies of remaining polysaccharides will be discussed. As such, BSG is an excellent feedstock for bioprocesses; due to its status as a waste product, it is a economically viable option for an industrial-scale substrate (Mussatto, 2014). The key limitation for using BSG effectively are preservation and storage (Mussatto and Roberto, 2006).

The storage of produced BSG is of concern; many of the microbiota resident on the BSG following wort production remain viable (Mussatto and Roberto, 2006) and additional microbes from the surrounding environment can colonise this feedstock (Bianco et al., 2020). After wort production, these microbes grow in this medium, supported by the high-water content of these grain wastes (Ikram et al., 2017) forming a dynamic ecosystem immediately after the production of this waste. These have the effect of metabolising the biomass comprising the BSG, altering its chemical composition over time and affecting the reproducibility and yields of subsequent fermentations using poorly-stored BSG (Bianco et al., 2020)

Pre-treatment of BSG aims to further improve the nutrient content of BSG using a variety of approaches (Mussatto and Roberto, 2006). Dilute acid hydrolysis, alkaline pre-treatment, microwave assisted treatments and steam hydrolysis were methods previously explored in BSG pre-treatments (dos Santos et al., 2015; Mussatto et al., 2008). Many of these pre-treatments also have the effect of sterilising BSG, improving both storage and the cultivation of anoxic bioprocesses (Jonglertjunya et al., 2014). Inhibiting and reducing compounds such as furans (especially hydroxymethyl furan) and coumaric acid (Ding et al., 2018; Liu et al., 2019) might be produced from different pre-treatments along with the liberation of desirable carbon sources e.g. lignocelluloses, cellulose and hemi-cellulose, starch and reducing sugars (Moreira et al., 2013; Mussatto, Dragone et al., 2007; Mussatto, Fernandes et al., 2007). The compounds mentioned above can inhibit fermentation completely (Jönsson and Martín, 2016).

An ultraturrex can be incorporated as a laboratory grade dispersing device that is used for homogenisation, emulsifying and suspending liquid solutions. The use of an ultraturrex for BSG treatment physically breaks down BSG particle size and makes it more soluble in water. An autoclave is a sealed vessel that is pressurised by the production of steam from heating water inside the pressure rated vessel. Autoclaves are efficient tools for sterilising media and equipment. Depending on the settings of temperature and time, the autoclave can sustain very high temperatures and pressures for a select duration for the intended purpose (Macheiner et al., 2003). The action of steam sterilization under high pressure and temperature can have physical and chemical effects on the media. Hydrolysis pre-treatment strategies of BSG with successful solubilisation of starches have been carried out by manipulating the operating conditions of an autoclave (Parchami et al., 2021; Ravindran et al., 2018).

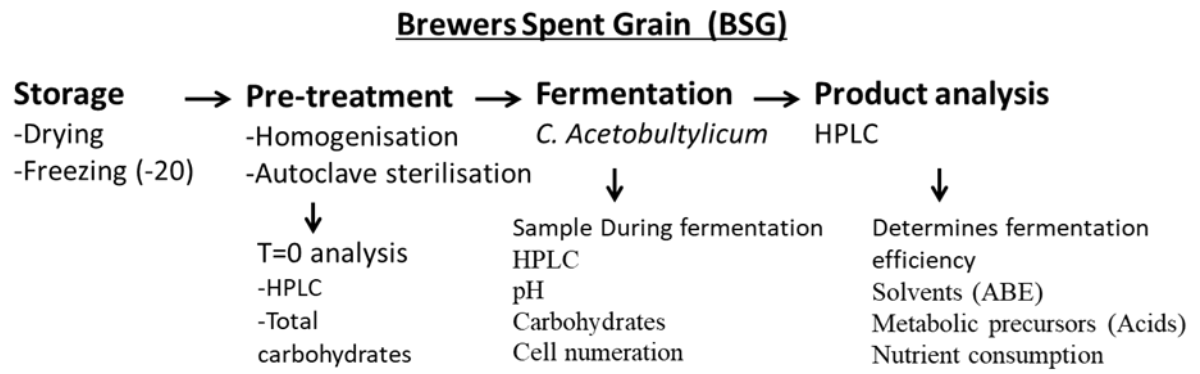

Figure 3.1: flow diagram of BSG processing for value additive solvent production from BSG used in Chapter 3.
As represented in Figure 3.1, the Kinetics of the fermentation of chemically defined media B (MB), Sterile Brewers Spent Grain (SBSG) and Non-Sterile Brewers Spent Grain (NSBSG) are measured and compared to highlight trends and themes for further experimentation. Fermentation parameters including, OD600. Total carbohydrates, pH, glucose, butyric acid, acetone and butanol were measured in the attempt to identify trends the ABE fermentation process. Adapted from (Barber et al., 1979 ; Long et al., 1983 ; Mussatto, 2014 ; Plaza et al., 2017)

The BSG nutritional value must be characterised for fermentation products to be analysed in order to track fermentation and measure metabolite production efficiency and ultimately yield. To do this, the BSG has to be stored in a manner that does not degrade the feedstock such as drying or freezing. The BSG must be treated (homogenised and autoclaved) so that the environment (pH) is conducive for the microbes (cell numeration) to access the nutrients (carbohydrates) and produce the desired products. The only way to ensure that the environment is conducive for efficient product formation is to track multiple parameters that show the kinetics through the course of fermentation of the analysed t=0 BSG substrate. Utilising HPLC method can show carbohydrates, metabolic precursors and solvents tracking progression of the fermentation from substrate to desired products.

3.2 Aims

The application of industrial microbes to produce value-added products requires a nutrient source which is sustainable and economically feasible. The overall aims of Chapter 3 were to evaluate the efficiency of raw sustainable waste.

The aim of the study in this Chapter is achieved by the determination of the kinetics of ABE fermentation utilizing different feedstocks, tracking carbohydrate consumption, organic acid and solvent production within select parameters. After nutrient characterisation of these sources, fermentation will be compared to chemically defined Media B from Chapter 2 of this thesis, both comparing the kinetics of the fermentations and comparing the overall yields of the ABE solvents produced. The major problem faced when attempting to produce biosynthetic products from a fermentation and determine the efficiency at different time is the effect of simple processing on the nutrient profile and subsequent fermentation process.

3.3 Methods and Materials

3.3.1 Reagents and Apparatus

Unless otherwise stated, all reagents were of technical grade (i.e. $\geq$ 95 %) or higher.

Silica gel and Phenol were sourced from Merck.

P2 trace element solution was formulated as detailed in Chapter 2 Section 2.3.2

All reagents for media preparation were also described in Chapter 2 section 2.3.

An Ultraturrex (Z722561) IKA® ULTRA-TURRAX® disperser tool was employed, specifically the stainless steel dispersing element designed for the T-10 basic disperser with a 10 mm diameter (Sigma), for the purpose of homogenizing the Brewers' Spent Grain (BSG).

3.3.2 Source and storage of brewery wastes

The BSG samples were sourced from beer mash from the third-year microbiology brewing practical to be processed for moisture content and fermentation.

BSG was collected from a pale ale recipe, which was comprised of 80% pale malt, 10% Caramunich 1 malt and 10% Munich Malt. The beer was mashed at 65 °C for one hour and 76 °C mashed out for 10 minutes before undergoing the lautering and sparging processes, where the remaining fermentable sugars are rinsed off. At this point the Malt is now considered BSG.

All the BSG utilised in this study was stored for moisture content analysis and fermentation studies respectively. The BSG was stored in 2 kg sealed polypropylene buckets at -20 ℃ until required for fermentation studies. BSG mass was recorded directly from the mashtun and stored in 50ml tubes at -20℃ until samples were dried for moisture content analysis.

3.3.3 Moisture content analysis

To determine the moisture content of the BSG samples were taken from the Microbiology third year brewing practical, three different samples of wet BSG were taken using 50 ml falcon tubes, before storing the remainder as detailed above.

The BSG was removed from the 50ml tube, placed on tinfoil and dried at 60 °C for rapid desiccation Alternatively, the BSG was dried in a SiO_2 desiccation chamber for 48 -72 hours without applied heat or electricity usage. The mass of the wet BSG was recorded and compared to the mass of the dry BSG to determine moisture content of raw BSG. (Mallen & Najdanovic-Visak, 2018)

3.3.4 Formulation of fermentation media

Media B was formulated as described in Chapter 2, Section 2.4.2. P2 was formulated as described in Chapter 2, Section 2.3.2.

For fermentation using BSG, the bucket containing BSG was taken out of the freezer and left sealed at room temperature to thaw for 30minutes. BSG-based media from this stored sample was prepared by adding water at a ratio of 100 ml for every 120 g of wet BSG. P2 was filter-sterilised

using 0.22 µm cellulose acetate filters and added at a ratio of 0.1% $^{v}/_{v}$ to the mixture of BSG and water.

The resulting mixture was homogenised using the ultraturrex for 1-2 minutes. The Ultraturrex vessel was sterilised with 70% ethanol and rinsed in sterilised water before use. After a pH measurement of the mixture was taken, no manipulation of pH range of 6-6.5 was required.

At this point the homogenised BSG was split into two different treatments namely, Sterilised brewers spent grain (SBSG) and non-sterilised brewers spent grain (NSBSG). NSBSG was processed immediately for inoculation following homogenisation, while SBSG was autoclave-sterilised (30 min, 121 °C) and cooled before the addition of P2 mentioned above and further processing explained below.

De-aeration was done by placing the media in the vacuum chamber for 60 minutes as in Chapter 2, Section 2.3.5. Media was inoculated immediately with methods described in Chapter 2, Section 2.3.5.

3.3.5 Construction, operation, and sampling of the batch fermentations

The setup of the operation was carried out as described for *C. acetobutylicum* fermentations described in Chapter 2, Section 2.3.5. Assembly and inoculation methods are also described in Chapter 2 Section 2.3.7.

The culture was allowed to incubate, without retrieving samples, for a minimum of 48 hours before the first measurement was taken once this was understood from reviewed data from Batch *C. acetobutylicum* fermentation experiments presented in this Chapter.

To remove samples, sterilized 20ml test tubes were placed inside an anaerobic chamber and de-aerated using N_2. A 5 ml pipette was used to transfer 4.5-5 ml of *C. acetobutylicum* broth for analysis. After sampling, the test tubes were removed, and the chamber was sealed and de-aerated again. The samples could then be subdivided into the test tubes for immediate processing of OD_{600} and pH measurements. Where samples for spectrophotometric assays such as total carbohydrates (phenol sulphuric acid) were stored in 1.5 ml tubes at 4°C and samples intended for HPLC analysis stored in 1.5 ml tubes at -20°C.

3.3.6 *Ex situ* assays of samples obtained during fermentation

The batch fermentations were used to compare *C. acetobutylicum* fermentations utilising chemically defined media and Brewers spent grain (BSG)**.** The BSG was either sterilized or non-sterilized. The chemically defined media that was selected was media B (MB), which contained precise measurements of nutrients including carbohydrate sources in the form of glucose and starch.

To obtain samples at various time points during the fermentation process starting from t=0 hours, multiple samples were collected at specific intervals, namely t=48, 72, 96, 172, 196, and 240 hours. Ensuring an anaerobic environment was crucial for these sampling procedures. The chamber was loaded with the necessary items, including flasks, equipment such as pipettes and pipette tips, and 1.5 ml Eppendorf tubes for storing the samples. Subsequently, the chamber was securely sealed and subjected to a nitrogen (N_2) purge before opening the flasks containing actively fermenting *C. acetobutylicum*.

For each sample obtained in Section 3.3.5, the following assays were conducted.

Total carbohydrate analysis conducted by phenol sulphuric acid assay is described in Chapter 2 Section 2.3.8 (duBois et al., 1956).

The HPLC method described in Chapter 2 section 2.3.9 was used to determine the concentration of glucose, butyric acid, acetone/ethanol and butanol (Vohra et al., 2015).

3.3.7 Statistical analysis and treatment

Experimental Batch fermentation parameters (n = 4 for all media) were only considered to be significantly different to the n = 1 control for each media namely MB, SBSG and NSBSG, if the control measurement was outside of the range of the calculated average ± 95 % confidence interval range of the experimental batch fermentation media. The 95% confidence interval was calculated using the t statistic, due to the low number of replicates used (Greenland et al., 2016).

The statistics used to compare individual parameters e.g. extracted parameters from experimental replicates for different media in batch fermentation studies were compared to one-another using one-way ANOVA analysis for each parameter in order to identify significant differences. If a

significant difference was identified by ANOVA, a Tukey HSD *post-hoc* test was conducted to identify which pairs of samples were significantly different from one-another, using a Holm-Bonferroni correction (Staffa and Zurakowski, 2020). For all statistical tests, the level of significance, α, was set to 0.05.

3.4. Results and discussion

3.4.1 Initial characterisation of BSG and growth media

Moisture content of BSG

BSG was dried and was determined to have a moisture content of 72 ± 4% as represented in Table 3.1. The findings represented here are consistent with other reports (Mallen & Najdanovic-Visak, 2018) that report 70-80% moisture content in raw BSG.

Table 3.1: The mean moisture content of BSG from wet and dry mass.

Sample	Wet mass (g)	Dry Mass (g)	Moisture content (%)
Average	20.20±1.05	5.57±0.47	72.31±3.78

Table 3.1 presenting the wet mass (g) of BSG when acquired from the mashtun, the dry mass (g) and the determination of the moisture content of BSG when it is produced as waste in the brewery.

3.4.2 Analysis of media for ABE batch fermentation

To mitigate oxygen entry, the choice to use the following sampling method in the context of the de-aeration step for NSBSG and SBSG, was made. The bulk of the BSG media was processed for fermentation of SBSG and NSBSG therefore a single sample to represent each media controls and replicates was taken for t=0. As a result of this sampling method the mean and standard deviation of each experimental replicate and controls at t=0 was not calculated. From this rationale the t=0 for the control represents the t=0 for the experimental replicates. Below is a HPLC chromatogram of all the media used for ABE batch fermentations prior to fermentation of *C. acetobutylicum* for media SBSG, NSBSG and MB.

The Figures 3.2 and 3.3 below present a comparison of the HPLC chromatograms of standards used to determine compounds of interest (glucose, butyric acid, acetone/ethanol and butanol) and the normalised peaks identified by RI HPLC in *C. acetobutylicum* fermentation of different media.

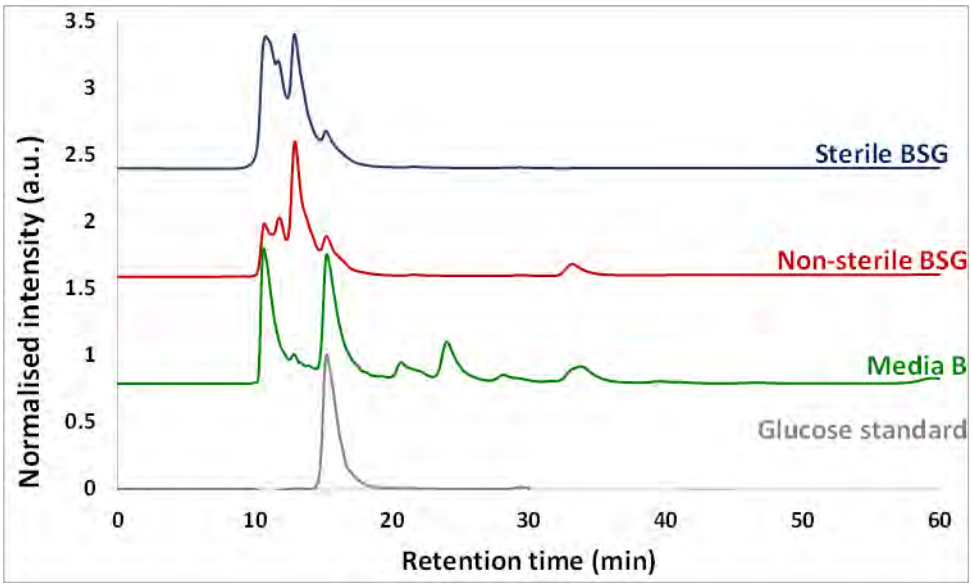

Figure 3.2: HPLC chromatograms of initial media prior to fermentation. 25 g/L of glucose included as standard.

Figure 3.2 is a comparison of the HPLC chromatograms of different media (SBSG, NSBSG and MB) used in this study prior to inoculation equivalent to t=0 and the indication of the glucose standard for presence of carbohydrates.

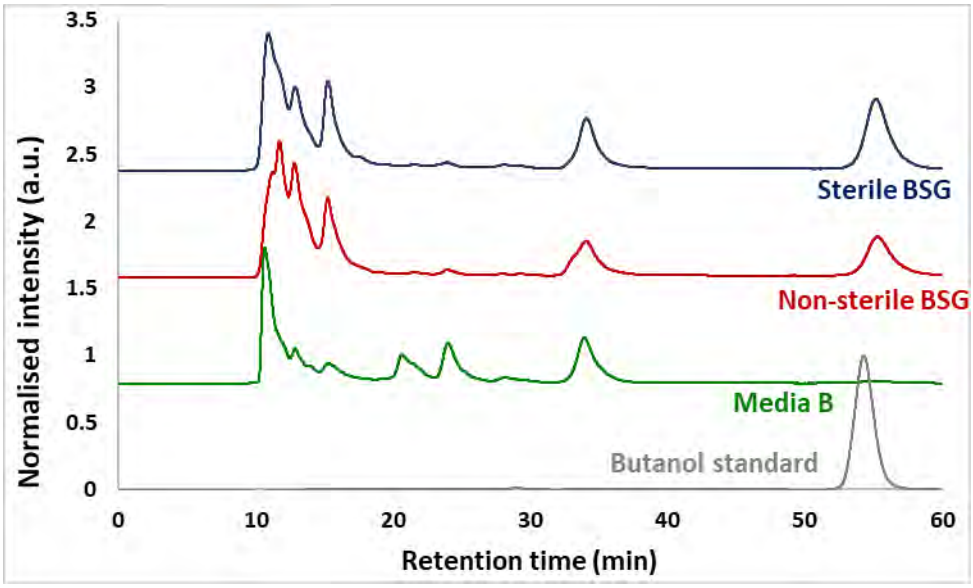

Figure 3.3: HPLC chromatograms of media following 48 hours of fermentation using *C. acetobutylicum*. 30 g/L of butanol included as standard.
Figure 3.3 is a comparison of the HPLC chromatograms of different media (SBSG, NSBSG and MB) used in this study after t=48 hours of fermentation and the indication of the butanol standard for the presence of metabolic products during solventogenic phase of *C. acetobutylicum* fermentation.

HPLC chromatograms were obtained for comparable media in *C. acetobutylicum* fermentation (Figures 3.2 and 3.3, which represent chromatograms obtained at t=0 and t=48). The consumption of glucose present in Media B t=0 was evident, and this medium showed the formation of butyric acid (peak at 22 minutes, standard not shown). Additionally, evidence of hydrolysis activity on the lignocellulosic SBSG and incurred release of glucose was observed in the t=0 chromatogram for SBSG. This resulted in changes in the media composition, as indicated by changes in complex peaks between 10 and 20 minutes. It is evident that NSBSG at t=0 also had glucose present in smaller quantities than MB along with complex carbohydrates as seen in SBSG. Butanol formation was evident in the SBSG and NSBSG.

Several observations were made during the course of the experiment, including the removal of glucose from Media B and the formation of butyric acid. Additionally, the release of glucose and evidence of amylase/cellulose activity in the BSG resulted in a change in the media composition, as evidenced by changes in complex peaks between 10 and 20 minutes. Furthermore, the formation of butanol was evident in both types of the BSG.

From the studies conducted in Chapter 2, it was expected that MB would produce comparable metabolic products to fermentation of alternative media SBSG and NSBSG when detected by HPLC method. In contrast to the standards presented in Chapter 2, Section 2.4.6 the chromatograms obtained from the initial media formulation show a complex profile, in line with their expected carbohydrate complexity. Simple carbon-based organic compounds any extractable component from the media (including alcohols, sugars, fatty acids, amino acids and some of their polymers) present in the sample analysed by the refractive index HPLC detector capable of passing through the column are expected to be represented in the above chromatograms. In terms of ABE analysis, the HPLC protocol (the column used, extraction procedure and mobile phase applied to the media components has been previously validated to detect the compounds of interest (Vohra et al., 2015).

In addition to HPLC-based determination of components, the initial physicochemical parameters of the different media were compared using a variety of assays. Table 3.2 below summarises their findings.

Table 3.2: Physicochemical assays of media prior to inoculation for fermentation

Parameter	**Assayed component**		
	Media B	**NSBSG**	**SBSG**
***In situ* measurements**			
pH	6.500	6.06	6.06
HPLC analysis			
Glucose content (g/L)	11.75	0.965	6.406
Butyric acid content (g/L)	n.d.	n.d.	n.d.
Acetone / ethanol content (g/L)	n.d.	n.d.	n.d.
Butanol content (g/L)	n.d.	n.d.	n.d.
Assays conducted			
Total carbohydrate content (g/L)	49.00±28.02	4.37±0.04	25.84±0.0032

n.d. – not detectable

Table 3.2 above is an overview of the batch fermentation media tested at t=0 hours. Collective analysis of the different media indicates comparatively that MB contains the most carbohydrates with both the highest total carbohydrate content (49.00±28.02 g/L) and glucose determined by HPLC (11.75 g/L) at t=0 hours, compared to either BSG sample: NSBSG's assayed glucose (0.965 g/L) and total Carbohydrate content (4.3651±0.04 g/L) remained the lowest, while and SBSG showed increased carbohydrate content both by HPLC (6.406 g/L) and Total Carbohydrate content (25.84±0.0032 g/L). It is expected that the higher concentration of glucose present in MB would result in higher total solvent yields. Additionally, the treatment differences for BSG, namely media NSBSG and SBSG indicate that the SBSG contains more glucose and soluble carbohydrates than NSBSG (Wilkinson et al., 2015). This may be contributed to the additional sterilising step where an autoclave was used that may have caused the BSG to undergo hydrothermal pretreatment that has been described as starch liberation depending on the operating conditions (Parchami et al., 2021; Ravindran et al., 2018). Albeit the BSG was retrieved from a small-scale batch conducted by non-professional brewers, source and brewing process has been established to contribute significantly in the chemical composition of BSG(Mussatto, 2014; Parchami et al., 2021; Ravindran et al., 2018).

Media B fermentation was formulated to provide a baseline control fermentation of chemically-defined media to compare the accuracy of the assays for efficient ABE fermentation. Gasses produced during fermentation were trapped as bubbles in the grain particles, separating the solid matter from the liquid at different times during the fermentation and making it difficult to take consistent samples. The composition of MB included methylene blue, unlike the SBSG and NSBSG media, which can affect the standard deviation and reliability of the results. The subsequent presentation of total carbohydrates was normalised because of the variations encountered when comparing the different media containing grain particles or oxygen indicator such as methylene blue on the basis of total carbohydrates, all subsequent measurements of total carbohydrates using the phenol-sulphuric acid assay were normalise to the uninoculated control at t=0 to the initial values, which were set at 100 %.

3.4.3 Comparison of fermentation kinetics and yields across the tested culture media

Fermentation experiments were intended to profile the trends of *C. acetobutylicum* for solvent production with the prepared feedstocks, namely the chemically defined media B, MB; the Non-sterile brewers spent grain, NSBSG; and the Sterilized Brewers spent grain, SBSG.

The represented data in tables 3.3 and 3.4 indicate the highest concentration of solvent produced by the different feedstocks during solventogenesis at 72 hours and post-solventogenesis at 196 hours. These are highlighted sample times during the length of the 240-hour experiment.

As previously mentioned, the sampling method for the BSG media relied on a single sample at t=0 for control and experimental replicates. It should be noted that the value for the t=0 for the control of SBSG and NSBSG is the same value for the experimental replicates. The graphs can be viewed within the kinetic parameters which track the progress of fermentation, carbohydrates consumption, organic acid production and solvent production, in that order.

The graphs below were analysed to detect the onset of the solventogenic phase, which typically occurs between 48 to 72 hours and is denoted by the symbol "α" in the graphs presented in Figures 3.4-3.6. The post-solventogenic phase, which occurs between 192 to 240 hours, is marked with the symbol "Ω" for the graphs in Figures 3.3-3.5.

Graphs below shows the measurements over the full duration of 240 hours. The averages represented in the graphs as solid lines are compared to the trend of the un-inoculated negative control as dashed lines run in parallel to the replicate samples. Error bars indicate the 95% CI range for experimental fermentations (n =4). Regions encompassed by grey boxes indicate a difference between the n =1 control fermentation and the experimental fermentations greater than the 95% CI of the experimental fermentations. The backdrop with the colouration of green, red and orange, following that sequence, represents the duration of acidogenic phase, solventogenic phase and post solventogenesis respectively.

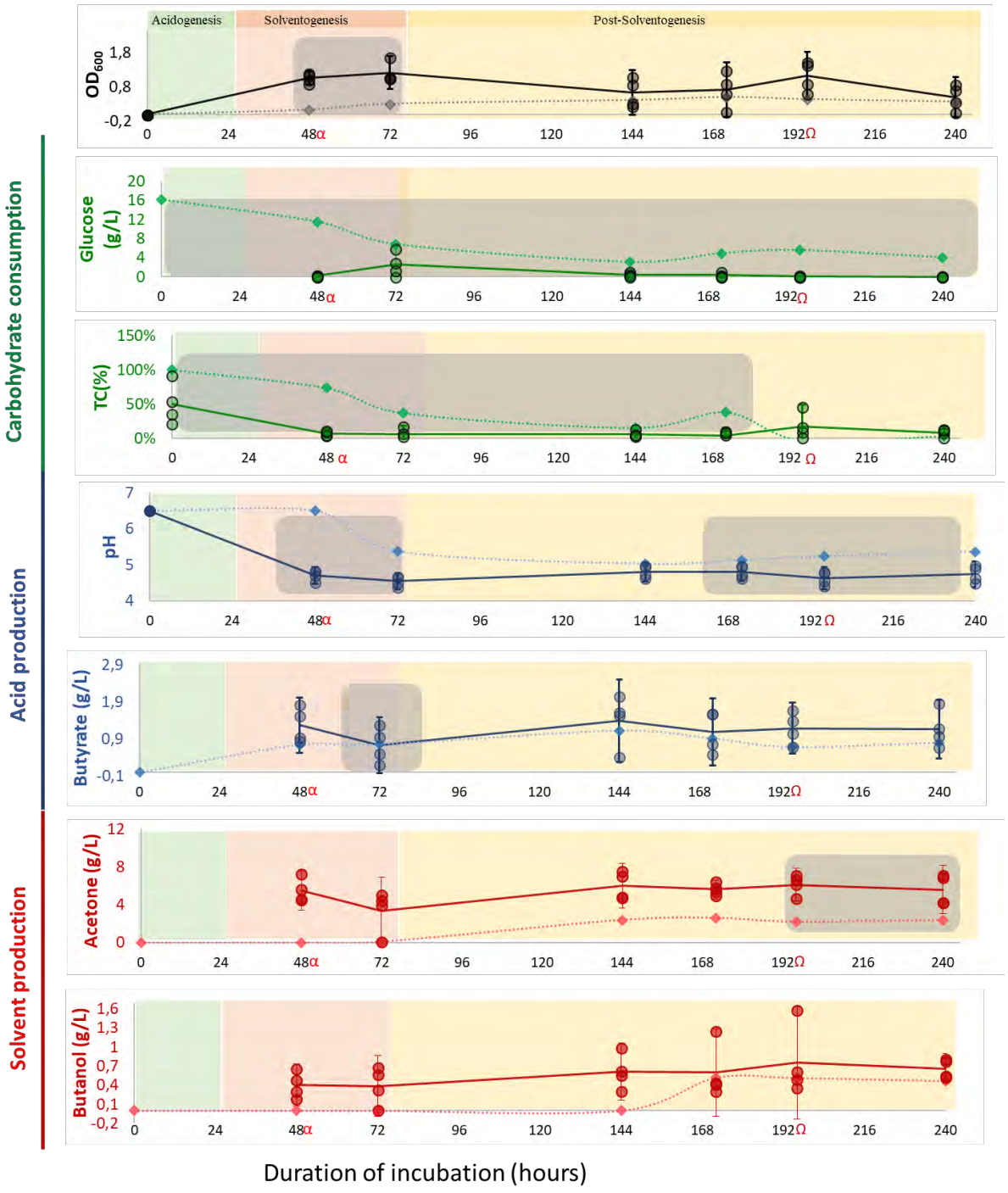

Figure 3.4: Responses and metabolites obtained at baseline Media B batch fermentation. Graphs represent *C. acetobutylicum* fermentation parameters for MB: the OD_{600}; the consumption of carbohydrates during operation (Total Carbohydrates, Total Carbohydrate content and HPLC-assayed glucose); changes in organic acid content (*in-situ* sampled pH and HPLC-assayed butyric acid content) and the production of solvent compounds (butanol and acetone/ethanol content).

An increase in the turbidity of the uninoculated control fermentation is evident for the Media B fermentation ("OD_{600nm}", Figure 3.4). This is largely evident in all subsequent fermentations and further on in microbial fuel cells in Chapter 4 (e.g. Section 4.3.1). This is attributed by inadvertent contamination of the fermentation during *in situ* pH measurements and/or during removal of samples for subsequent analyses, as detailed in Section 3.3.6. Due to the formation of solvent precursors ("butyric acids") and solvents ("butanol" and "acetone") within the uninoculated control, this contamination is largely attributed to cells from the *C. acetobutylicum* culture transferred within the anaerobic chamber, rather than environmental contamination.

It is crucial to emphasize that the reduction of carbohydrates, including Total Carbohydrate content (TC%) and glucose, from 48 hours, corresponds to the decline in pH and rise in turbidity, as well as the production of metabolites such as butyric acid and acetone. Very low overall yield production rates of butanol were achieved for media B (0.403 ± 0.362 g/L) peaking after solventogenic phase compared to 60 g/L butanol yield from initial glucose concentration of 9.9 g/L (Kheyrandish et al., 2015). The high production of acetone in media B could be a result of the presence of the reducing agent methylene blue (Ballongue et al., 1986).

This fermentation did not follow the expected kinetics explained in literature (Kheyrandish et al., 2015; Monot et al., 1982). Although not statistically-significant, the second peak seen in the OD_{600} trend over 240 hours is not expected and can possibly indicate cells that are not in solventogenic phase but rather vegetative phase from spore germination. The production of solvents occurred earlier in the fermentation than anticipated, which can be attributed to the inoculation rate and the mixed maturity of cells in the inoculum used. A similar phenomenon was described by Kheyrandish et al., (2015) when the seed inoculum was raised from 3 % to 5 % and inoculated with cultures containing 10 % actively fermenting cells. Alternatively, the OD_{600} measurement may be distorted by the presence of methylene blue later in the duration of the experiment when oxygen contamination occurs (Kheyrandish et al., 2015).

During fermentation especially the first 48-72 hours, the chemically defined media MB would retain an opaque yellow colouration. On the contrary, when the samples were aliquoted outside of an anaerobic environment, the samples in the wells of the 96 well plate would begin to oxidise and develop a visible green/blue colour both indicating oxygen entry into the media once outside the chambers. Interestingly the reaction did not occur as fast for samples taken during the first 72 hours

of fermentation compared to later sampling over the 240-hour incubation duration. The headspace of the fermentation flasks also exhibited similar reaction which seemed to occur more rapidly as sampling rate increased following sampling after 72 hours.

Figure 3.5 below represents the individual parameters of ABE fermentation kinetics for NSBSG. From the OD_{600} kinetics over 240-hour fermentation, it can be noted that there is an increase in cell biomass which increases to a maximum at 72 hours before constantly decreasing at a steady rate for the remainder of the duration of fermentation. Interestingly the carbohydrates such as glucose increase after t=0 where they are depleted following the maximum OD_{600} values at 72 hours of fermentation.

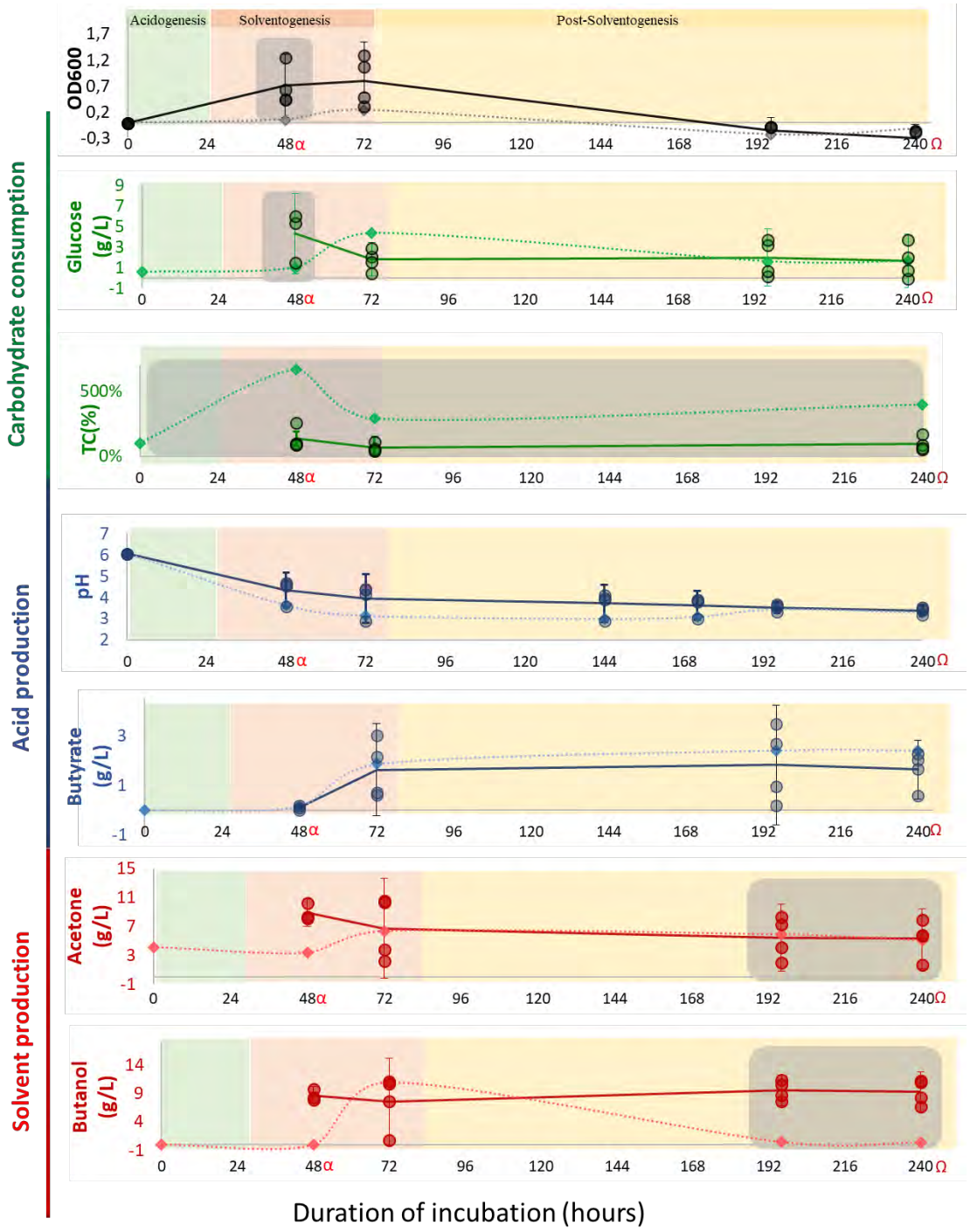

Figure 3.5: Graphs of change in measured parameters obtained during fermentation of NSBSG by *C. acetobutylicum*.

Graphs represent *C. acetobutylicum* fermentation parameters for NSBSG: the OD600; the consumption of carbohydrates during operation (Total Carbohydrates, Total Carbohydrate content and HPLC-assayed glucose); changes in organic acid content (in-situ sampled pH and HPLC-assayed butyric acid content) and the production of solvent compounds (butanol and acetone/ethanol content).

When NSBSG was used as fermentation media (Figure 3.5), the pH decreased rapidly from the initiation of fermentation and continues to decrease at a less rapid rate from 72 hours. The presence of butyric acid increased rapidly from 48 hours to 72 hours before reaching a plateau, which slowly decreased after 192 hours of fermentation. It is important to note while butyric acid and butanol were present in the media from 48 hours, there is no trend here to suggest their metabolic association until 192 hours, where a slight decrease in butyric acid and increase in butanol is noticed. The maximum yield of acetone was produced at 48 hours of fermentation which rapidly decreased thereafter.

The possible presence of thermophilic microbes such as *Bacillus* in the NSBSG are likely to be the cause of the rapid decrease in pH and low production of ABE fermentation products such as butyrate and butanol (Robertson et al., 2010). Although the BSG was placed into -20℃ for storage following the brewing process which produced it, the BSG is still highly susceptible to enzymatic attack and microbial metabolism (Bianco et al., 2020).

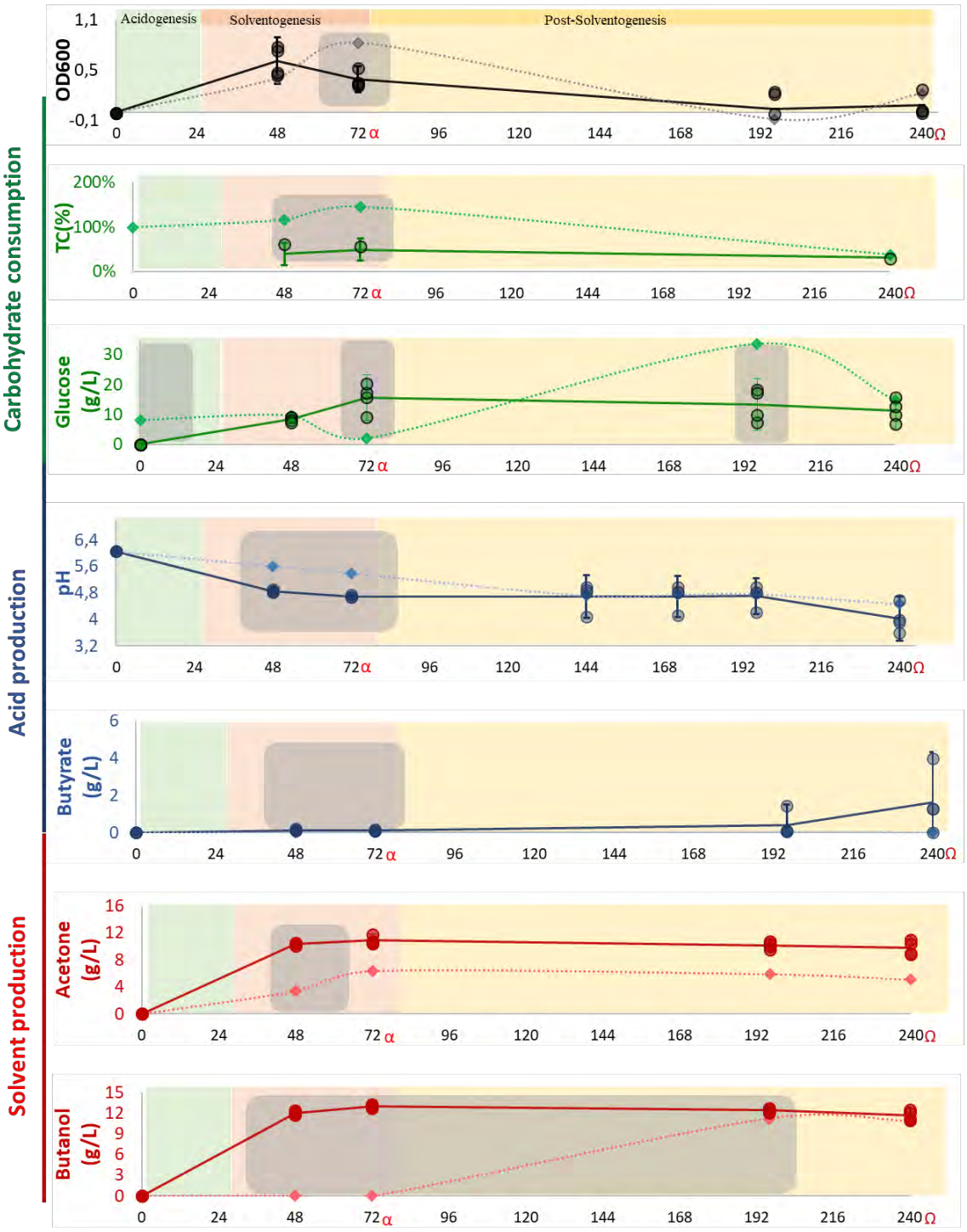

Figure 3.6: representing the individual parameters of ABE fermentation kinetics for SBSG. Graphs represent *C. acetobutylicum* fermentation parameters for SBSG: the OD_{600}; the consumption of carbohydrates during operation (Total Carbohydrates, Total Carbohydrate content and HPLC-assayed glucose); changes in organic acid content (in-situ sampled pH and HPLC-assayed butyric acid content) and the production of solvent compounds (butanol and acetone/ethanol content).

Significant production of solvents was obtained with the use of sterile BSG (SBSG), described in Figure 3.6: butanol and acetone/ethanol increase significantly from t=0 to t=48 and continue to increase at 72 hours of solventogenic fermentation. At this point (72 hours) solvent concentrations reach a plateau and slowly begin to decrease over the duration of post solventogenic fermentation from 196 to 240 hours. Higher overall yield production rates of solvents were achieved in the fermentation of SBSG compared to the other media, reaching a maximum at 72 hours of fermentation.

Solventogenesis during the fermentation of SBSG by *C.acetobutylicum* continues for 24 hours, from 48 hours of incubation to 72 hours of incubation. The increase seen here is not comparable to the trend in the fermentation kinetics of solvent production seen in the fermentation of MB and NSBSG.

Interestingly the production of butanol metabolic precursor, butyric acid did not increase after 48 hours of fermentation correlating to the expected pH drop. It can be seen that the carbohydrates increase from t=0 to t=72 hours and steadily decreasing for the remainder of the fermentation, similar to the trend seen in NSBSG (Figure 3.5).

Unlike the kinetics seen for the OD_{600} of MB (Figure 3.4), both SBSG and NSBSG show a rapid decline of OD_{600} following solventogenic phase. Trend seen in the OD_{600} measurement over 240-hour incubation period is characteristic of *C. acetobutylicum* ABE fermentation with sporulation initiation and cell lysis. The increased concentration of ABE metabolic products and the production of bacteriocin by *C. acetobutylicum* (ATCC 824) cause cell lysis and solvent toxicity (Petitdemange, 1985).

The sterilization treatment with the use of an autoclave operated at 15 psi and 121 ℃ may have contributed to the hydrolysis of the BSG increasing the availability of starch for amylolytic saccharification by *C.acetobutylicum* resulting in the increase of glucose at t=48 compared to t=0 (Ravindran et al., 2018).

Table 3.3: Comparative analysis of the tested batch fermentations during the solventogenic phase (48-72 hours)

Name	Time	Cell growth	Nutrient consumption		Organic acid production		ABE production	
		OD600	TC otal arbohydrate content (%)	Glucose(g/L)	Butyric acid (g/L)	pH	Acetone / Ethanol (g/L)	Butanol (g/L)
MB	48	1.068 ± 0.211	7 ± 16♦	0.232 ± 0.285	1.27 ± 0.946	4.705 ± 0.29♦	5.47 ± 2.06	0.403 ± 0.327
NSBSG	48	1.27 ± 0.192†‡	134 ± 66†	4.305 ± 3.88‡*	1.62 ± 1.89†*	4.34 ± 1.43♦*	8.92 ± 1.86†‡	8.659 ± 1.564†‡
SBSG	72	0.389 ± 0.156†	49 ± 31	15.512 ± 7.561†	0.11 ± 0.058†	4.69 ± 0.06	10.92 ± 0.961†	12.970 ± 0.381†
Statistical analyses								
ANOVA results		F (2,9) = 109.0 $p = 4.912\times10^{-7}$	F (2,9) = 7.19 $p = 0.01362$	F (2,8) = 24.60 $p = 0.0003827$	F (2,8) = 20.55; $p = 0.0007044$	F(2,9) = 1.73; $p = 0.2317$	F (2,8) = 247.4; $p = 6.409\times10^{-8}$	F (2,8) = 659.1; $p = 1.324\times10^{-9}$

Statistical annotations:

* - indicates range of the 95% CI of measured parameters are significantly different to uninoculated controls operated in parallel

† - indicates parameter significantly different to defined media (MB). Determined by Tukey HSD post hoc testing ($p < 0.05$)

‡ - indicates parameter significantly different to SBSG; Tukey HSD testing

♦ - means of measured parameter significantly different to media before inoculation at t=0 Tukey HSD testing

Table 3.3 above is a comparison of the fermentation parameters which describe the kinetics of *C. acetobutylicum* metabolism of different media during solventogenesis t=48-72 hours. The solventogenic fermentation kinetics of the lignocellulosic substrate BSG after different treatments, NSBSG and SBSG, are presented in comparison to chemically-defined MB. It can be seen from Table 3.3 that solventogenesis is initiated within the first 48 hours of fermentation of all media. The highest yield of butanol was recorded for fermentation of SBSG (12.970 ± 0.381 g/L) which is indicated as significantly higher than NSBSG (8.659 ± 1.564 g/L) and MB (0.403 ± 0.327 g/L). It can also be seen that a significantly higher concentration of acetone/ethanol produced from the fermentation of SBSG (10.92 ± 0.961 g/L) compared to that of (NSBSG (8.92 ± 1.86 g/L) and MB (5.47 ± 2.06 g/L). The concentrations of solvent indicated in table 3.3 for SBSG fermentation by *C. acetobutylicum* (ATCCC 824) is very high compared to previous studies of *Clostridium* fermentation of BSG reported i.e. 6.6± 0.8 g/L (Plaza et al., 2017), but are within range of optimal fermentation 13 to 15 g/L butanol and 26.64 g/l of total solvent.

Table 3.3 also indicates that the glucose is depleted in MB (0.232 ± 0.285 g/L) fermentation at t=48 hours whereas SBSG (15.512 ± 7.561 g/L) has significantly higher concentrations to both of the other tested media, including NSBSG (4.305 ± 3.88 g/L). In this study, the depletion of glucose did not correlate with increase in butyric acid or butanol.

Although SBSG has a very high concentration of glucose and butanol at t=72 hours, there are significantly lower concentrations of butyric acid recorded for SBSG (0.11 ± 0.058 g/L) compared to MB (0.232 ± 0.285 g/L) and NSBSG (0.11 ± 0.058 g/L), indicating efficient conversion of this to fuel compounds. However, the OD600 values for SBSG (0.389 ± 0.156 g/L) are significantly lower than both NSBSG (1.27 ± 0.192 g/L) and MB (1.068 ± 0.211 g/L). The depletion of glucose and higher butyric acid and OD600 measurements indicate acidogenesis and cellular growth occurring, but a suppression of solventogenesis thereafter. On the contrary, the high concentrations of butanol and glucose associated with low OD_{600} measurements in SBSG indicates a successful ABE fermentation and the death of cells after solventogenesis.

Table 3.4: Comparative media analysis of batch fermentations in their post-solventogenic phases (192-240 hours hours)

Name	Time	Organic acid production	Cell growth	Nutrient consumption		Organic acid production	ABE production	
		pH	OD600	Total Carbohydrate content (%)	Glucose (g/L)	Butyric acid (g/L)	Acetone/ ethanol (g/L)	Butanol (g/L)
MB	192	4.620±0.381	1.124 ± 0.702*	17±39*	0.076±0.242	1.191±0.855*	6.106±1.708	0.755±0.885*
NSBSG	240	3.390±0.318*†‡	-0.299±0.322*†	93±107†	1.658±2.600*‡	1.632±1.498*	5.293±4.144*‡	9.262±3.52†
SBSG	240	4.025±0.836*†	0.109±0.293*†	31±17*	11.251±5.94*†	1.651±2.677*	9.793±1.655†	11.639±1.172*†
Statistical analyses								
ANOVA results		F (2,9) = 19.44; $p = 0.0005$	F(2,8) = 18.95; $p = 0.0009$	F(2,9) = 5.7; $p = 0.0244$	F (2,9) = 26.37; $p = 0.0001723$	F (2,9) = 0.227 ; $p = 0.8011$	F (2,9) = 7.656 ; $p = 0.01142$	F (2,9) =138;2 $p = 3{,}617\times10^{-6}$

Statistical annotations:

* - indicates range of the 95% CI of measured parameters are significantly different to uninoculated controls operated in parallel

† - indicates parameter significantly different to defined media (MB). Determined by Tukey HSD *post hoc* testing ($p \leq 0.05$)

‡ - indicates parameter significantly different to SBSG; Tukey HSD testing

Table 3.4 is a comparison of the fermentation parameters which describe the kinetics of *C. acetobutylicum* metabolism of different media during Post-solventogenesis i.e. t=192-240 hours. The OD600 for NSBSG and SBSG was significantly lower than that of the chemically defined media at this sample time during the fermentation, this however may have been as a result of methylene blue oxidizing before the sample was taken, as discussed above.

During post-solventogenesis, both SBSG and NSBSG had significantly higher butanol concentrations compared to the chemically-defined Media B (Table 3.4) While Media B had essentially negligible concentrations of butanol (0.755±0.885 g/L), SBSG had the highest peak butanol concentration of (11.639±1.172 g/L), closely followed by NSBSG (9.262±3.52 g/L).

The chemically defined media, MB had considerable concentrations of acetone/ethanol (6.106±2.146 g/L) similar to that of NSBSG, where SBSG (9.793±1.655 g/L) had significantly higher concentrations of acetone/ethanol compared to other media. SBSG produced the highest total solvents for the batch fermentation of BSG by *C. acetobutylicum* at 21.439±2.87 g/l similar to that reported ±20 g/L in literature (Plaza et al., 2017).

The measurements of pH at this time indicate the effect of the ubiquitous microbial consortium in the spent grain as it is produced after mashing in the brewing process. Media B and SBSG had similar pH values within an ideal pH above 4.5 for Clostridial solvent production and NSBSG showed lower pH of 3.53 at 196 hours**.**

The carbohydrate measurements following 0 hours decreased significantly. Represented are very low levels during solventogenic phase at 72 hours and non-detectable concentrations in the post solventogenic phase at 196 hours. In contrast to the measurement made using the phenol sulphuric acid assay the HPLC method provides a greater resolution when applied in conjunction with other assays. The Glucose concentration determined by HPLC indicates significantly higher glucose for SBSG (11.251±5.94 g/L) compared to NSBSG (1.658±2.600 g/L) and chemically defined media B which showed the bacteria had mostly depleted glucose at 72 hours but increased slightly at 196 hours. Amylolytic activity may have metabolized low concentrations of starch present in the MB after more readily available glucose was depleted.

The constitution of the carbohydrates in the BSG treatments is more complex compared to the chemically defined media B. Media B comprised glucose and potato starch as the carbohydrate sources, readily-quantifiable using the phenol sulphuric acid assay where no data was collected for NSBSG and SBSG at 196 hours. Any pre-treatment process requires different infrastructure for processing and offer different downstream challenges (Ravindran et al., 2018).

Comparing Tables 3.3 and 3.4, a decrease in total solvent production from 48 hours to 240 hours is evident; peak values of solvents occurring between 72 and 196 hours of fermentation.

3.5 Conclusions

The rate of production of solvent was most active during the first 72 hours of fermentation for SBSG (12.970 ± 0.381 g/L). The production of solvent for SBSG expressed the highest yields of solvents Acetone/ethanol and butanol in the first 72 hours which decreased thereafter. Overall, MB fermentation yielded negligible amounts of butanol as (0.755±0.885 g/L), but generated quantifiable concentrations of acetone/ethanol (6.106±2.146 g/L) which peaked during post solventogenesis. BSG, especially SBSG, proved to be a viable feedstock for butanol and acetone/ethanol production, in line with previous investigations reported in literature.

The processing and storage of BSG is an additional cost for brewery operations. The fermentation of BSG by *Clostridium* can be a form of preservation and mitigate the need for energy intensive processes such as drying. The presence of BSG in the proximity to a food production facility increases risk as the source of infection. The reducing sugar and the high moisture content make BSG easily fermentable and unstable if not treated before storage. Intentional inoculation of *Clostridium* for ABE fermentation could be a viable option for breweries to treat their waste. BSG is a viable feedstock for ABE fermentation and may provide greater value to the traditional waste processing in the brewing industry.

Hydrothermal pre-treatment such as autoclaving of BSG results in increased solubility of starches by 83% (Parchami et al., 2021). The higher glucose and solvent concentrations in SBSG compared to NSBSG in this study support this increase in starch availability. BSG, however, is a complex lignocellulosic carbohydrate that may contribute other metabolites which may interact with the ABE biosynthesis during ABE fermentation. These forms of carbohydrate sources could be

explored as standards to adequately measure a wider range of complex carbohydrates and feedstocks respectively. *C. acetobutylicum* has been explored in the production of cellulases (López-Contreras et al., 2004; Fierobe et al., 2012). More research is needed into the catabolism of the carbohydrates and lignocellulosic and cellulosic carbohydrates in bacterial solvent producing fermentations.

Taking into consideration the non-pathogenicity of *C. acetobutylicum*, it may be possible to maintain the use of the waste by traditional disposal means following the value-added process of ABE solvent production. More research is needed in the incorporation of bacterial fermentations for value added products without the termination but rather the evolution of traditional waste utilization streams. This study focuses on the feedstocks and their effect on solvent production efficiencies only.

Chapter 4: The influence of MFC configurations on ABE production by *C. acetobutylicum*

4.1 Introduction

As detailed in Chapter 3, the ability of *C. acetobutylicum* to metabolize BSG to produce organic acids and alcohols such as acetate and butanol can have significant environmental and economic benefits (Abd-Alla et al., 2019). To further improve the economic sustainability of ABE production, the use of microbial fuel cells (MFCs) as low-cost interventions to further improve solvent production by *Clostridium acetobutylicum* were investigated and discussed in this Chapter.

A major metabolic bottleneck for the production of organic acids and alcohols in Clostridia is the oxidative decarboxylation of pyruvate to form Acetyl-CoA, predominantly during acidogenesis (Girbal et al., 1995). Depending on the intracellular reduction potential of available reductants during fermentation, the metabolism of pyruvate and hydrogen production can be facilitated by the electron-carrying cyclic ferredoxin hydrogenase pathway to form NADH (Demuez et al., 2007; Ding et al., 2018). Following acidogenesis, the biosynthesis of alcohols during ABE solventogenesis (Chapter 1 SECTION 1.1.6 and Figure 1.2) requires the accumulation of NADH from the glycolysis pathway and the associated intermediate metabolite (Su et al., 2020) produced during acidogenesis before organic acids are reassimilated and converted to alcohols (Luo et al., 2016a).

The intracellular NADH levels influence which products are subsequently formed as acetyl Co-A can either: directly undergo reduction to form ethanol (via the oxidation of two NADH), be released as acetate (ATP-forming); or enter the solventogenic pathway via thiolase; the formed acetoacetyl-CoA from the latter process forming either acetone (via fermentation of acetate), butanol (oxidising four NADH) or butyric acid (oxidising two NADH). The pathway of butyrate to form the butanol precursor, butyryl-CoA, is dependent on the same acetoacetate-butyryl-CoA transferase enzyme (Chapter 1, Figure 1.2) (Buehler and Mesbah, 2016b; Jiang et al., 2009; Lütke-Eversloh and Bahl, 2011).

The regulation of pH is often considered critical in determining ABE yields, as this influences both the activity of the key enzyme hydrogenases (Jiang et al., 2014; Khanna et al., 2011) and the reducing potential surrounding cells, which in turn affects energy-generating processes (Grupe et al., 1992; Girbal et al., 1995). Solventogenic metabolism can be triggered in nutrient-limited *Clostridia* cultures by either the addition of organic acids such as acetic acid or any other means of specifically lowering pH within the culture vessel (Al-Shorgani et al., 2018; Gao et al., 2016; Li et al., 2014b), or by supplying the cells with reducing potential in other ways e.g. increasing the NADH concentration within cells through metabolic engineering in bioreactors (Demuez et al., 2007; Vasconcelos, Girbal and Soucaille, 1994). The production of solvents by *C. acetobutylicum* traditionally tracks the metabolic phase shift during fermentation by means of pH fluctuation or acid production and/or internal sequestration of these acids for solvent production (Dürre 2005; Luo et al. 2016b; Xue et al. 2013).

Central to the rationale for the work in Chapter 4 is the concept of electrochemical control of solventogenesis and acidogenesis. While not fully understood, as reported by Finch (2011), when *Clostridia* are cultured in microbial fuel cells, they are capable of producing a voltage, indicating electrical connection between the attached cells' metabolic activity and the electrode. In this report, the different phases of fermentation i.e. acidogenesis and solventogenesis, were preceded by an increase in voltage difference between the electrodes of the MFC.

Similar to other bacteria, it is proposed that the metabolism of *C. acetobutylicum* can be linked to a MFC through the production of metabolites that serve as electron donors for the electron accepting bio-anode (Logan, 2009; Read et al., 2010). The physiological parameters of *C. acetobutylicum* fermentation enable the transport of electrons via extracellular electron transfer (EET), demonstrating multiple mechanisms for electron transport in C. *acetobutylicum* (Read et al., 2010).

From the understanding that electrons can be transported out of the cell by exoelectrogenesis, the possibility of transporting electrons by endoelectrogenesis from the cathode to cathode respiring bacteria was explored. This study was intended to explore whether supplying electrons to *C. acetobutylicum* cells (by connecting them to an MFC system at a bio-cathode) or withdrawing electrons (as a bio-anode) during metabolism could alter the extent and the form of solvents produced during fermentation. *Enterobacter cloacae,* an organism commonly applied to bio-anode

MFC studies (Abd-Alla et al., 2019; Khanna et al., 2011) was also explored as to whether it could function as a co-cultured organism to supply bio-cathodic *C. acetobutylicum* with electrons (Choi and Sang, 2016; Kim and Kim, 1988; Kracke et al., 2016; Schievano et al. 2016).

Electrically-connecting the cells to the electrode should change the metabolic properties of the clostridia and result in increased yields similar to the provision of metabolic precursors or reducing agents which direct the metabolic flux in the direction of the most energy-efficient pathways (Matta-el-Amouri et al., 1985) The accumulation of NADH as previously discussed is a direct rate-limiting reaction for the production of alcohols (ethanol and butanol) from their respective aldehyde precursors (Figure 1.2). The cofactor NADH is required to catalyse the reaction of butyraldehyde to butanol (Liao et al., 2019). Unlike the glycolysis pathway of pyruvate to form the complex Acetyl-CoA where NADH is produced, acetoacetate formed by the coenzyme A transferase from Acetyl-CoA requires an electron acceptor such as acetone. Acetyl-CoA is the activator molecule for the reverse ferredoxin reduction reaction catalysed by NADH-ferredoxin oxidoreductase enzyme. Manipulation of the concentration of intracellular metabolites in this manner is expected to affect the electron flux and fermentation kinetics, which determine the biosynthesis of microbial products (Matta-el-Amouri et al., 1985).

Similarly, Exoelectrogenic fermentation at a bioanode provides the bacteria with an electron acceptor facilitating metabolism of the organic substrate and producing a potential difference across a load. The dependence of the metabolism in the presence of an electrode may indicate the ability to act as an electron acceptor or donor depending on the metabolic needs and system configuration, in turn increasing fermentation efficiency (Choi and Sang, 2016; Rabaey and Rozendal, 2010; Shin et al., 2002; Venkata Mohan et al., 2014).

C. acetobutylicum and *E. cloacae* have both been shown to be promising candidates for use in MFCs due to their ability to produce metabolic products that can exchange electrons with the MFC system. While *C. acetobutylicum* has been shown to be highly efficient in the biosynthesis of an industrially relevant products, *E. cloacae* has been shown to exhibit high electron transfer rates, production of relevant metabolic products and to utilize a wide range of substrates. The combination of these two bacteria in a MFC system could result in a synergistic effect and lead to increased power generation, as well as improved substrate utilization and product formation.

As demonstrated in Chapters 3 and 2, the analytic method selected for quantification of ABE fermentation products as described by (Vohra et al., 2015) successful detection and quantification, although time-consuming, is possible for butyric acid, ethanol and butanol, even in complex media. The concentrations of each metabolic depend on the physiological parameters of fermentation where ethanol is produced in the lowest stoichiometric ratios compared to acetone and butanol (3:6:1 A:B:E). The prioritisation of acetone production over ethanol, reiterates the fact that the concentration of metabolic precursors is responsible for the flux towards dehydrogenation of aldehydes to produce alcohols or acetone from acetate which requires less intracellular NADH (Gao et al., 2016; Hassan et al., 2015; Jiang et al., 2009; Luo et al., 2016b).

Batch fermentation experiments discussed in Chapter 2 and Chapter 3 identified limitations in sampling and analysis of *C. acetobutylicum* ABE fermentation *in vitro*. Contamination was present in the uninoculated controls as a result of sampling and measurements for essential fermentation kinetic such as pH changes for the duration of incubation.

Not only does the MFC system remove the necessity for measurements that disturb the fermentation, but it grants the ability to provide a real time measurement of the fermentation kinetics by monitoring power output as a proxy for metabolic activity, increasing efficiency and feasibility (Finch et al., 2011) and eliminating the need to open containers to monitor activity. An added benefit to the total efficiency of the system would be the controlled manipulation of the bi-phasic metabolism for increased yield of select products during fermentation.

By harnessing the exoelectrogenic capacity of *C. acetobutylicum* and the endoelectrogenic capability of *E. cloacae*, it may be feasible to establish a synergistic fermentation process that operates without the need for supplementary power sources to facilitate electron acceptance and donation. From the concept of bacterial exoelectrogenesis in MFCs, the ability to accept extracellular electrons can be explored by microbial electrolysis cells for endoelectrogenic bacteria (Moscoviz et al., 2016). The electrons can be supplied by any power source if the redox imbalance in the fermentation system can be met through electrofermentation (Kracke et al., 2016).

4.2 Aims

The aim of this study was to determine the efficacy of using *C. acetobutylicum* in a MFC configuration by utilising the exoelectrogenic ability of *C. acetobutylicum* to alter solventogenesis pathways during culture. The comparative investigation of the fermentation kinetics observed in two scenarios is explored: one in which fermentations were established in the previous Chapter 3, and the other in which *C. acetobutylicum* is attached to an electrode in an MFC.

4.3 Methods

4.3.1 Reagents and apparatus

Unless otherwise stated, all reagents were of technical grade (i.e. $\geq$ 95%) or higher.

Unsubstituted iron (II) phthalocyanine (FePc; ≥82%); potassium ferricyanide ($K_3[Fe(CN)_6]$ ≥82%); potassium ferrocyanide ($K_4[Fe(CN_6)]$ ≥82%) were sourced from Sigma-Aldrich. Carbon black (grade N375) was sourced from Orion Engineered Carbon, South Africa. Carbon paper (SpectraCarb 2050A-0850) was sourced from the Fuel Cell Store (United States of America).

Phosphate Buffer (PB) solution was formulated by 0.25M PB solution of Na2HPO4 and NaH2PO4 using NaOH to titrate pH 7.4 Chemically-defined Media B was formulated as detailed in Chapter 2, Section 2.3.4 SBSG and NSBSG media were prepared as detailed in Chapter 3, Section 3.3.4.

The anaerobic inoculation and fermentation chambers constructed during this research (Chapter 2, Section 2.3.2) were used to assemble, inoculate, and subsequently operate MFCs.

Ultrasonication was conducted by immersing sample containers in an Elmasonic P ultrasonic bath filled with milliQ water, sweeping the frequency between 37 and 80 kHz.

All other reagents and apparatus used were as detailed in Chapter 2, Section 2.3.1.

Electronic programable power supply (Bio-rad) was utilised as an external power source for the constant provision of a select voltage during electro-fermentations when the configuration of a microbial electrolysis cell was explored.

Iodophor as detailed in Chapter 2, Section 2.3.1

The anaerobic fuel cell chamber was adapted from a modified sandblasting cabinet, modified as detailed in Chapter 2.

4.3.2 General fuel cell construction

All MFC experiments were conducted using modified 250 ml Schott bottles in a H bridge MFC design. To mitigate gas permeability and the effect of the electrode/lid interface, a 10 mm hole was made in the Schott bottle lid of the anodic and cathodic chambers and a rubber grommet was installed into each hole.

MFCs were subsequently assembled and constructed as detailed by (Mshoperi, Fogel and Limson, 2014) A circular piece of Nafion® PEM (3 cm diameter) served as the PEM. Before use, PEMs were successively boiled for 1 hour each in the following liquids: 3% $^{v}/_{v}$ H_2O_2, water, 5% $^{v}/_{v}$ H_2SO_4 and finally water. Prepared PEMs were stored in water until used. The Nafion® PEM was placed between the anodic and cathodic chambers and clamped together. A total of 150 ml of anolyte and catholyte were loaded into the vessels.

Electrodes were prepared similar to details by (Mshoperi et al., 2014) Both anodes and cathodes were formed by cutting 2×2 cm sections of carbon paper, piercing each in one corner and threading a 15 cm section of carbon fibre through the hole to serve as an electrode lead.

Anodes were functionalised by 30 minutes' ultrasonication in a 2.5 mg/ml suspension of N375 carbon black in ethanol. Cathodes were functionalised by ultrasonication in a 2.5 mg/ml suspension of N375 carbon black in ethanol for 30 minutes, followed by sonication in a 5 mg/ml suspension of FePc in ethanol for 30minutes. Functionalised electrodes were dried in sterile air (within an operating laminar flow chamber) until used.

After electrodes were immersed in the anolytes and catholytes, the lead was pulled through the rubber grommet and a caulking gun was used to seal the electrode leads and maintain anaerobic conditions in the chambers. Microbial fuel cells were then placed into the fermentation chamber.

A 10Ω resistor was used to connect the anode and the cathode. Holes were made through the lids of the flask as to port the electrodes through n airtight grommet. 10mm rubber gromets were used

to provide a seal between the lid and a modified bung from the tip of a caulking gun from the hardware store.

4.3.3 Specific MFC configurations tested

Similar to the ferrmentations described in Chapter 3 Section 3.3.5, all MFCs were assembled and operated as a batch of n =4 independent measurements. One of these was sacrificed to act as a control MFC – either lacking the initial microbial inoculum (uninoculated control) or consisting of an inoculated and assembled MFC that lacked an electrical connection between the anode and cathode via the external circuit (unconnected control).

Depending on the intention of the experiment and the redox reaction expected, the catholyte was not always electron-donating; in some experiments a biocathode including media and bacterial culture (e.g. MFC configuration 2) was used.

The schematics below are different electro fermentation configurations for electron transfer both in and out of the cell depending on the bacteria acting as electron donors or as electron acceptors. The configuration depicted Chapter 1 Section 1.1.8 Figure 1.3 (showing the anodic metabolism of glucose), is identical to MFC-1 with the addition of *C. acetobutylicum* as the bacteria and are compared to the set up and theoretical operations of the configurations of MFCs described in the schematics which follow.

The schematics for MFC 2 ,3 and MEC represent very distinct changes in configuration which further describe the differences of the other MFC experiments. It is essential to understand the differences between MFC 1, 2, 3 and MEC as all other MFC experiments are only minor variations of these configurations. These were constructed to explore the possible electron pathways possible through the use of *C. acetobutlylicum*, comparing and its ability to perform the role of an electron donor, or of an electron acceptor. The transfer of the electron is donated from the fermentation of feedstock (glucose), or an external power supply. The electron acceptor can be in the form of a chemical electron acceptor or electron accepting bacteria connected to the cathode. *C. acetobutylicum* was applied as a possible bio-anode MFC 1, 3, 4, 5, 6 and 8; while it was applied as a potential bio-cathode in MFCs 2, 7 and in the MEC configuration.

MFC 1 was constructed with simplicity in mind for the purpose of comparing media utilization for different ABE fermentation methods. The anolyte consisted of MB inoculated with C. *acetobutylicum*, while the catholyte was made up of 0.25M PB solution. In MFC 2, the anolyte consisted of CNM supplemented with 1 mM $K_4[Fe(CN_6)]$ ferrocyanide, which was added to the anolyte for the oxidation reaction of $Fe(CN_6)^{4-}$ to $Fe(CN_6)^{3-}$, providing the bio-cathode cultured *C. acetobutylicum* with an electron. The cathode was prepared the same way as in MFC-1. MFC 3 utilized chemically defined media feedstock for C. *acetobutylicum*, which was electrically connected to a cathode immersed in a catholyte containing 0.25 mM PBS with an additional 1 mM Fe $(CN_6)^{3-}$ ferricyanide added to the catholyte.

MFC 4 was prepared with an unconnected control and uninoculated control for comparison of fermentation kinetics and SEM analysis of the bacterial colonization of the electrodes. CNM was used as the feedstock for E. *cloacae* inoculated into the cathodic chamber without FePC electrode treatment, and the anolyte was the same as MFC-1. MFC 5 utilized chemically defined media anolyte inoculated with C. *acetobutylicum*, while the catholyte was uninoculated CNM. MFC 6 used SBSG as a feedstock for the anode, with the addition of methylene blue in the same concentration as described for MB. The catholyte was prepared the same way as MFC-1.

MFC 7 utilized an anode prepared in the same way as MFC-1, with the catholyte consisting of uninoculated CNM and the addition of 1 mM $K_4[Fe\ (CN_6)]$. In MFC 8, SBSG was prepared in the same way as MFC-6 and used as a substrate for *C. acetobutylicum* in an anodic electrolysis configuration connected to a CNM catholyte containing cathode with 1 mM $K_4[Fe(CN_6)]$ added as an oxidant.

In the Microbial electrolysis cell (MEC) experiment, Media B (MB) was used as a substrate which included methylene blue as an electron transfer mediator for C. *acetobutylicum*, and a power supply of 100mV was generated by a controllable power supply. A 100 Ω resistor was connected to the positive terminal and an AgCl reference electrode in 3M KCl was used in the configuration presented by fig Figure 4.1C and sterilized with iodophore. A cover was created from a 5ml pipette tip for the anode and cathode to ensure they did not wrap around each other or the reference electrode.

To understand the theoretical electron pathway across the load (resistor) the Figure 4.1 is presented below. Although the substrate and catholyte changes between different MFC configurations

changes as described above, the effect of the reducing agents and redox potentials created either by the electron transfer from the anode or bio-anode can have a direct interaction with the metabolism flux and correlating potential voltage resulting from the system configuration. The order of MFC nomenclature using numerical values e.g MFC-1,2,3 etc. indicates the deduced changes in configuration of MFC fermentations to control the redox potential of either cathode or anode to have a causal effect on the electron flux in *C. acetobutylicum* ABE fermentation of different substrates

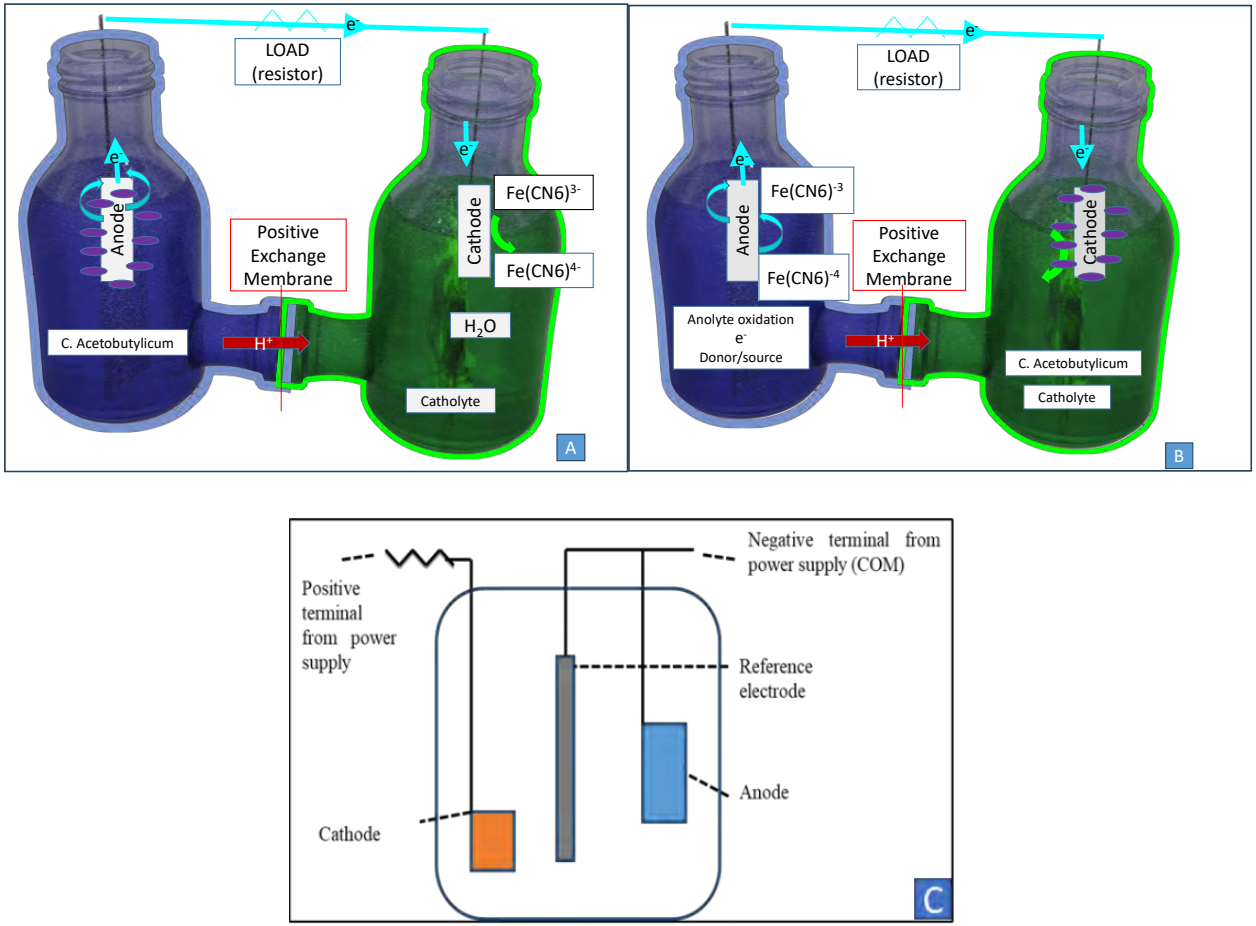

Figure 4.1: Schematics of MFC-3(A), MFC-2/7/8(B) and MEC (C) diagram electron path and configuration of fermentation with regards to the electrode chambers.

The above schematics describe how redox reactions occur in MFCs 2 and 3 depending on the bio-electrochemical redox potential induced by the addition of reducing/oxidising equivalents $Fe(CN_6)^{3-}$/ $Fe(CN_6)^{4-}$ added to cathode or anode for MFC 2/7/8 vs MFC 3 respectively. The Figure 4.1 above describes the connection of the reference electrode connected to the anode and a programmable power supply connected in series with a load resistor to the cathode for the MEC experiment.

4.3.4 Sampling of MFCs during operation

Measurement of potential voltage between anode and cathode was conducted with the use of the multimeter placing the negative and positive terminals across the resistor.

For sample analysis, 20ml test tubes were sterilised and placed inside the MFC chamber before de-aerating with N_2. A 5ml pipette was used to aliquot 4.5-5ml of *C. acetobutylicum* broth for analysis. Following the completion of sampling the experiments, the test tubes were removed, and the chamber was sealed and de-aerated again. The test tubes could now be used to subdivide the samples for immediate processing of OD_{600}, pH, total carbohydrates or to be stored in 1.5ml tubes at -20℃ for later HPLC analysis.

For SEM analysis refer to Chapter 2. Fixing step is altered by selecting a small piece of the electrode, placing it inside a sealable 50ml falcon tube and carrying out the procedure.

Sample rate during the 240 Hour experiments was varied towards the final experiments, Microbial fuel cell experiments for solvent and spore production. Some data points i.e., 144, 172 and in some cases 48 and 240, were not measured in some sets. The times were chosen from previous studies and to correlate to the fermentation kinetics of *C. acetobutylicum* in a MFC as explained previously described (Finch et al., 2011).

4.3.5 Data analysis

Experimental MFC parameters (n = 3 for MFC configuration 1; n = 4 for all others) were analysed and reported as was detailed for the fermentation reactions in Chapter 3, Section 3.3.7, with the same comparisons between the experimental MFCs and the $n = 1$ control and the same comparison between batches of different MFC 79ontent79ation. The additional parameter of potential difference was included as a further parameter.

4.4 Results and discussions

4.4.1 Comparison of different power and fermentation kinetics between the main tested MFC configurations

The graphs below (Figures 4.2, 4.3 and 4.4) show the MFC fermentation measurements for MFC 1, 2, 3 and 6 of selected times 0, 48, 72, 144, 172, 196, 240 over the full duration of 240 hours. This compares the differences in electrical potential difference output by the MFC (mV), carbohydrates content (glucose levels), organic acid production (pH and butyric acid production) and solvent production (butanol/acetone) over the operation of the MFC. The averages, un-inoculated negative control and experimental replicates are represented in the graphs with each symbol explained in the graph legend. Regions encompassed by grey boxes in the potential difference indicate a difference between the n =1 control fermentation and the experimental fermentations greater than the 95% CI of the experimental fermentations.

Error bars indicate the 95% CI range for experimental fermentations (n =4 and n= 3). The backdrop with the colouration of green, red and orange, following that sequence, represents the incubation duration of acidogenic phase, solventogenic phase and post solventogenesis respectively.

Subsequent graphs were analysed for evidence of the culture entering the solventogenic phase (usually between 48-72 hours, indicated with the symbol “α” in these graphs shown in (Figures 4.2. 4.3 and 4.4). Timeframes corresponding to post-solventogenic phases (between 192-240 hours) are annotated with the symbol “Ω” in the graphs shown (Figures 4.2. 4.3 and 4.4).

Figure 4.2 below represents the changes to the MFC system tracked during operation of MFC-1 (the baseline MFC) as the first example of the kinetics of MFC-assisted fermentation.

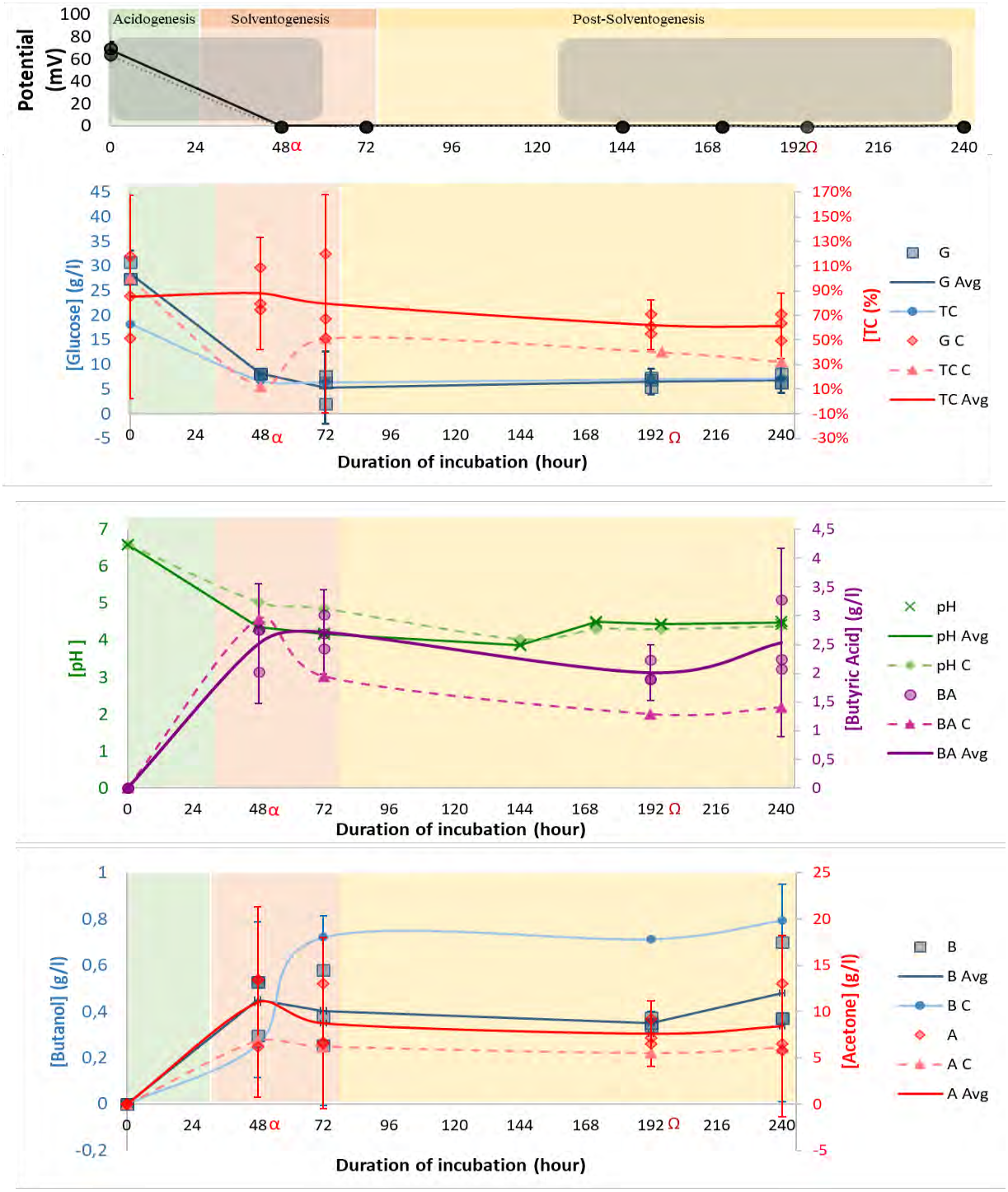

Figure 4.2: Responses and metabolites obtained at baseline MFCs (MFC 1 configuration) Graphs represent: the observable potential difference at the resistor connecting anode and cathode (Potential); the consumption of carbohydrates during operation (Total Carbohydrate content and HPLC-assayed glucose); changes in organic acid content (*in-situ* sampled pH and HPLC-assayed butyric acid content) and the production of solvent compounds (butanol and acetone/ethanol content).

The fermentation kinetics for MFC 1 act as a baseline for the remainder of the MFC study for the comparison of ABE fermentation efficiency between different MFC configurations and ABE fermentation method.

For comparison of the fermentation kinetics of the different substrates, certain samples were selected for comparison with other MFC configurations, compared in Tables below (Tables 4.3). In general, the regions between 48-72 hours and 192-240 hours were selected for solventogenesis and post solventogenesis respectively. The decrease in pH and increase in butyric acid and solvent production correlates with the depletion of carbohydrates (both Total Carbohydrate content and glucose) after 48 hours, similar to the fermentations discussed in Chapter 3 (Section 3.4.2 and 3.4.3).

While detectable power was initially obtained between 0 and 48 hours (Figure 4.2), the MFC-assisted fermentation did not follow the kinetics expected in previous reports (Finch et al., 2011; Monot et al., 1982). Compared to those, the potential difference obtained in MFC-1 did not exhibit the two large peaks at either the acidogenic or solventogenic phase shifts respectively; conversely, it decreased throughout MFC operation. Some of this may be attributed to the difference in the state of the inoculum used: these studies inoculated with spore stocks, whereas this study utilised revived and actively growing bacteria as the inoculum. The inoculum used for this research would have a mixed population of cell states, preventing a distinct shift in the population from being measured using potential shifts.

While there is evidence that the inoculated *C. acetobutylicum* in the baseline MFCs were actively metabolising (the rapid consumption of glucose and other carbohydrates, and the production of organic acids evident in Figure 4.2), overall, a very low yield butanol was observed for MFC 1, peaking between 48-72 hours. The HPLC measurements for MFC 1 indicated a high production of acetone using MB as substrate which could be a result of anodic respiration, or the presence of the reducing agent methylene blue as introduced in Chapter 2 mentioned in Chapter 3 (Ballongue et al., 1986).

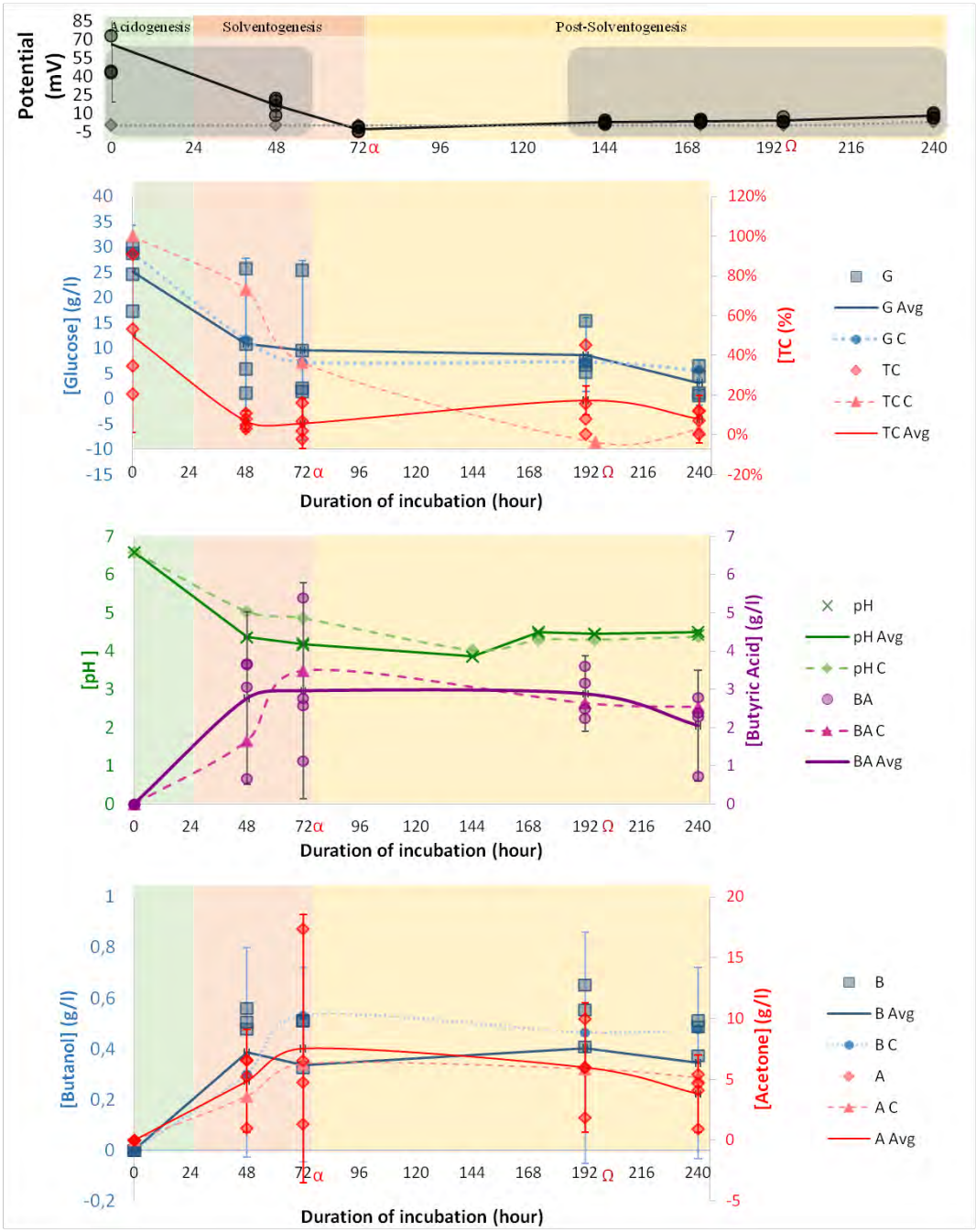

Figure 4.3: Responses and metabolites obtained at MFC 2 configuration with the addition of 1 mM $K_4[Fe(CN_6)]$ to the CNM anolyte

The graphs depict various aspects including the potential difference at the resistor connecting the anode and cathode (referred to as Potential), the amount of carbohydrates consumed during operation (Total Carbohydrates and HPLC-assayed glucose), variations in organic acid levels (determined through in-situ sampled pH and HPLC-assayed butyric acid content), and the production of solvent compounds (butanol and acetone/ethanol content).

MFC-2 is the baseline experiment directly comparable to MFC-1 for one of five MFC fermentation experiments for the metabolic flux manipulation of *C. acetobutylicum* ABE fermentation kinetics cultured in a bio-cathode configuration, namely, MFC 2, 4, 7, 8 and MEC, which explore fermentation kinetics of *C. acetobutylicum* under conditions where the electrons are supplied by an external source (Kim et al., 2015).

MFC configuration 2 was constructed to test the capability of the MFC system to supply *C. acetobutylicum* with exogenous electrons during operation. Similar to Finch et al (2011), CNM was used as both anolyte and catholyte, but *C. acetobutylicum* was inoculated into the cathodic chamber and $[Fe(CN_6)]^{4-}$ was added to the anolyte to act as an electron donor, similar to previous studies (Choi & Sang, 2016; Koch et al., 2017).

The intention of culturing *C. acetobutylicum* in a biocathode was to utilise the exoelectrogenic ability of the bacteria; suppling the reducing power in the form of electrons, to manipulate metabolic fluxes towards increased alcohol production (Choi & Sang, 2016). It was expected that, if the *C. acetobutylicum* bio-cathodic complex received reducing power equivalents from the cathode, this would substantially alter the metabolite profile of the fermentation during MFC operation.

No substantial difference in fermentation profile is evident when comparing MFC-2 to the baseline. The fermentation characteristics for most parameters for MFC-2 (Figure 4.3) followed similar trends to that of MFC-1 (Figure 4.2) compared to the baseline MFC, an increased variation in the measured parameters (especially concentration of metabolites) between experimental replicates is evident, as indicated by the larger extent of 95% CI error bars and dispersion of individual values.

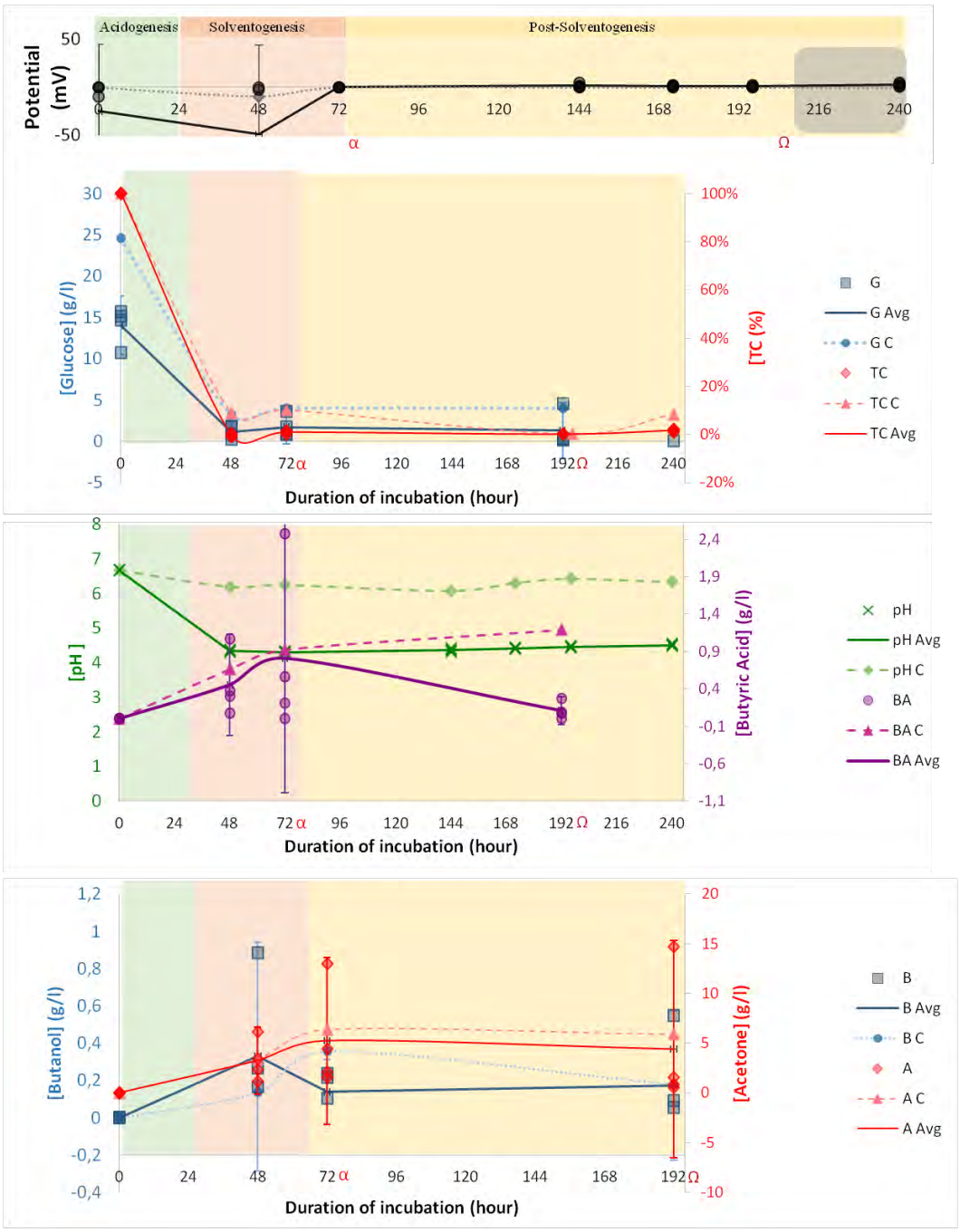

Figure 4.4: Responses and Metabolites Obtained at MFC 3 Configuration with the Addition of 1 mM $[Fe(CN_6)]^{3-}$ to the CNM Anolyte

The graphs illustrate different elements, such as the Potential, which is the observable potential difference at the resistor linking the anode and cathode, the amount of Total Carbohydrates and glucose consumed during operation as determined by HPLC analysis, changes in organic acid levels indicated by in-situ pH measurements and HPLC-assayed butyric acid content, and the production of solvent compounds, including butanol and acetone/ethanol content.

MFC 3, indicates the impact of the addition of 1 mM $K_3[Fe(CN_6)]$ to the 0.25 mM PBS catholyte for the metabolic flux manipulation of *C. acetobutylicum* ABE fermentation kinetics with the chemically defined media MB as substrate (Kim et al., 2015; Kim and Kim, 1988a).

Theoretically the reduction reaction of $[Fe(CN_6)]^{3-}$ to $[Fe(CN_6)]^{4-}$ acts as an electron acceptor in the cathode for the transferred electron by exoelectrogenic ability of *C. acetobutylicum* during anodic respiration (Choi and Sang, 2016; Kim and Kim, 1988a). The aim of this experiment aligns with the aims of this Chapter in the attempt to redirect the metabolic flux of *C. acetobutylicum* by altering the redox potential by the addition of available reducing powers in the form of chemical mediators or electron transfer between bacteria and electrodes.

A negative potential voltage is recorded in the early phase of fermentation from t=0 and increasing rapidly after 72 hours, indicating that electrons are flowing towards the cathode unlike the MFC-1 and 2 kinetics. The increase after 72 hours may be the point at which the ferricyanide is reduced. The negative potential difference measured as millivoltage is associated with the rapid depletion of glucose. Unlike what is seen in MFC 1 and 2 where glucose is not depleted, the carbohydrates in MFC 3 rapidly metabolised in 48 hours. The fermentation kinetics for MFC 3 witness a rapid depletion of carbohydrates from t=0 to t=48 synonymous with the rapid decrease in pH and increase in acid production and solvent production. The increase in butyric acid production after 72 hours does not indicate an association pH which plateaus from 72 hours and does not increase as seen in MFC 1 and 2.

4.4.2 Cross-comparison of obtained MFC parameters during solventogenic and post-solventogenic phase.

The Tables below (Tables 4.1 and 4.2) respectively compare the parameters sampled from the operated MFCs when their solventogenic and post-solventogenic stages are reached.

Table 4.1: Comparison of solventogenic phase parameters obtained when operating MFCs (48-72 hours)

Name	Time	MFC function	Nutrient consumption		Organic acid production		ABE production	
		Volts (mV)	Total Carbohydrate content (%)	Glucose(g/L)	Butyric acid (g/L)	pH	Ethanol/Acetone (g/L)	Butanol (g/L)
MFC-1	48	0±0‡	87.7 ± 20,86	8.143 ± 0.0843*‡	2.513 ±0.474*‡	4.327 ± 0.054‡	11.00 ± 4.69*‡	0.451 ± 0.153*‡
MFC-2	72	-3.3 ± 2.0‡	2.47 ± 2.6‡	9.572 ± 10.929*	2.959 ± 1.743*‡	4.175 ± 0.023‡	7.523 ± 6.783*	0.337 ± 0.236*
MFC-3	72	0±0	0.25 ± 0.49‡	1.743 ± 1.255‡	0.809 ± 1.108*	4.305 ± 0.020‡	5.234 ±5.182*	0.138 ± 0.106*
MFC-4	72	8.0 ± 15.7*	1.06 ± 0.18‡	1.740 ± 0.882*	0.048 ± 0.093*†	4.315 ± 0.034‡	3.893 ± 2.082‡*	0.484 ± 0.361*‡
MFC-5	48	-15.5 ± 21.4	128.6 ± 51.9*	0.172 ± 0.209‡	0.201 ± 0.054†	4.853 ± 0.031†	1.972 ± 0.984*†	0.397 ± 0.199
MFC-6	48	-3.75 ± 0.94	-13.99 ± 0.52‡	1.013 ± 1.189*	0.196 ±0.202*†	4.34 ± 0.134‡	0.293 ± 0.574†	0.033 ± 0.064
MFC-7	72	-2.75 ± 12.4*	88.34 ± 87.77‡	1.839 ± 1.952	0.138 ± 0.157 †	4.805 ± 0.076†‡	0.798 ± 0.973†	0.256 ± 0.299
MFC-8	72	5.75 ± 3.3‡	n.d. †	4.962 ± 3.575*‡	0.049 ± 0.097†	4.603 ± 0.036†‡	2.687 ± 1.424‡	2.407 ± 1.298*†‡
MEC-2	48	12.7 ± 21.0*	-3.60 ± 0.20‡	0.003 ± 0.003‡	n.d.	4.693 ± 0.086*†‡	2.551 ± 1.799†‡	0.420 ± 0.292‡
Statistical analyses								
ANOVA results		F(8,26)= 1.68; $p = 0.149$	F (8,26) = 56.15 ; $p = 1.885\times10^{-14}$	F (8,26) = 2.745 ; $p = 0.02437$	F (8,26) = 9.08; $p = 7.165\times10^{-6}$	F (8,26) = 55.23; $p = 2{,}305\times10^{-14}$	F (8,26) = 3.577; $p = 0.00634$	F (8,26) = 8.014; $p = 2.116\times10^{-5}$

Statistical annotations:

* - indicates range of the 95% CI of measured parameters are significantly different to uninoculated controls operated in parallel

† - indicates parameters significantly different to MFC 1. Determined by Tukey HSD *post hoc* testing ($p \leq 0.05$)

‡- means of measured parameter significantly different to that of the initial fermentation parameters t=0 of the same sample, Tukey HSD

n.d. not detectable

The table above is a snapshot of MFC fermentation parameters at the peak of t=48-72 hours solventogenesis for the MFC fermentation experiments. The data is extracted from the 240hour experiments that were conducted for all MFCs 1-8 and MEC and the time frame from which the data was extracted from was indicated by the symbol α in the graphs for MFC 1, 2 and 3 or Figures 4. Metabolites in the form of butyrate and solvents for MFC 1-4 are significantly higher to that of the negative control for each experiment. MFC 1, 4, 8 and MEC 2 present values significantly higher concentration to the initial measurements at t=0 for butyric acid, acetone and butanol production. Butyrate yields and pH indicated a general capacity for further solventogenesis, as organic acids (chiefly butyric acid) act as precursors to solvent production (Buehler and Mesbah, 2016a). The highest yields during solventogenesis were obtained in MFC-1 (2.513 ±0.474 g/L) and MFC-2 (2.959 ± 1.742 g/L); all other operated MFC configurations produced lower levels of this metabolite: many of the remaining tested configurations (MFC-4 to MEC-8) produced significantly less than the baseline MFCs.

While not very consistent, MFC configurations producing lower butyric acid also tended to have higher pH levels when sampled: the *in situ* measured pH values varied from a low of 4.327 ± 0.054 for MFC-2 to a maximum of 4.805 ± 0.076 for MFC-5. pHs below 4.5 are considered to be optimal for the onset of solventogenesis (Vasconcelos et al., 1994): of the tested MFC configurations, only MFC-1, MFC-2, MFC-3, MFC-4 and MC-6 produced this level of acidity. Taken together indicated that, apart from the ferricyanide-supplemented CNM in MFC-2, all other interventions resulted in lowered acidogenic behaviour by the MFCs compared to the baseline.

Although butanol yields for MFC were low in general, the highest butanol yield for all MFCs was detected for MFC 8 (2.407 ± 1.2982 g/L) which was significantly higher than from the negative control of MFC 8, the butanol yields of baseline MFC 1 and from t=0 butanol concentration for MFC-8. The acetone/ethanol production in MFC 1 is significantly higher to MFC 5, 6 and 7 which delivered very low yields of acetone/ethanol. The butanol yield at 72 hours of incubation for MFC 8 is significantly lower to that of MFC- 1 at 48 hours. The highest acetone/ethanol yields where recorded by HPLC method for MFC1(10.998 ± 4.688 g/L). Acetone/ethanol yields for MFC 2 (7.523 ± 6.783 g/L) closely followed by MFC 3 (5.234 ±5.182 g/L) were not significantly lower than MFC-1.

Table 4.2: MFC fermentation media comparison of Post-solventogenic phase (192-240 hours)

Name	Time	Cell growth	Nutrient consumption		Organic acid production		ABE production	
		Volts (mV)	Total Carbohydrate content (%)	Glucose(g/L)	Butyric acid (g/L)	pH	Acetone/Ethanol (g/L)	Butanol (g/L)
MFC-1	192	-0.67 ± 0.65	62.33 ± 9.15	6.57 ± 1.17*	2.008 ± 0.218*	4.513 ± 0.058	7.624 ± 1.619*	0.352 ± 0.023
MFC-2	192	4.00 ± 1.96	0.86 ± 0.11*†	8.67 ± 4.50*	2.878 ± 0.608*†	4.453 ± 0.00‡	5.967 ± 3.262*	0.404 ± 0.281*
MFC-3	192	1.25 ± 1.23*	0.31 ± 0.60*†	1.29 ± 2.09*†	0.108 ± 0.115†	4.45 ± 0.02‡	4.391 ± 6.744*	0.172 ± 0.248*
MFC-4	192	13.25 ± 9.24*	0 ± 0‡†	0.77 ± 0.51*†	0.406 ± 0.430*†	4.405 ± 0.028‡	1.685 ± 1.106‡	0.223 ± 0.142*
MFC-5	192	12.25 ± 13.86*	0 ± 0‡†	0.07 ± 0.037†	0.012 ± 0.024†	4.945 ± 0.054†	1.705 ± 1.356†	0.328 ± 0.300*
MFC-6	240	3.50 ± 6.85*	7.83 ± 2.47*†	1.27 ± 1.32*†	0.141 ± 0.126*†	4.908 ± 0.141†‡	1.475 ± 1.366 ‡	1.845 ± 1.770*
MFC-7	192	3.75 ± 4.40*	0 ± 0‡†	0.01 ± 0.01*†	0.099 ± 0.143*†	4.765 ± 0.036*†	0.698 ± 0.599†	0.298 ± 0.306*
MFC-8	192	5.50 ± 4.56*	n.d	1.59 ± 0.53*	0.021 ± 0.034†	4.503 ± 0.027‡	1.072 ± 0.236*‡	1.005 ± 0.124*†
MEC	240	24.75 ± 33.01*	-3.93 ± 0.24†‡	n.q.	n.q.	4.585 ± 0.085	n.q.	n.q.
Statistical analyses								
ANOVA results		F (8,26) = 1.391 ; p = 0.2467	F (7,23) = 230.21 ; $p < 2.236\times10^{-16}$	F (7,22) = 10.11; $p = 1.263\times10^{-5}$	F (7,22) = 50.72; $p = 4.049\times10^{-12}$	F (8,26) = 39.6; $p = 1.236\times10^{-12}$	F (6,19) = 9.000 ; $p = 9.873\times10^{-5}$	F (6,19) = 4.237 ; p = 0.0071

Statistical annotations:

* - indicates range of the 95% CI of measured parameters are significantly different to uninoculated controls operated in parallel

† - indicates parameters significantly different to MFC 1. Determined by Tukey HSD *post hoc* testing ($p \leq 0.05$)

‡- means of measured parameter significantly different to that of the initial fermentation parameters t=0 of the same sample; Tukey HSD

n.d. not detectable **n.q.** – not quantifiable (only one sample was processed for this time for this sample)

Grey blocks are not included for statistical processing other than *

The highest acetone yields recorded by the HPLC method during post-solventogenesis (192-240 hours) was retained by MFC1 (7.624 ± 1.619) and MFC2 (5.967 ± 3.262), with MFC3 (4.391 ± 6.744) closely following, but without any significant differences among them.

Solvent concentration following solventogenic phase (Table 4.1) steadily declined during post-solventogenesis for all MFC's. The aim of this study was to enhance solvent production by manipulating the fermentation by culturing *C. acetobutylicum* in different electron donating or receiving configurations. Although the majority of the MFC experimental fermentations did not yield desirable quantities of solvents, changes were caused by the electrochemical configuration associated with the respective experiments. From the previous studies in Chapter 2 and 3 it can be said that the conditions were not favourable for successful ABE biosynthesis as a result of the composition of the media containing methylene blue.

Unlike the preparation for SBSG and NSBSG for the batch fermentation study in Chapter 3, methylene blue was added in addition to the BSG media in the MFC experiments. The results for MFC 4, 5, 7 and MEC indicated very little with regards to the electrochemical link to solvent production due to the poor production of anticipated metabolites. The rise in mV for MFC 4, 5 and 7 is likely a result of oxygen contamination in the system after operating for 196 hours. The reason for the variation was a result of the gap of data that was lost on the stolen HPLC computer. Nevertheless, from these studies specifically referring to results from MFC 1, 2, 3, 6 and 8 it can be said that changes in electrical configuration of MFC's inoculated with *C. acetobutylicum* grown on different media do have a direct effect on the metabolic products produced. From the above findings it can be said that the possibility of manipulating ABE fermentation with MFC's is worth re-investigating.

4.4.3 SEM analysis of biofilm formed on electrodes during MFC operation.

Electron transfer in MFCs in a biocathode or bioanode between the bacteria and the electrode follows different pathways and methods respectively, depending on the bacteria (Choi & Sang, 2016). It is difficult to understand the mechanisms which are expressed on the physiological level in order to understand the mechanism which *C. acetobutylicum* cells transfer electrons. The ability of bacteria to exchange electrons in and out of the cell can be done using different mechanisms

which includes morphological structures such as pili that connect directly to the electrode during the fermentation (91onten and Nevin, 2013; Rabaey and Rozendal, 2010). The images in Figure 4.5 below present a visual representation of the of the morphological structures formed by *C. acetobutylicum* in a MFC during anodic use.

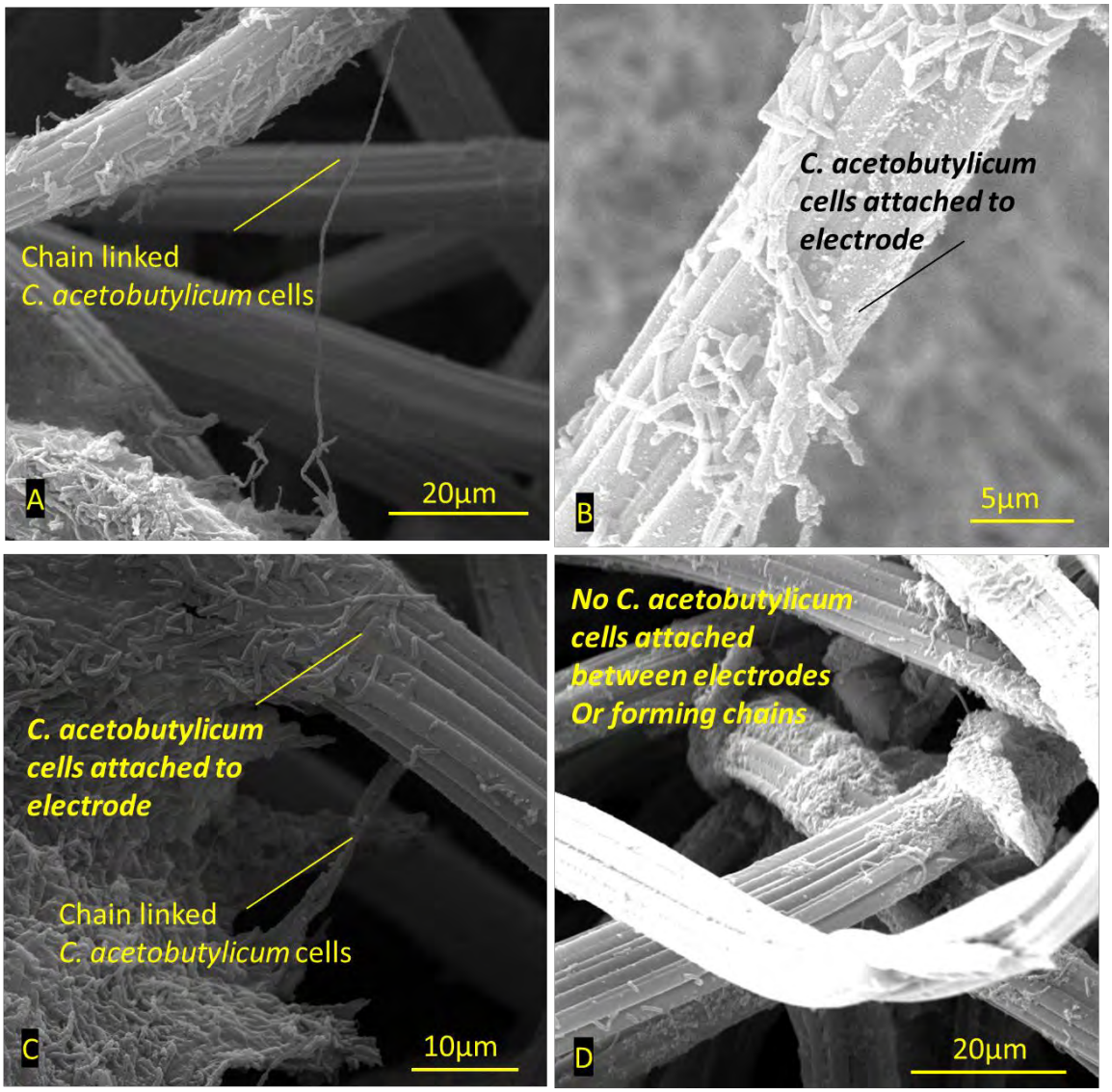

Figure 4.5: Scanning electron micrographs of biofilms cultured on electrodes used for MFCs. Images A and C are taken from MFC 2, Image B from MFC 3 and image D from MFC 4 unconnected control.

C. acetobutylicum cultured in an MFC configuration using carbon-graphite electrodes display different morphological biofilm structures when electrically connected. The biofilm formed by *C. acetobutylicum* in electrically-connected MFCs is made up of many individual cells in between

extracellular polymetric substances (EPS), similar to other reports (Choi & Sang, 2016). From the images in Figure 4.5, it can be seen that *C. acetobutylicum* forms chain linked structures, indicating that they might be capable of direct electron transfer between cells and the electrodes, as was explored in Section 4.3.1 above.

Figure 4.5 indicates structure of the biofilm formed when *C. acetobutylicum* is conFigured for electro fermentation. The findings from Finch et al, (2011) indicate the metabolic connection to the electrode by distinguished peaks for metabolic phase shifts. Although the findings in this study cannot distinguish the connection between *C. acetobutylicum* and the electrode, changes in potential voltage were recorded and Figure 4.5 indicates chain like biofilm structures in electrically connected fermentations compared to non-connected.

4.4.4 Comparison of optimum fermentation- and MFC-based interventions and their effect on solvent production

C. acetobutylicum was selected for this project for the study of solvent production in the ABE fermentation. The ability to both act as an anode-respiring bacteria and metabolise recalcitrant organic matter was the main area of focus for increasing yield and efficiency of the ABE fermentation. The complex physiological responses from the ABE system are simplified at best by comparison of the most efficient forms of solvent production by fermentation, in this research. The table below represents a comparison including highest ABE solvent yields respectively, for the baseline chemically defined media MB, MFC-1 fermentation and SBSG batch ABE fermentation.

Table 4.3: Comparison of parameters of optimum MFC and batch fermentation at points of highest solvent concentration (48-72 hours)

Name	Time	Nutrient consumption		Organic acid production		ABE production	
		TC (%)	Glucose (g/L)	Butyric acid (g/L)	pH	Acetone/ethanol (g/L)	Butanol (g/L)
MB	48	7 ± 16†	0.23 ± 5.06†	1.27 ± 0.946†	4.71 ± 0.29†	5.47 ± 4.48†	0.40 ± 0.36†
MFC 1	48	87.7 ± 20.86	8.14 ± 0.08	2.513 ± 0.474	4.33 ± 0.05	11.00 ± 4.69	0.45 ± 0.15
SBSG	72	49 ± 31†	15.51 ± 9.55†	0.114 ± 0.073†	4.69 ± 0.06†	10.92 ± 1.21	12.97 ± 0.48†
Statistical analyses							
ANOVA results		$F(2,8)= 32.2$ $p=1.5\times10^{-4}$	$F(2,8) = 27.3$; $p = 2.678\times10^{-4}$	$F(2,8) = 39.3$; $p = 7.279\times10^{-5}$	$F(2,8) = 17.2$; $p = 0.0012$	$F(2,8) = 31.9$; $p =0.542\times10^{-4}$	$F(2,8) = 7992$; $p = 6.3\times10^{-14}$

Statistical annotations:

† - indicates parameters significantly different MFC 1 by Tukey HSD post hoc testing ($p < 0.05$)

Table 4.3 presents a comparison of the overall highest solvent concentrations produced by MFC and batch ABE fermentation methods at 48-72 hours. MFC-1 produced the highest butyric acid concentrations (2.513 ± 0.474 g/L), significantly higher than either MB (1.27 ± 0.95) or SBSG (0.114 ± 0.073). However, the excess of butyric acid did not produce significantly higher productions of butanol (0.45 ± 0.15 g/L), compared to MB (0.403 ± 0.362 g/L); both producing significantly lower than SBSG (12.97 ± 0.478 g/L). There was no statistical difference between the acetone concentration for MFC1 (10.99 ± 4.69 g/L) and SBSG (10.92±1.21 g/L), comparatively MB (5.467 ± 4.478 g/L) produced significantly less acetone.

The study suggests that MFC-1 may be a more efficient method for producing high concentrations of butyric acid and expressed the lowest pH and results in improved acetone and ethanol production compared to the fermentation of MB alone. Overall, SBSG showed promise as a method for producing high levels of solvents in the batch fermentation method.

With all methylene blue containing feedstocks for ABE fermentation, including MB batch and MFCs 1-8, there is an increased flux towards the production of acetone/ethanol rather than butanol and, in some cases, suppression of solventogenesis. The metabolic flux of *C. acetobutylicum* towards acetone production is significantly different for different configurations of MFC and appears to not depend strongly on the media used as feedstock in the MFC (MFC 6 containing SBSG, but not resulting in strong solvent formation). Methylene blue was not added to SBSG and NSBSG but it was added to MFC-1-8, including MFC6 which utilized SBSG as the primary feedstock.

Although the change in sampling times was made for later experiments from MFC6-MFC-8 the results from HPLC analysis indicate that the efficiency of the ABE fermentation continued to decrease. This can be a result of the maintenance practices applied when preserving *C. acetobutylicum* and the loss of solventogenic ability after successive generations (Adler and Crow, 1987; Assobhei et al., 1998).

4.5 Conclusions

All the MFCs were complex to operate. The complexity is derived from the sensitivity of the systems in key areas where reactions occur, namely at the PEM and the electrodes. The operation of such MFCs including bacterial processes and sampling is hindered by the lack of a modular, operator friendly design. Ports for taking samples should be independent and not providing more than one function as to avoid disruption and accidental disassembly. For an anaerobic fuel cell, the above-mentioned changes are imperative to avoid constant problems, including oxygen contamination.

The concentrations of solvents or acetone/ethanol decreased from solventogenesis t=48-72 hours to post solventogenesis t=192-240 hours similar to the solvent production kinetics of the batch fermentations in Chapter 3. Although comparable concentrations of acetone/ethanol were record for acetone/ethanol, SBSG produced significantly higher concentrations of ABE solvents overall. The base line MFC-1 experiment did reach significantly higher acetone/ethanol than the baseline batch experiment MB (5.47 ± 4.48 g/L) indicating the improved solventogenic ability of *C. acetobutylicum* fermentation of the same media MB containing methylene blue, in a MFC configuration. From the high levels of ethanol/acetone concentrations produced for most of the MFC configurations in this study, such as for MFC-1(11.00 ± 4.69 g/L), MFC-2 (7.523 ± 6.783 g/L) and MFC-3 (5.234 ±5.182 g/L) indicates that the acetone/ethanol producing pathways were favoured, in turn indicating that very low concentrations of NADH were accumulated in the MFCs in this study (Kim et al., 2015; Li et al., 2014a).

It would be beneficial for the construction of the MFC to be designed for operation under anaerobic conditions with simultaneous automated sampling that does not affect operation. Operating within an anaerobic chamber did aid the ability to ensure an anaerobic environment; however, it does not allow for flexible control of the MFC systems. For example, for MFC-1, the lack of oxygen during operation prevented the expected cathodic half-reaction from occurring; given the use of PB Solution as the catholyte, it is unclear what the electron acceptor in this system was.

The introduction of electrochemical electron acceptors and donors in the form of Ferricyanide as one option was tested as an anaerobic alternative to aerobic cathodes however the flexibility of what needs to be a dynamic system is rather restricted. It is imperative that individual anodic chambers can be de-aerated and anaerobicity can be modulated between separate MFC's. A

dedicated anaerobic bioreactor with dedicated ports for sampling and relative measurements that do not disturb the fermentation would be a priority that could be addressed in future studies. While affecting the redox potentials that control the metabolism of *C. acetobutylicum* is possible very little success in enhancing production of butanol by manipulating the redox dependant switches driving the hydrogen flux in the favour of the butanol pathway, was achieved.

The use of MFC system as a biosensor for the biphasic metabolism shift in *C. acetobutylicum* ABE fermentation correlates to the oxidation of metabolic precursors by select dehydrogenases for acidogenesis and solventogenesis respectively (Finch et al., 2011).

The decrease in butanol output and increase in acetone, was not attributed to oxygen contamination, as enzymatic activity of hydrogenase enzyme complexes involved in the production of solvents are disturbed for H_2O_2 metabolism (O'brien and Morris , 1971). Alternatively, the culture consisting of mixed maturity of *C. acetobutylicum* cells may cause additional stressors from different nutrient requirements at different phases of the life cycle. Without the accumulation of NADH during exponential growth phase and Acidogenesis when cultured from spore, intracellular physiological conditions of the culture did not provide the environment for the metabolic phase shift for viable solvent producing cells within the consortium. According to (Kheyrandish et al., 2015) the addition of P2 and higher actively fermenting inoculums can decrease the time to complete lag phase. The majority of NADH and acid accumulation occurs at this time. The time intervals for the voltage measurements that would indicate the expected correlating peaks for the biphasic fermentation (Finch et al., 2011) may have not been sufficient to track the rapid change.

Lastly, it has been discovered that *C. acetobutylicum* pure culture undergoes a form of senescence after successive sub culturing with the loss of its ability to mature to a spore forming, solvent producing culture (Assobhei et al., 1998). The reduction of butanol ethanol and acetone seen in later experiments could be attributed to this. The majority of the MFC experiments were conducted more than six months from the initial germination of the commercial spores and inoculation for experimentation of solvent production was not carried out by growth of culture from spore form. In saying this, the inoculated culture, was a consortium of cells which were of different maturities and undergoing different life cycles. Losing the ability to produce alcohol does not imply the inability to carry out metabolic activities such as acid production from carbohydrates. The

consortium of *Clostridial* cells that are cultured together may include cells that have both lost their ability to produce solvents and those that have retained solventogenic traits. The production of acids by cells that do not produce solvents, favour the production of acetone precursors such as acetate which co-incidentally reduces abundance of NADH to drive alcohol synthesis in remaining cells which still synthesize ABE (Jiang et al., 2009; Luo et al., 2016b).

Chapter 5: Conclusions, Limitations and Future Research

5.1 Summary of findings

The aim of the study was to improve the efficiency and sustainability of solvent production in ABE fermentation by using *C. acetobutylicum* in electro fermentation and its ability to convert brewers spent grain to value-added products. The study aimed to track specific fermentation parameters using various techniques to better understand the kinetics of the fermentation under different conditions. Solvent toxicity and autolysis, like oxygen contamination threaten the efficiency of solvent production.

This study achieved the aim to propagate *C. acetobutylicum* from spores and compare different media for solvent production studies and culture maintenance. A low-cost anaerobic chamber was designed, manufactured, and tested to achieve this aim. The main objective of the chamber design was to create an airtight environment that is sealable to oxygen contamination and operator friendly. However, sampling in a small, enclosed area was found to be the most challenging design flaw. Maintaining an efficient fermentation operation with the correct hardware and facilities can reduce the impact of uncontrollable variables resulting from the system design.

The Gram staining method was used to determine the purity of the culture and for cell enumeration, but SEM was used for morphological characterization. Chemically defined media were developed and four amendments were applied to the CNM supplemented using P2 micronutrient solution. Growth kinetics were measured by OD600 and HPLC. A decrease in cell density was observed, which was attributed to solvent toxicity and bacteriocin production. Multiple parameters, such as pH, were measured to identify trends in fermentation kinetics. The shift from the acidogenic phase to the solventogenic phase was observed when the pH reached a minimum between 48-72 hours.

HPLC was used for analysis of the different parameter that describes the kinetics of the fermentation in terms of carbohydrate consumption and product synthesis. A successful Method for HPLC was refined for adequate detection of the compounds interest that are consumed (glucose) or produced (butyric acid, acetone/ethanol and butanol). Increasing column oven temperature and decreasing flow rate in HPLC analysis for ABE fermentation produced a chromatogram with a greater separation resolution of peaks for the standards that could determine concentration using linear regression following the log-log transformation.

Experiments in Chapter 2 studied *C. acetobutylicum* fermentation kinetics in chemically defined media, characterizing metabolite production and sporulation using various techniques. Sporulation media B was chosen for future experiments and referred to as media B (MB). P2 was added for micronutrient supplementation, aiding in fermentative ability. HPLC analysis confirmed key fermentation parameters could be detected, but acetone and ethanol were not separable. Methods for measuring fermentation kinetics in *C. acetobutylicum* were established for culture maintenance and quantification of metabolic products and precursors.

Chapter 3 aims to evaluate the efficiency of raw sustainable waste as a nutrient source for ABE fermentation by determining its kinetics and comparing it to chemically defined Media B from The moisture content of BSG was found to be 72 ± 4%, consistent with previous reports. HPLC chromatograms of the media before and after fermentation showed glucose consumption, formation of butyric acid, amylase/cellulose activity, and butanol production. The physicochemical parameters of the media were compared, and MB was found to have the most glucose 11.75 g/L at t=0 compared to NSBSG (0.965 g/L) and SBSG (6.406 g/L). The phenol sulphuric acid assay indicated distorted results from grain particles in NBSG (4.3651±0.04), SBSG (25.84±0.0032) media and methylene blue from MB (49.00±28.02) respectively. The level of error could have possible been reduced by increasing sample size greater than n=3 or n=4 for replicates of individual samples.

The Solvent producing efficiency *C. acetobutylicum* was investigated by comparing kinetics of the select parameters when cultured on different media. The results showed significant differences in growth characteristics and product development between the chemically defined media MB and both treatments of Brewers spent grain. The fermentation was tracked over the full duration of 240 hours compared to the trend of the un-inoculated negative control. The study identified contamination as a significant issue in the experiment due to the formation of solvent precursors and solvents within the uninoculated control. Overall, the BSG media both produced significantly higher concentrations of ABE solvents. Where MB (0.403 ± 0.327 g/L) batch fermentation produced almost no butanol, NSBSG (8.659 ± 1.564 g/L) and SBSG (12.970 ± 0.381 g/L) produced significantly higher concentrations. Acetone/ethanol was detected in MB (5.47 ± 2.06 g/L) and NSBSG (8.92 ± 1.86 g/L) batch fermentation where significantly higher concentrations

of acetone/ethanol were achieved by SBSG (10.92 ± 0.961 g/L). The highest Solvent concentrations were achieved by SBSG at 72 hours of *C. acetobutylicum* fermentation.

It can be seen from Table 3.3 that solventogenesis is initiated within the first 48 hours of fermentation of all media. The highest yield of butanol was recorded for fermentation of SBSG (12.970 ± 0.381 g/L) which is indicated as significantly higher than NSBSG (8.659 ± 1.564 g/L) and MB (0.403 ± 0.327 g/L). It can also be seen that a significantly higher concentration of acetone/ethanol produced from the fermentation of SBSG (10.92 ± 0.961 g/L) compared to that of (NSBSG (8.92 ± 1.86 g/L) and MB (5.47 ± 2.06 g/L). The production of acetone from the presence of methylene blue in Media B may have blocked the redox-dependent regulatory switch such as Thiolase, to solventogenesis.

Following solventogenesis over the course of the 240 hour experiments the concentration of solvents decreased significantly after 192 hours during post solventogenesis. Interestingly SBSG retained increased concentrations of glucose following the production of toxic solvent levels, indicating that the fermentation was inhibited by product formation as opposed to the exhaustion of resources. Comparatively SBSG produced significantly higher concentrations of ABE compared to NSBSG, however the pre-treatment for SBSG required more energy input compared to very little processing of the NSBSG. This indicates the efficacy of the storage methods used; however, no comparison was made on the feasibility of the treatment compared to the value added products retrieved.

The aim of the study in Chapter 4 is to explore the efficacy of using *C. acetobutylicum* in a MFC configuration to alter solventogenesis pathways during culture. The study compares fermentation kinetics observed in two scenarios: one where fermentations were established in a previous Chapter 3, and the other where *C. acetobutylicum* is attached to an electrode in an MFC.

The analysis shows the inoculated *C. acetobutylicum* actively metabolizing media with a low yield of butanol observed. The graphs were used to identify the culture entering the solventogenic phase and the post-solventogenic phase.

The fermentation kinetics of MFC-1 act as a baseline for the comparison of ABE fermentation efficiency between different MFC configurations and ABE fermentation methods. The MFC-1 (11.00 ± 4.69 g/L), MFC-2 (7.523 ± 6.783 g/L) and MFC-3 (5.234 ±5.182 g/L) experiment did

reach significantly higher acetone/ethanol than the baseline batch experiment MB (5.47 ± 4.48g/L). Although no increased yield of solvent was noticed for MFC-2 compared to baseline MFC-1, the reverse configuration of *C. acetobutylicum* cultured in a biocathode did witness negative voltage which was not seen for the bioanode configurations.

The concentrations of solvents or acetone/ethanol decreased from solventogenesis t=48-72 hours to post solventogenesis t=192-240hours similar to the solvent production kinetics of the batch fermentations in Chapter 3. Although comparable concentrations of acetone/ethanol were record for acetone/ethanol, SBSG produced significantly higher concentrations of ABE solvents overall. The SBSG and the NSBSG batch fermentations yielded higher concentration of both acetone and butanol compared to MFC 6 which utilized SBSG as the feedstock. The MFC configuration in general, yielded much lower concentrations of total solvent (acetone/ethanol and butanol) produced compared to the simple Batch fermentation.

The base line MFC-1 experiment did reach significantly higher acetone/ethanol than the baseline batch experiment MB (5.47 ± 4.48g/L) indicating the improved solventogenic ability of *C. acetobutylicum* fermentation of the same media MB containing methylene blue, in a MFC configuration. From the high levels of ethanol/acetone concentrations produced for most of the MFC configurations in this study, such as for MFC-1(11.00 ± 4.69 g/L), MFC-2 (7.523 ± 6.783 g/L) and MFC-3 (5.234 ±5.182 g/L) indicates that the acetone/ethanol producing pathways were favoured, in turn indicating that very low concentrations of NADH were accumulated in the MFCs in this study.

MFC-1 outperformed MB in terms of butyric acid production, yielding significantly higher concentrations (2.513 ± 0.474 g/L), whereas MB (1.27 ± 0.95) and SBSG (0.114 ± 0.073) lagged behind. Surprisingly, despite the elevated butyric acid levels in MFC-1, it did not result in significantly greater butanol production (0.45 ± 0.15 g/L) compared to MB (0.403 ± 0.362 g/L), with both falling short of SBSG (12.97 ± 0.478 g/L). Additionally, MFC-1 and SBSG exhibited comparable acetone concentrations (10.99 ± 4.69 g/L and 10.92±1.21 g/L, respectively), while MB yielded significantly lower acetone levels (5.467 ± 4.478 g/L). In conclusion, MFC-1 shows promise as a more efficient method for generating high butyric acid concentrations, with potential benefits in acetone and ethanol production compared to MB fermentation alone.

The simplicity and ease of setting up the batch fermentation decreases complexity and time taken to prepare the fermentation. The Batch fermentation is more efficient as it requires less time and technical input for higher yields of solvent compared to the tedious and low solvent yielding MFC fermentations irrespective in the change of feedstocks. For future considerations this may be mitigated with the development of infrastructure allocated for the purpose of MFC anaerobic fermentation.

The intention of the thesis is to highlight a value additive waste feedstock to the ABE fermentation technology that can be utilized in parallel to existing waste streams of BSG within the Brewing fields and possibly utilized by brewers with their transferable existing knowledge. The complexity of MFC and yield are a major deterrent compared to the simplicity of batch fermentation. The theoretical outcome of an operation ABE MFC bioreactor still holds great promise for commercial production application.

5.2 Future recommendations/research

Running multi-day *C. acetobutylicum* fermentations experiments in the traditional MFC configuration, using a multimeter every 3-6 hours for multiple 240hour experiment cannot adequately measure voltage changes related to metabolic change. For instances where voltage changes can occur in a hour or two before or after taking the measurement. Automation or automated data logging is essential in biotechnology.

BSG waste can be used as animal feedstock after microbial fermentation, and more research is needed on incorporating bacterial fermentations for value-added products. The study only focuses on feedstocks and their effect on solvent production efficiency. (Parchami et al., 2021 ; López-Contreras et al., 2004 ; Fierobe et al., 2012)

If the study had to have taken place in conjunction with a commercial brewery and research conducted in parallel to the brewing process, a more directly applicable method would have been developed for the brewing industry. Basic sterilizing of the grain can significantly increase yields of solvent it is however not completely necessary if the facility or feasibility of the sterilization of BSG is not viable. The majority of breweries have a battle with downstream processing following the constant production of BSG. Worldwide the uses and methods of disposal are completely

diverse often with very little return. With a set intention and a predictable outcome of product from the BSG, the practice of waste disposal or processing of BSG waste does not have to be a burden but rather a positive feedback incentive for increased beer production.

From the data presented in this study it can be concluded that BSG is an excellent feed stock for *Clostridium acetobutylicum* and more research is needed with ABE fermentation studies conducted in conjunction with brewing operations and anaerobic electro fermentation configurations such as MFC's.

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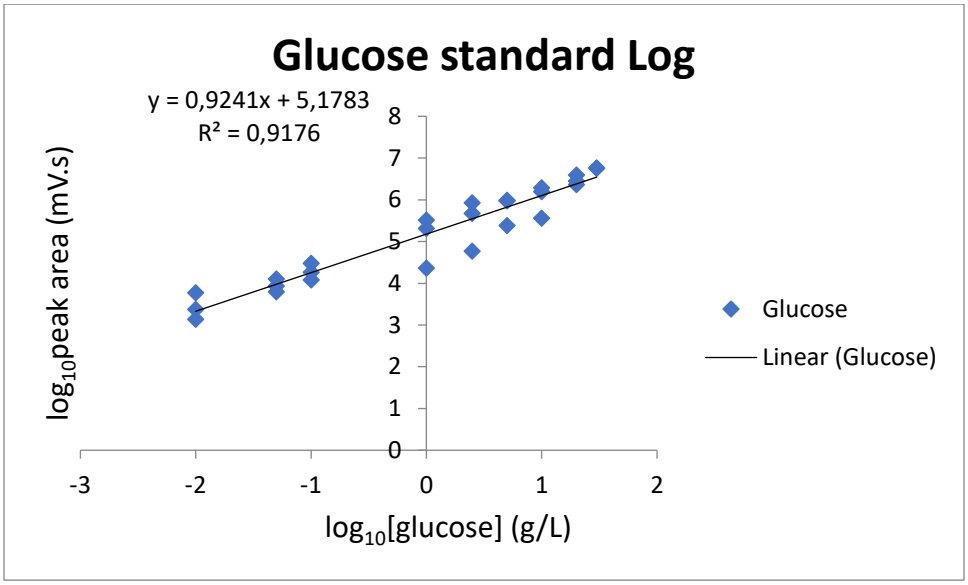
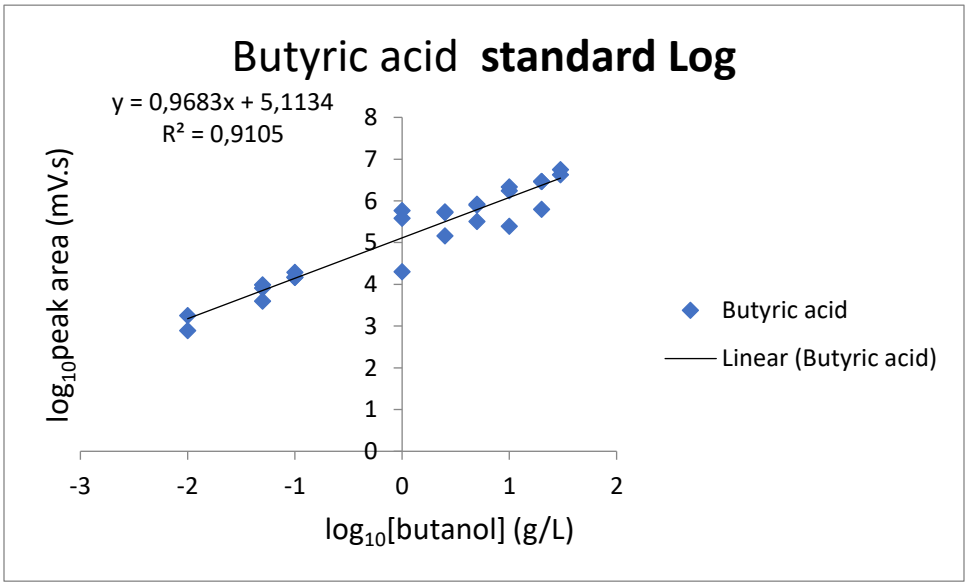
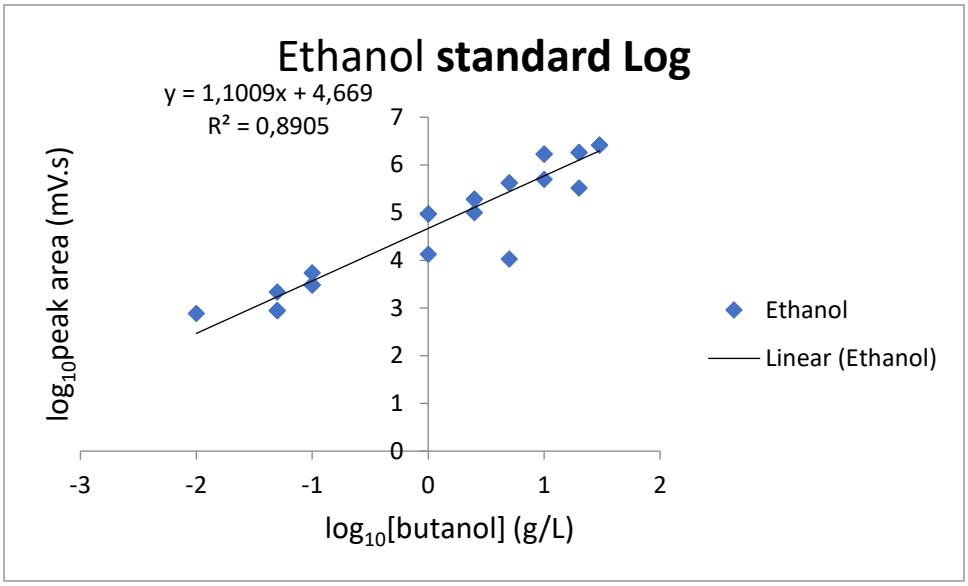
Appendices



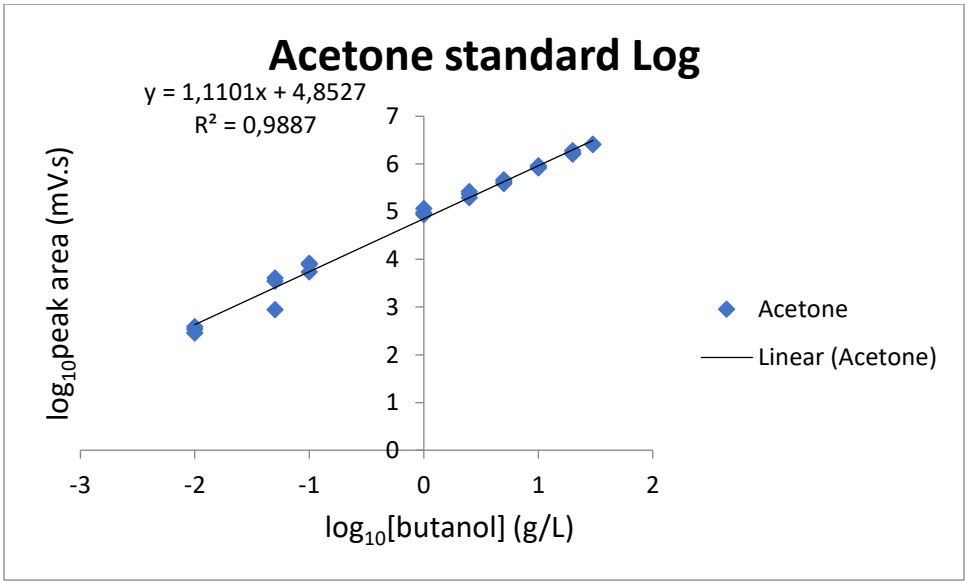
Appendix A: Logarithmic plot of HPLC glucose standard.



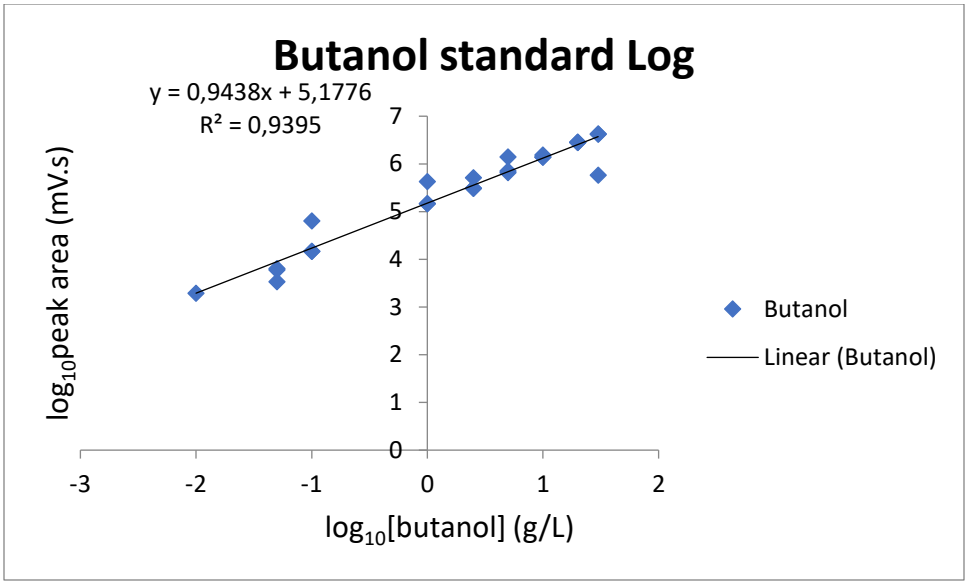
Appendix B: Logarithmic plot of HPLC Butyric acid standard.



Appendix C: Logarithmic plot of HPLC ethanol standard.



Appendix D: Logarithmic plot of HPLC Acetone standard.



Appendix E: Logarithmic plot of HPLC Butanol standard.