

Effect of Crude Glycerol from Biodiesel Production on the Performance and Anaerobic Metabolism of Catalysts in a Glycerol Oxidizing Microbial Fuel Cell

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Abstract

Use of waste glycerol as fuel in microbial fuel cells (MFCs) would result in the production of valuable metabolites and electricity, to the benefit of biodiesel operations. In this research, the effect of salt and other compounds found in waste glycerol from biodiesel production on the metabolism and performance of three cultures (*Escherichia coli* W3110, *Propionibacterium freudenreichii* ssp. *shermanii* and mixed culture AR2), used as anodic catalysts in an MFC was studied. MFC experiments were performed in parallel with serum bottle fermentations to allow for comparison of glycerol consumption and metabolite yield.

The effect of salt content on the performance of all three cultures was positive in most cases and negligible in others. Using waste glycerol with an increased concentration of other compounds (other than salt) only reduced the performance of AR2, however an inhibitory effect on the rate of glycerol consumption was observed with both AR2 and *P. freudenreichii* ssp. *shermanii*. For all strains, the rate of glycerol consumption was slower in MFCs than in fermentations as a result of the electrochemical environment; the yield of various metabolites also differed.

Resumé

L'utilisation du glycérol comme carburant dans des piles à combustible microbiennes (PCM) produira des métabolites et de l'électricité, au profit des opérations de production du biodiesel. Dans cette recherche, l'effet du sel et d'autres impuretés du glycérol provenant de la production de biodiesel sur le métabolisme et les performances de trois cultures (*Escherichia coli* W3110, *Propionibacterium freudenreichii* ssp. *shermanii* et la culture mixte AR2), utilisés comme catalyseurs anodiques dans un PCM a été étudié. Les expériences de PCM ont été réalisées en parallèle avec des fermentations en flacon à sérum afin de permettre la comparaison de la consommation de glycérol et le rendement de métabolites.

L'effet de la concentration du sel sur la performance des trois cultures a été positif dans la plupart des cas et négligeable dans les autres. L'utilisation de glycérol avec une concentration augmentée d'impuretés (autres que le sel) a seulement réduit le rendement de AR2, cependant un effet inhibiteur sur le taux de consommation du glycérol a été observée avec AR2 et *P. freudenreichii* ssp. *shermanii*. Pour toutes les cultures, le taux de consommation de glycérol était plus lent dans les PCM comparé aux fermentations en raison de l'environnement électrochimique; le rendement de divers métabolites différait aussi.

Statement of Contributors and Collaborators

I hereby declare that I am the sole author of this thesis. I am responsible for all experiments and subsequent data analysis presented in this thesis.

Dr. Kathlyn Kirkwood supervised this thesis project, providing guidance and editorial contributions to this written work.

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Nomenclature

μ	Electrochemical efficiency
A_{An}	Area of the anode (m^2)
b_e	Moles of electrons available per mole of substrate when complete oxidation is achieved
C_E	Coulombic efficiency
C_{int}	Number of electrons from a substrate that would be recovered as current if complete oxidation of the substrate was achieved
C_{rec}	Number of electrons from a substrate recovered as current
E	Potential achieved by a fuel cell (V)
E^0	Standard cell reduction potential (V)
E_{an}	Open circuit potential observed at anode
E_{cat}	Open circuit potential observed at cathode
E_{MFC}	Open circuit potential achieved by a microbial fuel cell (V)
F	Faraday's constant
G^0	Gibbs free energy at standard conditions ($Jmol^{-1}$)
g_1	Initial concentration of glycerol (gL^{-1})
g_2	Final concentration of glycerol (gL^{-1})
H_f°	Standard enthalpy of formation ($kJmol^{-1}$)
I	Current (A)
I_{An}	Current density $mA m^{-2}$
m_{Gly}	Mass amount of sodium contained in the glycerol waste, per gram glycerol (gg^{-1})
M_{HCl}	Molarity of the industrial strength HCl solution ($molL^{-1}$)
m_{HCl}	Average mass of HCl samples (g)
m_{HCl}	Mass amount of industrial strength HCl used (g)
m_{Na1}	Mass amount of sodium contained in the glycerol waste, per gram glycerol (gg^{-1})
m_{Na2}	Concentration of sodium per gram glycerol (dependent on concentration of catalyst used in biodiesel production) (gg^{-1})
M_S	Molecular weight of the substrate
MW_{Gly}	Molecular weight of glycerol ($gmol^{-1}$)
MW_{Na}	Molecular weight of sodium ($gmol^{-1}$)
MW_{NaCl}	Molecular weight of sodium chloride ($gmol^{-1}$)
MW_{NaOCH_3}	Molecular weight of sodium methoxide ($gmol^{-1}$)
MW_{oil}	Molecular weight of soybean oil ($gmol^{-1}$)
n	Number of electrons transferred with complete oxidation of the substrate

OCP	Open circuit potential (V)
OD	Optical density
OD ₆₀₀	Optical density observed at a wavelength of 600nm
P	Power
p ₁	Initial concentration of the fermentation product of interest (gL ⁻¹)
p ₂	Final concentration of the fermentation product of interest (gL ⁻¹)
P _{An}	Power density normalized to the area of the anode
p _{HCl}	Density of the industrial strength HCl solution (gL ⁻¹)
R	Gas constant
R _{ext}	External resistance (kΩ)
R _{int}	Internal resistance (kΩ)
S _f [°]	Standard entropy of formation (Jmol ⁻¹ K ⁻¹)
T	Temperature (K)
V _{NaOH}	Average volume of 0.09molL ⁻¹ NaOH used to neutralize samples (L)
x	Concentration of the compound of interest in the sample (gL ⁻¹)
x _e	Concentration of protein in a sample of <i>E. coli</i> W3110 (mgL ⁻¹)
x _{HCl}	Amount of hydrochloric acid used (g)
x _p	Concentration of protein in a sample of <i>P. freudenreichii</i> ssp. <i>shermanii</i> (mgL ⁻¹)
y	Peak area observed with HPLC analysis of the sample (nRIU)
y _e	Optical density observed at a wavelength of 600nm for an <i>E. coli</i> W3110 sample
y _p	Optical density observed at a wavelength of 600nm for a sample of <i>P. freudenreichii</i> ssp. <i>shermanii</i>
Y _{p/g}	Yield of product per gram of glycerol fermented (gg ⁻¹)
Δc	Change in substrate concentration over time
v	Volume of the anode

List of Abbreviations

Analytical techniques

HPLC	High performance liquid chromatography
RC DC®	Reducing agent detergent compatible
RID	Refractive index detector
TCD	Thermal conductivity detector

Microbial fuel cells

MFC	Microbial fuel cell
-----	---------------------

Metabolites

1,2-PDO	1,2-Propanediol
1,3-PDO	1,3-Propanediol
3-HPA	3- Hydroxypropionaldehyde
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CO ₂	Carbon dioxide gas
CoA	Coenzyme A
DHA	Dihydroxyacetone
DHAK	Dihydroxyacetone kinase
DHAP	Dihydroxyacetone phosphate
H ₂	Hydrogen gas
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamideadenine dinucleotide
PEP	Phosphophenol pyruvate
PFL	Pyruvate formatelyase
PYR	Pyruvate

Growth media

RCM	Reinforced clostridial medium
LB	Luria Bertani

Others

ATCC	American Type Culture Collection
FAME	Fatty acid methyl esters

Chapter 1. Introduction

1.1 Background

Glycerol, the high-volume co-product of biodiesel production, has recently been labelled as a waste stream due to the high cost of purification and a decrease in market value (Yazdani and Gonzalez, 2007); this has created a need for the development of new platforms for its use.

Microbial fuel cells (MFCs) are electrochemical devices that convert the chemical potential energy of a fuel to electrical energy via biocatalysis. Typically studied as a potential technology for waste water treatment, MFCs provide an environmentally sustainable option for the treatment of organic wastes. MFCs are similar to conventional fuel cells but differ in the nature of the anodic catalyst; in an MFC, microorganisms act as the anodic catalyst. In the anode chamber, fuel is oxidized by the microorganisms and the electrons released are accepted by the anodic electrode. Electrons then travel to the cathode through an external circuit where they combine with hydrogen ions and oxygen producing water. The anode chamber is kept anaerobic whereas the cathode is continuously sparged with air to maintain a high concentration of dissolved oxygen. In addition to releasing electrons, a variety of metabolites can result from the anaerobic fermentation of a fuel.

Bacteria used as catalysts in MFCs can be classified as exoelectrogenic or as non-exoelectrogenic. Bacteria classified as exoelectrogenic are capable of producing their own redox shuttles or participating in direct electron transfer. Bacteria classified as non-exoelectrogenic can still be used as catalysts in MFCs but require the addition of electron mediating compounds to the anolyte in order to achieve electron transfer (Schröder, 2007). Exoelectrogenic bacteria are preferred over non-exoelectrogenic bacteria since chemical mediators can be toxic, and expensive (Bullen et al., 2006).

An environmentally friendly option for the disposal of waste glycerol from biodiesel production involves its use as a fuel in MFCs. In previous work, when used as a catalyst in a dual chamber MFC, *Propionibacterium freudenreichii* ssp. *shermanii* (ATCC 9614), *Escherichia coli* W3110 (ATCC 27325), and mixed culture AR2 were shown to

anaerobically metabolize analytical grade glycerol and produce a current in the absence of external electron acceptors (Reiche, 2012), making them good catalyst candidates for a fuel cell using waste glycerol from biodiesel production.

Due to processing conditions, waste glycerol from biodiesel production is composed of a number of different components including water, free fatty acids, soaps, colour bodies and fatty acid methyl esters (FAME) (Santori et al., 2012). Commercial biodiesel results from the transesterification of vegetable oil triglycerides with methanol. This reaction is typically catalyzed by a homogeneous alkali catalyst such as potassium or sodium hydroxide or methoxide. Once the reaction reaches completion, the catalyst is neutralized with acid (typically hydrochloric acid), and the FAME separated from the glycerol waste. Because of their hydrophilic nature, most of the salts that result from the neutralization reaction collect in the glycerol phase. The concentration of salt in the glycerol phase is dependent on the type and concentration of catalyst used. The concentration of other components is dependent on the concentration and type of catalyst used in addition to the processing conditions and the quality and type of oil used. These components could be expected to affect the performance of biocatalysts in subsequent bioprocessing operations.

1.2 Hypotheses

Waste glycerol was expected to affect the different cultures in a culture-dependent manner.

FAME, free fatty acids and other compounds (except salt) found in waste glycerol, at elevated concentrations, will negatively affect the growth of *P. freudenreichii* ssp. *shermanii*, thereby reducing fuel cell performance from what was previously observed when analytical grade glycerol was used. Elevated concentrations of free fatty acids have been shown in some studies to negatively affect the growth of *Propionibacterium* (Boyaval et al., 1995); a decrease in growth can be directly related to a decrease in glycerol consumption, which ultimately results in a decrease in the number of reduction equivalents available at the anode at a given time.

FAME, free fatty acids and other compounds found in waste glycerol, at elevated concentrations, will not affect the growth of *E. coli* W3110. In a study conducted by Yazdani et al, (2008) *E. coli* was capable of anaerobically metabolizing waste glycerol from biodiesel production.

FAME, free fatty acids, salt and other compounds found in waste glycerol, at elevated concentrations, will affect the growth and catalytic performance of mixed culture AR2 from what was previously observed when analytical grade glycerol was used. Since AR2 is a mixed culture, it will likely evolve in the presence of waste glycerol; bacteria that are not negatively affected by free fatty acids, FAME, salt and other compounds should dominate.

1.3 Objectives and thesis overview

The principle objective of this research was to determine the effect of crude glycerol from biodiesel production on the performance of *E. coli* W3110, *P. freudenreichii* ssp. *shermanii*, and mixed culture AR2 as catalysts in an MFC. The research was conducted in three parts: use of waste glycerol from biodiesel produced using minimal catalyst, supplemented with sodium chloride and potassium chloride; use of waste glycerol from biodiesel produced using high catalyst concentrations; and metabolite analysis. The objectives for each part are summarized in Figure 1.1, and are described in greater detail (along with the experimental approach followed), in sections 1.3.1, 1.3.2, and 1.3.3. Due to limitations associated with fuel cell architecture, this research was primarily focused on comparing the performance of the different cultures when grown on different types of glycerol.

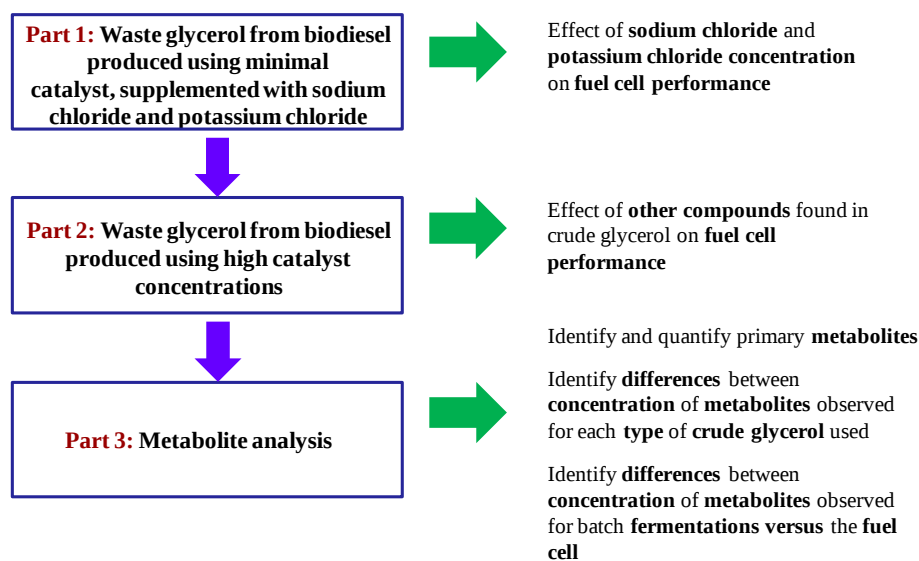


Figure 1.1: Studies conducted and their primary objectives.

1.3.1 Waste glycerol from biodiesel produced using minimal catalyst, supplemented with sodium chloride and potassium chloride (Chapter 4)

The primary objective of the work in this chapter was to determine the effect of sodium chloride and potassium chloride, at concentrations typically found in waste glycerol from biodiesel production, on the performance of *E. coli* W3110, *P. freudenreichii* ssp. *shermanii*, and mixed culture AR2 as catalysts in an MFC.

Sodium chloride and potassium chloride were added to concentrations set to mimic crude glycerol that would have resulted if 0.6wt%, 0.8wt%, 1.0wt% and 1.2wt% catalyst (based on the mass of oil) were used in the transesterification reaction, to crude glycerol obtained from biodiesel produced using minimal sodium methoxide catalyst. Maximum power density was the major parameter by which the performance of each strain was evaluated. The performances of fuel cells containing added salt were compared to that of an analytical glycerol control.

1.3.2 Waste glycerol from biodiesel produced using high catalyst concentrations (Chapter 5)

The primary objective of the work in this chapter was to determine the effect of crude glycerol, produced using 0.8wt% KOH and 0.8wt% NaOCH₃, on the performance of *E. coli* W3110, *P. freudenreichii* ssp. *shermanii*, and AR2 as catalysts in an MFC. Specific objectives include:

1. Neglecting salt content, determine if other compounds in crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ have an effect on fuel cell performance.
2. Identify whether the results obtained in Chapter 4 provide a good representation of fuel cell performance had crude glycerol been obtained from a process that used the catalyst concentrations noted.

Biodiesel was produced using potassium hydroxide and sodium methoxide as catalysts, at concentrations of 0.8wt%. The glycerol obtained from each reaction was then neutralized using concentrated HCl and stripped of methanol. The neutralized crude glycerol was used alongside the model crude glycerol constructed in Chapter 4 to mimic the salt content of glycerol that resulted from the use of 0.8wt% sodium methoxide or potassium hydroxide catalysts in biodiesel production. Maximum power density was the major parameter by which the performance of each strain was evaluated.

1.3.3 Metabolite analysis (Chapter 6)

The primary objective of this chapter was to identify, quantify and compare the metabolites resulting from the anaerobic fermentation of glycerol by *E. coli* W3110, *P. freudenreichii* ssp. *shermanii*, and AR2 in the anode of MFCs and in batch fermentations. Specific objectives include:

1. Identify and quantify major metabolites at various stages of growth
2. Identify any differences between the type and concentration of metabolites observed for each species of crude glycerol used

3. Identify any differences between the type and concentration of metabolites observed for batch fermentations versus the electrochemical environment of a fuel cell

Batch fermentation experiments were performed in parallel to the fuel cell experiments of Chapters 4 and 5, using the same medium. Batch fermentations were sampled at regular intervals and analyzed using HPLC in order to identify compounds that would have resulted from glycerol metabolism. Batch fermentation results were then compared to the results observed with MFCs.

Chapter 2. Literature Review

2.1 Glycerol from biodiesel production

The world energy demand is predicted to grow by 56% from 2010 to 2014 and concerns about the diminishing reserves and high cost of oil in addition to the environmental impact of fossil fuel generated pollution is expected to trigger an increase in the use of nuclear and renewable energies over the next three decades (U.S. Energy Information Administration, 2013).

Biodiesel, manufactured from plant or animal oils, is an environmentally friendly, renewable, nontoxic alternative to petroleum-based diesel fuel. The use of biodiesel in place of petroleum diesel is associated with lower emissions of CO, sulfates, and particulate matter and does not result in the net generation of CO₂; any CO₂ emissions resulting from the use of biodiesel can be directly related to the CO₂ that was fixed by the photosynthesizing plant (United States Environmental Protection Agency 2012). Furthermore, diesel engines can use biodiesel without undergoing any major modifications, making substitution simple. Worldwide, production of biodiesel has seen significant growth in the past decade, increasing from 20 thousand barrels per day in 2001 to 404 thousand barrels per day in 2011 (Figure 2.1). Growth of the biodiesel industry is expected to continue increasing in the coming years as a result of the influence of mandates and initiatives such as the Renewable Energy Directive (RED), which stipulates that renewable energies should have a minimum 10% share of the transport fuels market in Europe by 2020 (European Commission, 2013).

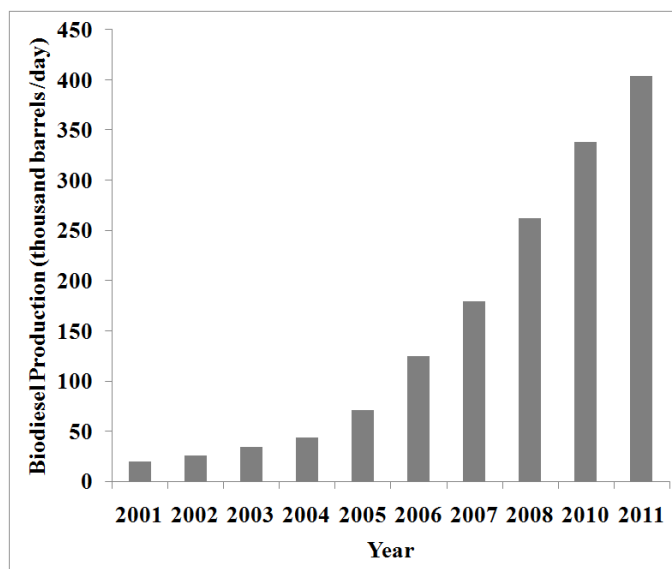


Figure 2.1: Increase in word biodiesel production capacity from 2001 to 2011 (Modified from U.S Energy Administration, 2011).

Glycerol is a co-product of biodiesel production, accounting for 10% of the total product yield. Growth of the biodiesel industry has resulted in a market surplus of glycerol that surpasses the requirements of conventional uses (Agliaro and Rossi, 2010). As a result of the surplus, the market value of refined glycerol has decreased from 0.70USD/lb in 2001 to 0.41USD/lb in 2012 (Agliaro and Rossi, 2010). The decrease in the market value of refined glycerol has made it no longer economically viable for many small to medium sized biodiesel operations to refine their crude glycerol streams, rendering what was once a valuable co-product a waste (Pachauri and He, 2006).

2.1.1 Commercial production of biodiesel through the transesterification of vegetable oils

Biodiesel is composed of FAME, and is produced through the transesterification of vegetable oil triglycerides with a low molecular weight alcohol (Figure 2.2). A triglyceride is an ester formed by reacting glycerol with three fatty acids. The three carbon atoms of the glycerol molecule provide a backbone off which fatty acid side chains are attached via ester bonds. When a mole of triglycerides is reacted with three moles of alcohol, one mole of glycerol and three moles of FAME result. Three consecutive reactions are required for complete conversion; triglycerides are first converted to diglycerides then monoglycerides

before glycerol can be liberated. A catalyst is typically required to increase the rate of reaction and yield.

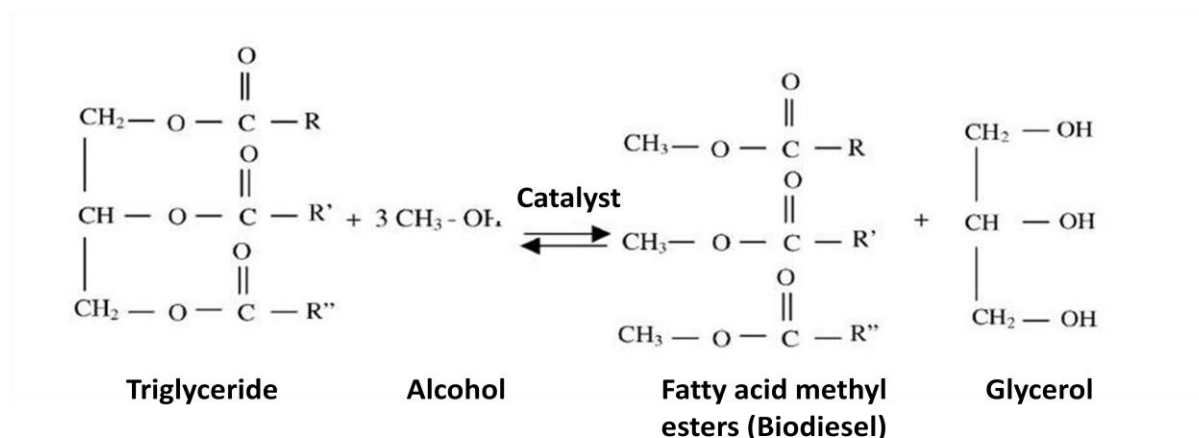


Figure 2.2: Biodiesel production through transesterification (Modified from (Leung et al., 2010)).

The transesterification reaction can be catalyzed by an alkaline, acid or enzymatic catalyst (Fukuda et al., 2001). Alkaline catalysts are typically used in the commercial production of biodiesel, except in the event that a feedstock contains elevated concentrations of free fatty acids or water, as their presence in the reaction mixture results in catalyst consumption via soap formation (saponification) (Santori et al., 2012). Alkaline catalysts promote fast reaction rates, high yields and produce less corrosive intermediates while requiring lesser amounts of excess alcohol and milder temperatures than acid catalysts (Ma and Hanna, 1999; Santori et al., 2012). Alkaline catalysts sodium methoxide and potassium hydroxide are widely used in industry. Methoxide catalysts are often more costly than their hydroxide counterparts but boast the advantage of not producing water upon dissolution (Ma and Hanna, 1999). Acid catalysts, which do not react with water or free fatty acids to produce soap, are often used as pre-treatment for feeds with a high free fatty acid content such as waste cooking oil (Santori et al., 2012).

Biodiesel can be produced in batch, or by a continuous process. Once the transesterification reaction has reached completion, the basic catalyst dissolved in the reaction mixture is typically neutralized using a strong acid, resulting in the production of

salt and water. FAME (organic phase) and glycerol (aqueous) are immiscible, and therefore can be separated based on the difference in their densities (Santori et al., 2012).

After separation, the FAME is often washed with water, to remove any salt and any glycerol that may not have been removed in the initial separation. The wash water is then combined with the glycerol stream; often the glycerol stream will be partially refined through the removal of methanol and water (Leung et al., 2010).

2.1.2 Components of crude glycerol from biodiesel production

The composition of crude glycerol from biodiesel production depends on the type of catalyst used, the transesterification efficiency, the presence of water and other compounds in the feedstock, and the recovery of methanol and catalyst. Depending on these factors, crude glycerol can contain varying amounts of methanol, salt, soap, free fatty acids, FAME, and water (Yang et al., 2012).

Transesterification efficiency is dependent on a variety of factors, including the concentration and type of catalyst used, the oil to alcohol ratio, the temperature at which the reaction is conducted and the reaction time (Ma and Hanna, 1999). Most of the salt produced through the neutralization of alkaline catalyst will accumulate in the glycerol phase. In waste glycerol, the ratio of salt to glycerol will increase as conversion decreases because more glycerol remains trapped in triglyceride, diglyceride and monoglyceride molecules.

Components in oil can include free fatty acids, water, phospholipids, sterols, odorants and coloured bodies. The concentration of these components is highly dependent on the degree of refining the oil has undergone. Common refining practices include degumming, deacidifying, bleaching, and deodorizing. Degumming of oils is common, and involves the removal of phospholipids. Oil is acidic in nature due to the presence of free fatty acids; neutralization with alkali converts free fatty acids to soap, which can be easily separated from the oil. Oil can be bleached and deodorized to remove any coloured bodies and odoriferous substances respectively; this is often the case with food grade products (Santori et al., 2012).

Separation of glycerol and FAME can be achieved directly in the reactor, in a settling tank, or through centrifugation (in the case of a continuous process). The presence of mono-, di-, and tri- glycerides in the final product can result in the formation of an emulsion between organic and aqueous phases, complicating separation and resulting in a greater concentration of organic matter in the glycerol phase (Santori et al., 2012). The use of homogeneous alkaline catalysts results in glycerol wastes with salt concentrations that range from 5wt% to 7wt% (Dow Chemicals, 2013).

Waste glycerol can be purified to varying degrees. In the simplest form of purification, waste glycerol is neutralized using a strong acid, and stripped of methanol which can be recycled back into the process (once water content is reduced to 0.1wt%) (Santori et al., 2012). Methanol can be removed by vacuum flash processes, to yield a stream with a glycerol content of approximately 85wt% (Leung et al., 2010; Santori et al., 2012); if a greater degree of purity is desired, vacuum distillation can be used alongside ion exchange to ensure the removal of volatiles, water, methanol, and dissolved salt (Santori et al., 2012). For operations that use potassium hydroxide as a catalyst, phosphoric acid can be used in place of hydrochloric acid to achieve neutralization of the glycerol phase. This reaction produces potassium phosphate which can later be recovered and used as fertilizer (Leung et al., 2010; Santori et al., 2012). Phosphoric acid is, however, more costly than hydrochloric acid.

2.2 Microbial fuel cells

2.2.1 Overview

MFCs function in a similar manner to conventional fuel cells, converting the chemical potential energy of a fuel to electrical energy. The primary difference between these two technologies lies in the nature of the anodic catalyst which in the case of an MFC is biological. In an MFC, oxidation of fuel in the anode chamber is accomplished by anaerobic catabolism where in the absence of oxygen, bacteria use the anode as a terminal electron acceptor. Electrons released in the anode chamber flow through an external circuit to the cathode where an electron acceptor is reduced (Figure 2.3). In addition to producing a

current, the anaerobic catabolism of a fuel also yields various metabolites, which will vary depending on the type of fuel and catalyst used.

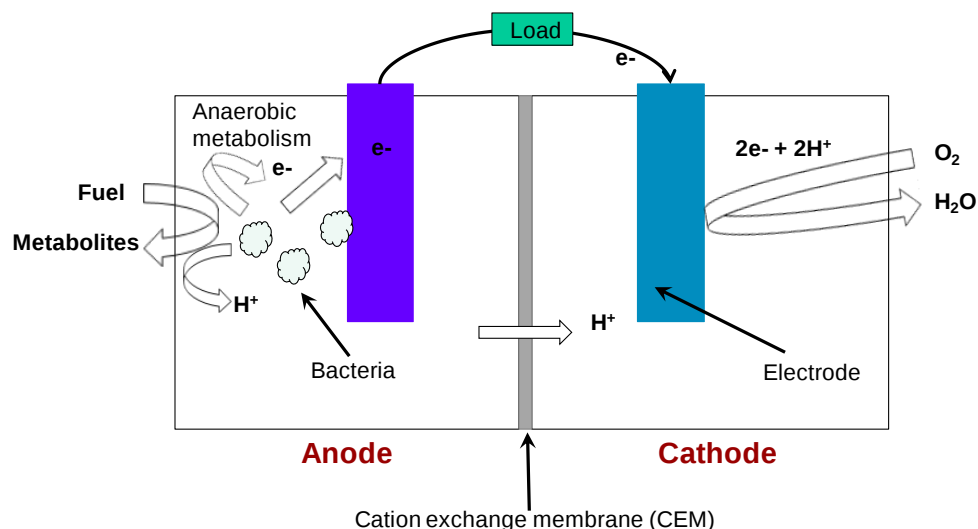


Figure 2.3: Current generation in a microbial fuel cell.

To generate current, bacteria used as anodic catalysts in MFCs must be capable of transporting electrons from their origin of generation to the anode. This can be accomplished by the direct transfer of electrons from a membrane bound cytochrome, or mediated through the use of redox active shuttles (Schröder, 2007). For direct electron transfer to occur, bacteria must be in direct contact with the anode. In some cases, bacteria have been shown to produce conductive appendages associated with membrane bound cytochromes called nanowires. These appendages allow bacteria at further distances from the anode to continue using it as a terminal electron acceptor (Logan, 2008; Logan et al., 2006; Schröder, 2007). Redox active shuttles, referred to as “mediators” in literature, achieve electron transfer by cycling from the bacteria where they are reduced, to the anode where they are oxidized (Schröder, 2007).

Bacteria classified as exoelectrogenic are capable of producing their own redox shuttles or participating in direct electron transfer. Bacteria not classified as exoelectrogenic can still be used as catalysts in MFCs but require the addition of electron mediating compounds to the anolyte in order to achieve electron transfer (Schröder, 2007).

MFCs have been developed to include a variety of architectures, most of which can be classified as either dual or single chamber.

Dual chamber MFCs typically feature liquid catholyte and anolyte solutions separated by a semi-permeable membrane or salt bridge (Logan, 2008). The membranes used in MFCs must facilitate the transport of charged species from the anode to the cathode and vice versa in order to maintain electroneutrality and pH. Maintaining electroneutrality is necessary to sustain the transport of electrons from the anode to the cathode. Maintaining a pH of 7 in the anode is necessary as bacterial metabolism and growth can be pH sensitive (Logan, 2008). Membranes also prevent the transport of oxygen from the cathode and fuel from the anode, both of which can reduce coulombic efficiency. Cation exchange membranes are often selected for use in MFCs as they help maintain the concentration of protons in the cathode by allowing the transfer of positively charged species (Logan, 2008). Dissolved oxygen is typically used as an electron acceptor in the cathode chamber as its standard potential is large and it can be obtained at minimal cost by sparging the catholyte with air. The use of ferricyanide as a catholyte has also been investigated and although it has been proven effective, it is not a sustainable option for industrial processes as it must be chemically regenerated after use (Du et al., 2007).

Single chamber fuel cells often consist of an anode chamber and an air cathode (Du et al., 2007). Incorporation of a membrane is not a necessity for single chamber cells as it is for dual chamber cells. Although membranes help control the diffusion of oxygen into the anode chamber, they also reduce the rate at which protons can be transported to the cathode therefore reducing the maximum power density that can be achieved (Liu and Logan, 2004). The greater concentration of oxygen observed in air than observed in water (0.0084mol/L and 0.0012mol/L at 30°C respectively (Haynes, 2013)) allows single chamber fuel cells to achieve power densities that are (on average) greater than their dual chamber counterparts, making them a more attractive option for future industrial applications.

The experimental results published in this thesis were generated using an H-type fuel cell. An H-type fuel cell is a dual chamber fuel cell that features a simple inexpensive design. The H-type fuel cell features two glass bottles (cathode and anode chambers) connected by a tube containing either a salt bridge or a membrane (Logan et al., 2006). The cathode and the

anode are suspended in the catholyte and anolyte respectively, and a complete circuit is formed by connecting the electrodes with a wire, placed outside the two chambers (Figure 2.4). The H-type fuel cell is well suited and widely used for basic parameter research such as examining power production with different bacterial strains, electrode materials or electrolyte compositions (Logan et al., 2006). The H-type fuel cell is also well suited for pure culture studies as it is autoclave compatible and is therefore easily sterilized. The high internal resistance observed by H-type fuel cells limits the maximum power density that can be achieved (Logan, 2008).



Figure 2.4: H-type fuel cell.

Materials selected for use as electrodes or as membranes can largely affect fuel cell performance. Materials selected for the anode should be conductive, non-corrosive, inexpensive and have a large specific surface area and porosity to maximize the area available for bacteria to colonize. The use of graphite rods, felts or brushes and other carbon based materials such as carbon cloth, paper, foam and reticulated vitreous carbon (RVC) is common. Carbon based materials are attractive as they possess good conductivity and have been proven well suited for bacterial growth (Logan, 2008). Materials selected for the cathode should be conductive as well as possess the ability to absorb oxygen in cases where it is used as an electron acceptor. Many of the carbon based materials used for the anode can be used for the cathode (Logan, 2008). To significantly improve the rate of oxygen reduction, carbon based cathodes can be loaded with a catalyst (Logan, 2008). Transition and

precious metals have both been proven to enhance the catalytic activity of the cathode, but at an added cost (Logan, 2008).

The membrane used to separate the cathode chamber from the anode chamber is an important component of dual chamber MFCs. Cation exchange membranes are the most popular choice for MFC applications although anion exchange membranes and bipolar membranes (cation exchange membrane bonded to an anion exchange membrane) have also been used (Kim et al., 2007).

Nafion 117TM fabricated by DuPont is the most used membrane in MFC research to date (Logan, 2008). NafionTM is a copolymer consisting of a polytetrafluoroethylene (PTFE) backbone and fluorocarbon side chains that terminate in sulfonic acid groups (-SO₃H). NafionTM was originally developed for use in conventional fuel cells, and functions as a cation exchange membrane; dissociation of the hydrogen ion from the sulfonic acid group leaves a negatively charged species (-SO₃⁻) capable of attracting positively charged species. As a result of differences in operating conditions, these membranes do not perform as well when used in MFCs. To support bacterial growth, the anolyte typically requires the addition of a variety of salts. Positively charged species such as Na⁺, K⁺, Ca²⁺ and Mg²⁺ are typically present in the anolyte at concentrations greater than 10⁵ times that of protons at neutral pH (Rozendal et al., 2006). As a result, charge neutrality is maintained through the transfer of metal ions from anode to cathode as opposed to protons. The rate of proton diffusion through the membrane of an MFC often limits power production (Liu and Logan, 2004).

At the present time, the rate of power generation experienced by MFCs is very low which in turn limits the number of applications for this technology (Franks and Nevin, 2010). MFCs are a suitable technology for powering small telemetry systems and wireless sensors that have minimal power requirements and are located in remote areas. MFCs are a suitable technology for the treatment of waste water and other organic wastes, as bacteria possess the ability to breakdown a variety of organic compounds (Franks and Nevin, 2010). MFCs can also be modified to generate hydrogen as opposed to electricity; however, the application of an external potential at the cathode is required to make this reaction thermodynamically favourable (Gadd, 2008). Benthic MFCs are being used to power meteorological buoys; these are the only MFCs that are currently being used commercially. These fuel cells take

advantage of the organic matter and bacteria naturally found in the sediment of the ocean floor; the anode remains buried while the cathode is exposed to dissolved oxygen in the water (Tender et al., 2002).

2.2.2 Performance measures

MFC performance can be evaluated by analysing a variety of parameters. The most used parameter in the evaluation of fuel cell performance is power density as the primary goal of an MFC is energy production. Parameters such as electrochemical efficiency, and coulombic efficiency and tools such as polarization curves can also provide a basis for comparison.

2.2.2.1 Potential

The potential achieved by a fuel cell (E) or battery is function of the external resistance (R_{ext}) of the circuit, and the current (I) (Equation (1))

$$E = IR_{\text{ext}} \quad (1)$$

The maximum potential (E_{MFC}) that can be achieved by any fuel cell or battery is equal to the difference in reduction potential observed at the cathode (E_{cat}) and the anode (E_{an}), (Equation (2)), when the cell is in open circuit (Logan, 2008).

$$E_{\text{MFC}} = E_{\text{cat}} - E_{\text{an}} \quad (2)$$

The maximum potential that can be achieved by any system is limited by thermodynamics. The standard cell reduction potential for any half-reaction can be calculated using Equation (3), where ΔG represents change in Gibbs free energy, n represents the number of electrons transferred with complete oxidation of the substrate, and F represents Faraday's constant (Logan, 2008).

$$E^0 = -\frac{\Delta G}{nF} \quad (3)$$

Through use of the Nernst equation (Equation (4)), where R represents the gas constant and T represents the temperature in Kelvin, the standard cell reduction potential can

be used to calculate the maximum reduction potential that can be achieved at the anode and at the cathode of an MFC under conditions that deviate from the standard. The activity of the products (reduced species) and reactants (oxidized species) affects the equilibrium reduction potential that can be achieved by a half-cell. The reaction quotient that appears in the Nernst equation uses the equilibrium concentration of products and reactants, both raised to their respective stoichiometric coefficients (Logan, 2008).

$$E = E^0 - \frac{RT}{nF} \ln \left[\frac{products^p}{reactants^r} \right] \quad (4)$$

The maximum potential that can be generated by an MFC can be difficult to predict. In an MFC, bacteria use the anode to dispose of reducing equivalents, which allows them to maintain redox balance. The concentration of reduced and oxidized species in the cell regulates the rate of electron transfer. Electron transfer is only thermodynamically favourable when the reduction potential of the oxidizing agent is greater than that of the reducing agent. The reduction potential of a given half reaction can change depending on the concentration of reactants and products, according to the Nernst equation (Equation (4)). For an MFC, when calculating the anode standard cell potential the biochemical processes that play a role in electron transfer would need to be addressed to yield an accurate prediction (Logan, 2008). This is obviously a very difficult task, and reduction potential at the anode is often calculated from the chemical equation that describes complete oxidation of the substrate.

The open circuit potential (OCP) represents the maximum potential observed experimentally, that can be achieved by the cell and is established at zero current and infinite resistance. If the potential achieved by the cell is limited by thermodynamics, the OCP should approach the theoretical value; this is often not the case due to various potential losses experienced by the cell (Note: $OCP = E_{MFC}$) (Logan, 2008). The nature of these losses will be covered in more detail in section 2.2.2.2.

Electrochemical efficiency is equal to the open circuit potential divided by the standard cell potential (Equation(5)). Although the standard cell potential is difficult to predict for MFCs, and therefore may not be highly accurate, this value can provide a basis to assess system performance (Logan, 2008).

$$\mu = \frac{OCP}{E^0} \quad (5)$$

2.2.2.2 Polarization curves

Polarization curves show the change in potential observed by a fuel cell as a function of current density (Figure 2.5). In MFCs, electrode overpotentials and ohmic losses are responsible for the decrease in potential observed with increasing current (Logan et al., 2006).

Ohmic losses are typically significant in MFCs and result from poor proton conduction and electron flow in the system. Resistance to proton flow can result from the presence of a membrane or the strength of the electrolyte solutions used in the cathode and anode chambers. Resistance to electron flow can result from the ineffective connection of electrodes to elements in the external circuit. The effect of ohmic losses on potential is most evident in the linear region of the polarization curve (Figure 2.5) (Logan et al., 2006).

Overpotential refers to the difference in reduction potential between the value calculated using thermodynamic principles (Equation (4)) and the value observed experimentally. Electrode overpotentials can be classified as activation losses, bacterial metabolism losses, and concentration losses (Logan et al., 2006).

Activation losses refer to the energy lost as heat from initiating oxidation and reduction reactions. These losses occur at the anode (transfer of electrons from a terminal protein), and at the cathode (transfer of electrons to an electron acceptor). The effect of activation losses on potential is most evident at low current densities (Figure 2.5) (Logan et al., 2006).

Bacterial metabolism losses result from the manner in which bacteria derive energy from substrate oxidation (Logan, 2008). Bacteria can derive energy from two metabolic processes: respiration and fermentation. Bacteria are able to generate more energy through respiratory metabolism than through fermentation. Respiratory metabolism involves the transfer of electrons released from substrate oxidation down a chain of progressively more electronegative protein complexes, where the final complex is known as the terminal electron

acceptor (Schröder, 2007). The greater the difference in reduction potential between the initial substrate and the terminal electron acceptor (the anode in the case of an MFC), the greater the energy gain for the bacteria and the smaller the amount of energy that can be harvested by the MFC (Logan, 2008).

Concentration losses result from a reduced rate of reaction at the anode and at the cathode due to insufficient flux of reactants and products. Insufficient flux of protons from the anode can result in regions of low pH which might affect metabolism and growth of bacteria. The effect of concentration losses are most evident at high current densities (Figure 2.5) (Logan, 2008).

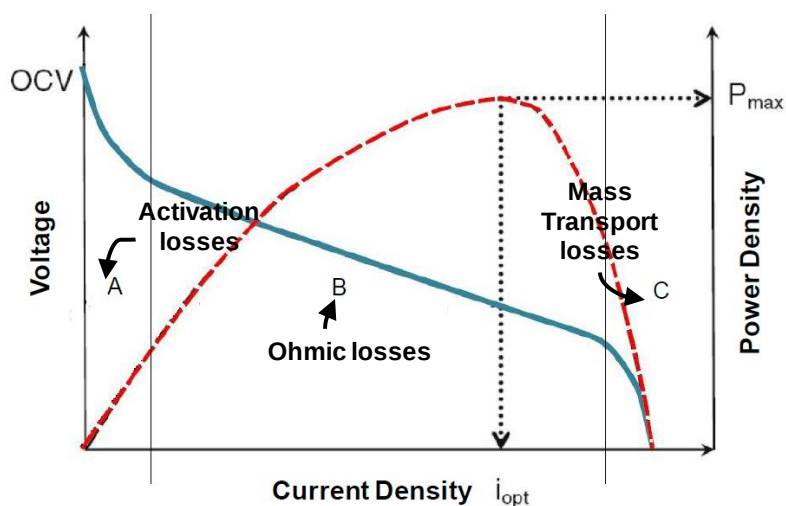


Figure 2.5: Polarization and power density curves (Modified from Logan, 2008)

The internal resistance of MFCs (R_{int}) can be easily calculated. The internal resistance of a system includes the contribution of ohmic losses and electrode overpotentials that vary with current, and is equal to the slope of the linear portion of a polarization curve (Figure 2.5) (Logan, 2008). At the moment, the contribution of ohmic losses and electrode overpotentials to the internal resistance of a cell cannot be separately quantified.

2.2.2.3 Power density

The amount of electrical energy produced by an MFC per second (power) can be calculated using Equation (6), where I represents current.

$$P = IE_{MFC} \quad (6)$$

MFCs come in a variety of sizes therefore power is often normalized to the surface area of the anode (A_{An}) to allow for easy comparison (Equation (7)). The surface area of the anode is used because it represents the area available for microbial growth which often affects the amount of power that can be generated. Alternatively, in cases where the surface area of the anode has little effect on the amount of power that can be generated by the cell (such might be the case when the cathode is much smaller than the anode), the surface area of the cathode or membrane (if present) may be used. Power can also be normalized to reactor volume instead of surface area; from an industrial stand point the size of a system is often correlated with cost (Logan, 2008).

$$P_{An} = \frac{IE}{A_{An}} \quad (7)$$

Power density curves show the change in power density as a function of current density; a power density curve can be derived from a polarization curve. The maximum power density that can be achieved by an MFC is equal to the value at the peak of its power density curve (Logan et al., 2006). Power density can be maximized by minimizing the rate at which potential decreases with increasing current.

2.2.2.4 Coulombic efficiency

Coulombic efficiency compares the number of electrons recovered as current (C_{rec}) to the number of electrons that would be recovered if all fuel oxidation produced current (C_{int}), as shown in Equation (8) (Logan et al., 2006).

$$C_E = \frac{C_{rec}}{C_{int}} \times 100 \quad (8)$$

The total coulombs recovered as current by the cell can be calculated by integrating current with respect to time. The total coulombs available in the substrate can be calculated using Equation (9), where Δc represents the change in concentration of the substrate over time, M_s represents the molecular weight of the fuel, v represents the volume of the anode

chamber, and b_e represents the moles of electrons available per mole of fuel when complete oxidation is achieved (Logan, 2008).

$$C_{int} = \frac{F b_e v \Delta c}{M_s} \quad (9)$$

Coulombic efficiency can be difficult to calculate for MFCs and can vary greatly; values between 2 and 50 % have been reported (Logan, 2008).

2.2.3 Glycerol oxidizing microbial fuel cells

A recent focus of MFC research is expanding the variety of feedstocks that can be oxidized in the anode chamber. Glycerol is a highly reduced compound and is available in large quantities (due to the growth experienced by the biodiesel industry), making it an attractive feed. Analytical grade glycerol and crude glycerol from biodiesel production have been used by several researchers as fuel in both single and dual chamber MFCs. Additionally, use of glycerol in enzymatic fuel cells and microbial electrolysis cells, both of which share similarities to MFCs, has been recorded.

Reiche (2012) and Nimje et al. (2011) both used analytical grade glycerol as fuel in an MFC. Using a two chamber MFC, Reiche (2012) found *P. freudenreichii* ssp. *shermanii*, *E. coli* W3110 and mixed culture AR2 (derived from compost) capable of producing current in the presence and absence of the external electron mediating compound resazurin. *P. freudenreichii* ssp. *shermanii*, *E. coli* W3110 and AR2 achieved power densities of 24.0mWm⁻², 5.5mWm⁻², and 24.5mWm⁻² respectively, in an electron mediated fuel cell and power densities of 14.9mWm⁻², 9.8mWm⁻², and 11.7mWm⁻² respectively, in a non-electron mediated fuel cell. Nimje et al. (2011) used analytical grade glycerol in a single chamber MFC employing *Bacillus subtilis* as anodic catalyst. Fed batch experiments were performed and after a lag period (required for the enrichment and development of an electrochemically active biofilm at the anode) the potential achieved by the cell increased after each feeding. The maximum OCV and power density achieved by the cell were 560mV and 600mWm⁻² respectively (Nimje et al., 2011).

Crude glycerol from biodiesel production contains a variety of components, the concentration and identity of which are dependent on the refining process. For the most part, the maximum potential achieved by a system using analytical grade glycerol was found to be greater than that achieved by the same system using crude glycerol. Crude glycerol has yet to be used as fuel in an MFC employing a pure culture catalyst.

Crude glycerol obtained from biodiesel production (61-63% glycerine), was used in a single chamber MFC with domestic wastewater inoculum (Feng et al., 2011). Maximum power densities of $487 \pm 5 \text{ mWm}^{-2}$ and $533 \pm 14 \text{ mWm}^{-2}$ were achieved with the use of crude and analytical glycerol respectively. The use of biodiesel as fuel in the same system resulted in a maximum power density that was 10 times less than that observed with analytical glycerol, implying that the bacteria present had difficulties metabolizing FAME (Feng et al., 2011). Two streams of crude glycerol, neutralized (56% methanol, 37% glycerol and 7% unknown compounds) and refined (80% glycerol, 8% methanol, and 12% unknown compounds), obtained from biodiesel production, were also used in a single chamber MFC with domestic wastewater inoculum (Sharma et al., 2011). Use of the neutralized stream resulted in a maximum power density of 1614 mWm^{-2} whereas use of the refined stream (neutralized then stripped of methanol) resulted in a maximum power density of 2340 mWm^{-2} . It was hypothesized that the reduced potential observed with the neutralized stream may be the result of catalyst deactivation at the cathode due to the high concentration of methanol. Less power was generated with the use of crude glycerol over analytical (4579 mWm^{-2}) (Sharma et al., 2011). Clauwaert et al. (2008) used crude glycerol from biodiesel production (80%v/v glycerol and 6.5%w/v Na_2SO_4) in an upflow MFC with a biocathode. No significant difference in performance was observed with the use of crude glycerol over analytical; maximum power densities of 82 Wm^{-3} and 93 Wm^{-3} were achieved, respectively. Both the anodic catalyst and the cathodic catalyst used in this study were not clearly identified (Clauwaert et al., 2008).

Biofuel cells rely on enzymes instead of whole cells to catalyze the oxidation reaction taking place at the anode. Pyrroloquinoline quinine-dependant alcohol dehydrogenase, pyrroloquinoline quinine-dependant aldehyde dehydrogenase, and oxalate oxidase were used

as anodic catalysts to achieve complete degradation of glycerol in a biofuel cell. A maximum power density of 13200mWm^{-2} was achieved (Arechederra and Minteer, 2009).

In a microbial electrolysis cell, hydrogen is produced instead of electricity. Oxygen is not provided at the cathode; instead, a potential is applied with a magnitude that is large enough to make the reduction of hydrogen ions (formed at the anode) thermodynamically favourable. Use of glycerol as fuel in microbial electrolysis cells has been investigated by several researchers. Important results of this work are summarized in Table 1.

Table 1: Use of glycerol as fuel in a microbial electrolysis cell.

Anodic catalyst	Fuel	Concentration	Applied potential (V)	Maximum H ₂ yield (mol/mol glycerol)	Reference
<i>Enterobacter aerogenes</i>	Crude glycerol	10gL^{-1}	0.395	0.74	(Sakai and Yagishita, 2007)
Anaerobic sludge	Analytical grade glycerol	$0.7\text{gL}^{-1}\text{d}^{-1}$	1	5.39 ± 0.90	(Escapa et al., 2009)
Domestic wastewater	Analytical grade glycerol	1gL^{-1}	0.9	3.9	(Selemba et al., 2009)
	Crude glycerol	1gL^{-1}	0.5	2.3	

2.3 Anaerobic metabolism of glycerol

Fermentation of glycerol can yield a variety of products, many of which have a market value greater than that of partially refined waste glycerol (Table 2). The production of 1,3-propanediol (1,3-PDO) via anaerobic fermentation is one of the most promising options for the biological conversion of waste glycerol (Yang et al., 2012). Fermentation of waste glycerol to 1,3-PDO has been studied in *Klebsiella pneumoniae* and *Clostridium butyricum*. 1,3-PDO has applications in the production of adhesives, polyesters, antifreeze, and UV-cured coatings (Pachauri and He, 2006; Yang et al., 2012). Propionic acid, the major product of fermentative metabolism by *Propionibacterium*, has applications as an antifungal agent in

foods, and can also be used in the production of perfumes, plastics and herbicides (Barbirato et al., 1997).

Table 2: Market value of partially refined glycerol waste from biodiesel production and select glycerol fermentation products

Compound	Price* (USD/lb)
1,3-PDO	0.8
Butanol	0.57-0.58
Glycerol	0.45
Ethanol	0.55
Propionic acid	0.46-0.49

*Prices obtained from ICIS, 2011

Biological conversion of waste glycerol presents a number of advantages such as greater product specificity, the use of mild processing conditions (temperature and pressure), and the ability to process wastes with high concentration of contaminants, over traditional chemical processes such as pyrolysis, steam gasification and catalytic treatments (Pathak et al., 2010; Yazdani and Gonzalez, 2007).

2.3.1 *Escherichia coli*

In the past, *E. coli* was shown incapable of anaerobically metabolizing glycerol in the absence of external electron acceptors (Lin, 1976; Quastel and Stephenson, 1925; Quastel et al., 1925). Gonzalez et al. (2008), were the first to show that *E. coli* was capable of anaerobically metabolizing glycerol in the absence of external electron acceptors; *E. coli* was shown to ferment glycerol in a in 1,2-propanediol (1,2-PDO) dependent manner. The production of 1,2-PDO allows *E. coli* to maintain redox balance during glycerol fermentation by consuming reduction equivalents generated through the production of cell mass (Gonzalez et al., 2008).

The pathway by which *E. coli* is believed to ferment glycerol is shown in Figure 2.7. Glycerol is oxidized to dihydroxyacetone (DHA) by glycerol dehydrogenase. DHA is then phosphorylated by dihydroxyacetone kinase (DHAK) to dihydroxyacetone phosphate

(DHAP). DHAK can be either phosphoenol pyruvate (PEP)-dependent or adenosine triphosphate (ATP)-dependent (Gadd, 2008; Gonzalez et al., 2008). DHAP can either enter glycolysis, or be metabolized to 1,2- PDO by an alternative pathway.

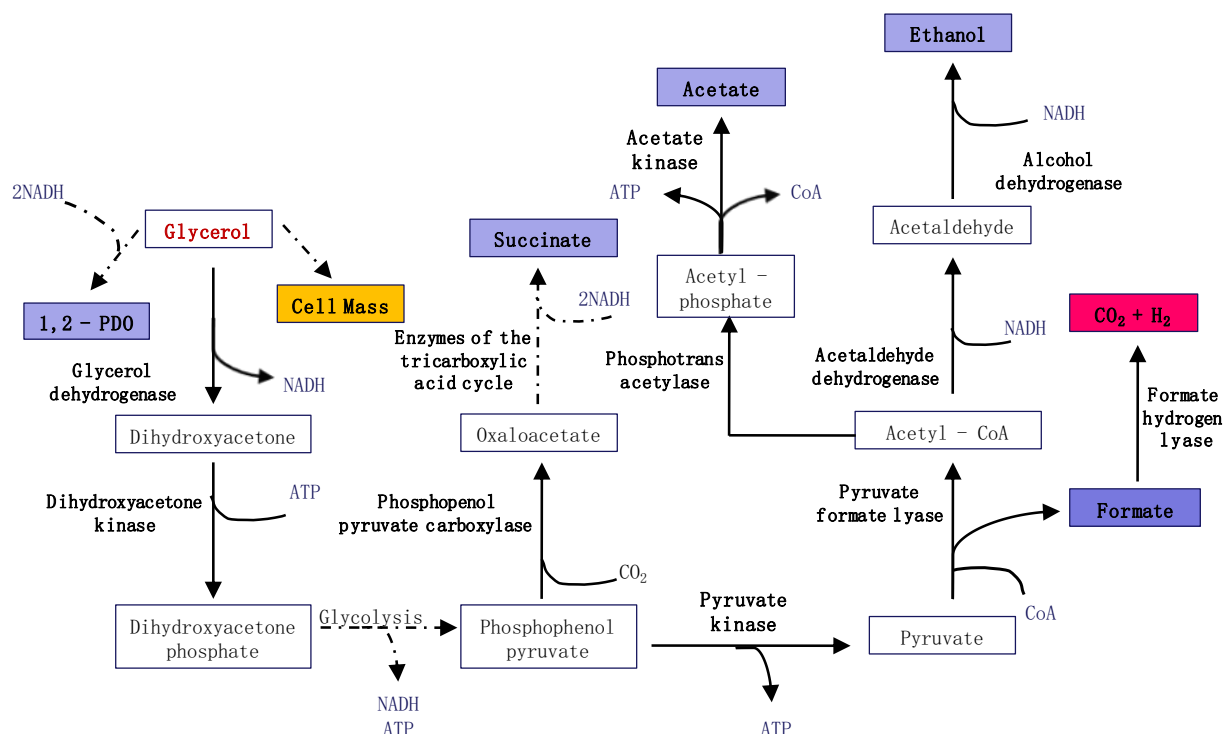


Figure 2.6: Pathways showing the anaerobic fermentation of glycerol by *E. coli*.

Via glycolysis, DHAP is metabolized to PEP by a chain of reactions catalyzed by triose-phosphate isomerase, 3-phosphoglycerate kinase, phosphoglycerate mutase, and finally enolase. For every molecule of DHAP that enters glycolysis, one molecule of NAD^+ is reduced to NADH and one molecule of adenosine diphosphate (ADP) is phosphorylated to ATP. PEP can then be reduced to succinic acid (succinate), or dephosphorylated to yield pyruvate, from which a variety of products including ethanol, acetic acid (acetate), formate, carbon dioxide (CO_2) and hydrogen (H_2) can be produced (Gadd, 2008).

In the pathway where DHAK is ATP-dependent, PEP is converted to pyruvate (PYR) by pyruvate kinase (Murarka et al., 2008). In the pathway where DHAK is PEP-dependent, conversion of PEP to PYR is coupled to the phosphorylation of DHA (Gonzalez et al., 2008).

Through the action of pyruvate formate lyase (PFL), pyruvate is metabolized to yield one molecule of formate and one molecule of acetyl-CoA. Formate can be oxidized to CO₂ through the action of formate hydrogen lyase, and acetyl-CoA can be metabolized to yield ethanol or acetic acid.

Acetyl-CoA is metabolized to acetic acid through the consecutive action of phosphotransacetylase and acetate kinase. The production of acetic acid from acetyl-CoA generates one molecule of ATP. Acetyl-CoA is reduced to ethanol through the consecutive action of acetaldehyde dehydrogenase and alcohol dehydrogenase. Production of ethanol from acetyl-CoA is coupled to the oxidation of NADH to NAD⁺.

The overall reaction for the production of ethanol or succinic acid from glycerol is redox-balanced. The production of ethanol is necessary for glycerol fermentation to proceed; production of ethanol is the only redox balanced pathway that also results in the generation of ATP via substrate level phosphorylation (Murarka et al., 2008). Due to the highly reduced nature of glycerol, ethanol is the primary product of glycerol fermentation (Dharmadi et al., 2006; Murarka et al., 2008) whereas lactic acid is the primary product of fermentation when glucose is used as the primary carbon source (Sawers and Clark). The amount of formate reduced to CO₂ was found to be dependent on the pH of the broth; greater conversion was realized at a slightly acidic pH of 6.5 (Dharmadi et al., 2006).

2.3.2 *P. freudenreichii* ssp. *shermanii*

The pathway by which *P. freudenreichii* is believed to ferment glycerol is shown in Figure 2.7. Glycerol is oxidized to DHAP, reducing one molecule of NAD⁺ to NADH and consuming the energy stored in the phosphate-phosphate bond of a single molecule of ATP (Gadd, 2008).

methylmalonyl-CoA. R-methylmalonyl-CoA then undergoes a rearrangement to yield S-methylmalonyl-CoA; this reaction uses vitamin B12 as a cofactor, which stabilizes the intermediate species. A carbonyl group is then transferred from S-methylmalonyl-CoA to pyruvate, to yield propionyl-CoA. This reaction is catalyzed by S-methylmalonyl-CoA pyruvate transcarboxylase, which uses biotin as a cofactor. Coenzyme A transferase catalyzes the final reaction, yielding propionic acid.

Propionic acid is the primary product of the fermentative metabolism of sugars by *P. freudenreichii*. Due to similar boiling points, separation of propionic acid from acetic acid is difficult. Due to the highly reduced nature of glycerol over conventional sugars, better selectivity towards propionic acid has been reported with *P. freudenreichii* ssp. *shermanii*, *P. acidipropionici* and *P. acnes* (Barbirato et al., 1997; Himmi et al., 2000; Wang and Yang, 2013).

2.3.3 Mixed culture AR2

1,3-PDO was observed in mixed culture fermentations by Reiche (2012), therefore, there are bacteria present in the mixed culture that ferment glycerol in a 1,3-PDO dependent manner. 1,3-PDO dependent fermentation is accomplished via parallel oxidative and reductive pathways. The reductive pathway involves the conversion of glycerol to 3-hydroxy propionaldehyde (3HPA), catalyzed by glycerol dehydratase, followed by the reduction of 3HPA to 1,3-PDO, catalyzed by 1,3-PDO dehydrogenase, and coupled to the oxidation of NADH to NAD⁺. In the oxidative pathway, glycerol is oxidized to DHA, catalyzed by glycerol dehydrogenase, and coupled to the reduction of NAD⁺ to NADH (Gonzalez et al., 2008). Finally, DHAK catalyses the phosphorylation of DHA, to DHAP, which can then enter glycolysis. Depending on the bacterial species, a variety of fermentation products including acetone, ethanol, formic acid, butanol, CO₂, H₂ and succinic acid, can be observed (Yazdani and Gonzalez, 2007).

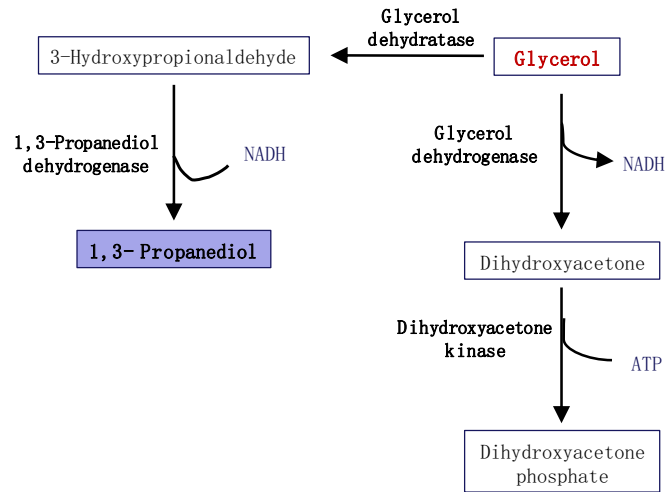


Figure 2.8: Oxidative and reductive pathways in the 1,3- PDO dependent fermentation of glycerol

Chapter 3. Materials and methods

3.1 Chemicals

Chemicals used and their suppliers are shown in Table 3.

Table 3: Purity of chemicals and their supplier

Chemical	Purity	Supplier
1,2- Propanediol	99.5%	Sigma Aldrich
1,3-Propanediol	98%	Sigma Aldrich
Acetic acid	99%	Fisher Scientific
Ethanol	98%	Fisher Scientific
Formic acid	>85%	Fisher Scientific
Glycerol	99.8%	Fisher Scientific
Hydrochloric acid	35-38%	Fisher Scientific
Methanol	99.8%	Fisher Scientific
Potassium hydroxide	>85%	Sigma Aldrich
Propionic acid	99%	Fisher Scientific
Sodium methoxide	25% in methanol	Sigma Aldrich
Sodium thioglycolate	>96.5%	Sigma Aldrich
Soybean oil	-	-
Succinic acid	99.5%	Fisher scientific
Sulfuric acid	1 N	Fisher scientific

3.2 Growth medium

Three varieties of growth medium (Reinforced Clostridial Medium (RCM), Luria-Bertani (LB) medium and M9 medium) were used. Before use, medium was sterilized via autoclave for 20 min, at 121°C and 21psi.

RCM (Oxoid) and LB medium (Fisher Scientific) were prepared using deionized water according to manufacturer's instructions. The formulation of basic M9 medium, shown in Table 4, was outlined by Sambrook et al. (1989). Depending on the culture used, basic M9 medium was enriched with glycerol and various nutritive supplements including Bacto tryptone (BD) and Bacto yeast extract (BD). M9 medium was prepared by boiling water, adding salts (listed in Table 4), adding glycerol and nutritive supplements, and sparging with nitrogen while the solution cooled to help maintain low oxygen content. Prepared M9 medium was stored in 125mL crimp cap serum bottles in 50mL and 100mL aliquots with nitrogen in the headspace. Sodium thioglycolate, an oxygen scavenger, was added to a concentration of 0.5gL^{-1} to medium prepared for use in fermentation experiments to help maintain anaerobic conditions.

Table 4: Amount of each compound required to yield 1L of basic M9 medium

Compound	Concentration in 1L of medium
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	12.8g
KH_2PO_4	3g
NaCl	0.5g
NH_4Cl	1g
1 M MgSO_4	100 μL
1 M CaCl_2	1mL
Deionized water	to 1L

Plates were prepared by mixing growth medium (either LB medium or RCM) with 15gL^{-1} Bacto agar (BD). Once prepared, plates were stored at 4°C .

3.3 Cultures

Bacterial strains *P. freudenreichii* ssp. *shermanii* (ATCC 9614), and *E. coli* W3110 (ATCC 27325), were purchased from the American Type Culture Collection. Strains were stored at -80°C ; *E. coli* W3110 was stored in LB medium, supplemented to a final glycerol concentration of 150gL^{-1} , and *P. freudenreichii* ssp. *shermanii* was stored in RCM,

supplemented to a final glycerol concentration of 150gL^{-1} . Monthly, *P. freudenreichii* ssp. *shermanii* and *E. coli* W3110 were streaked on RCM and LB plates, respectively. Both strains were grown anaerobically in an anaerobic jar containing a CO_2 generating pouch and an anaerobic indicator (all purchased from Oxoid). *P. freudenreichii* ssp. *shermanii* was grown at 30°C , and *E. coli* W3110 was grown at 37°C . Plates were stored at 4°C until use.

Mixed culture AR2 was cultivated from month old compost (collected from the University of Ottawa's mechanical compositing system), in a manner that supported the growth of bacteria with the ability to anaerobically metabolize glycerol (Reiche, 2011). Mixed culture AR2 was maintained by monthly transfer into 50mL of fresh M9 minimal medium, enriched with 20gL^{-1} glycerol and supplemented with 0.5gL^{-1} sodium thioglycolate; this culture was used as stock for experiments. Mixed culture AR2 was grown at room temperature.

3.4 Microbial fuel cell design

A schematic and photograph of the fuel cells used in this study are shown in Figure 3.1. MFCs were fabricated from two 250mL Pyrex bottles. Each bottle was fused with a straight tube adaptor ending in an O-ring joint, and was sealed with a plug seal cap (Fisher Scientific). Side ports sealed with butyl rubber crimp caps permitted the sampling of both cathode and anode. A NafionTM 212 membrane (9.6cm^2), held in place by two rubber O-rings and a clamp, was used to separate the anode and cathode chambers. Both anode (12.5cm^2) and cathode (50cm^2) were made of untreated carbon cloth (Fuel Cell Store). A titanium wire with a diameter of 0.5mm (VWR International) was used to connect the anode and cathode to a $1\text{k}\Omega$ resistor. Connections were secured with alligator clips. The anode chamber was completely sealed from the atmosphere to maintain anaerobic conditions while the cathode chamber was continuously sparged with air, filtered through a $45\mu\text{m}$ syringe filter (Fisher Scientific) to help maintain sterile conditions.

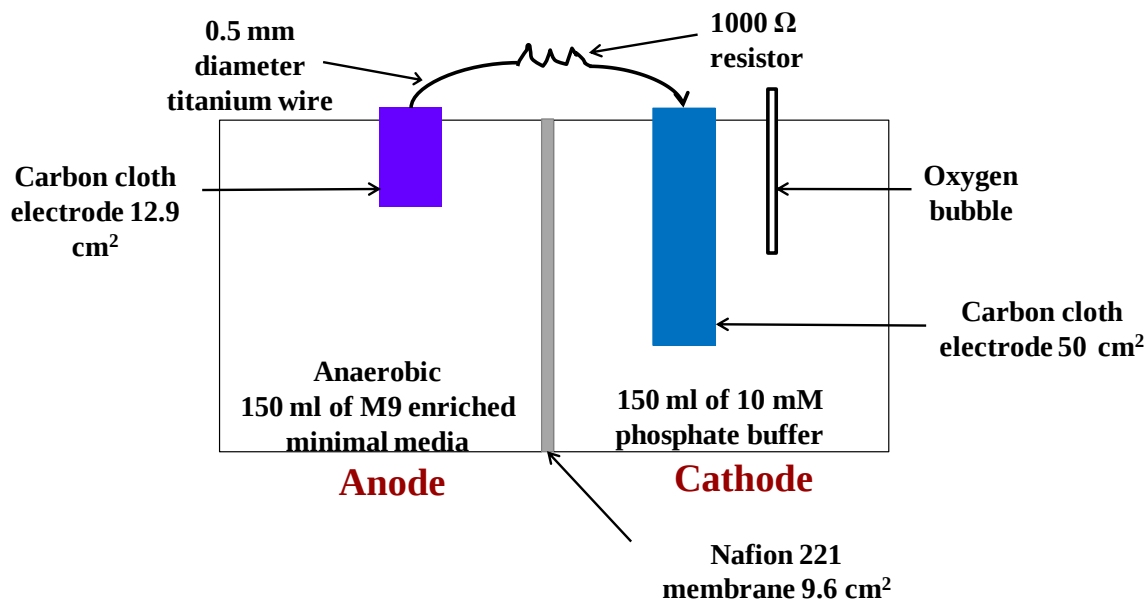


Figure 3.1: (Top) Schematic and (Bottom) photograph showing microbial fuel cell design

The fuel cells used in this study were designed by Reiche (2012). The fuel cells featured a simple inexpensive design, which allowed for the simultaneous operation of multiple units, and could be sterilized via autoclave, which was necessary for pure culture work. Although there were many benefits associated with the system used, the maximum power density that can be achieved with H-type fuel cells is limited as high values of internal resistance are typically observed.

3.5 MFC operation

The cathode chamber was filled with 150mL of 10mM phosphate buffer, and the anode was filled with 150mL of enriched M9 medium. Before use, fuel cells, phosphate buffer and enriched M9 medium were autoclaved for 20 min at 121°C and 21psi. After sterilization of the medium and the phosphate buffer had been achieved, they were added to the empty fuel cell, while still warm, to help prevent contamination. To the anode, sterile sodium thioglycolate was added to a final concentration of 0.5gL^{-1} to help maintain an anaerobic environment.

The 10mM phosphate buffer used in the anode was prepared by adding a 1M KH_2PO_4 solution to 1M K_2HPO_4 solution until a pH of 7.2 was achieved, then diluting this mixture 100x with deionized water.

NafionTM membranes were regenerated before use by boiling sequentially in 3% hydrogen peroxide, deionized water, 1N sulfuric acid, and again in deionized water. Each step took an hour to complete, making the entire procedure last a total of 4h. NafionTM membranes were stored in deionised water until use.

3.6 MFC and fermentation experiments

Fermentation and MFC experiments were conducted in parallel. Pre-culture procedures and the frequency of sampling differed for experiments conducted with pure cultures versus mixed cultures.

3.6.1 Pure cultures

From plates, a single colony was picked and grown overnight in a hungate tube. *P. freudenreichii* ssp. *shermanii* was grown in RCM and *E. coli* W3110 was grown in LB. In this step and all subsequent steps, *P. freudenreichii* ssp. *shermanii* and *E. coli* W3110 were grown at 30°C, and 37°C, respectively. In the morning, 0.5mL of culture was extracted from the hungate tube, and used to inoculate 125mL serum bottles containing 50mL of enriched M9 medium with the same composition as the experimental control of the anticipated study. Once active growth was observed, fermentations and MFCs were inoculated to a final

protein concentration of 0.1mgL^{-1} . Fermentations were performed in triplicate for each condition tested.

Fermentations and MFCs were sampled once daily, using sterile 23 gauge needles and sterile 1mL syringes. MFCs were sampled so that the concentration of glycerol and fermentation products in the anode could be monitored. Fermentations were sampled so that growth and the concentration of glycerol and fermentation products in the anode could be monitored. To assess growth, the optical density of each sample, at a wavelength of 600nm (OD_{600}), was measured using a spectrophotometer. Samples for HPLC analysis were centrifuged at 13,400rcf for 2 min, and supernatants stored at -20°C until use. In experiments where crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH_3 were used, supernatants were filtered with a $0.45\mu\text{m}$ syringe filter, before being stored at -20°C ; filtration was necessary for the removal of bacteria suspended in the organic phase.

During the day, the working potential of MFCs was measured every 1h to 4h across a $1\text{k}\Omega$ resistor. Polarization curves were generated once the potential observed reached a stable maximum.

3.6.2 Mixed cultures

Stock culture (0.5mL) was used to inoculate a 125mL serum bottle containing 50mL of fresh M9 medium enriched with 20gL^{-1} analytical glycerol and supplemented with 0.5gL^{-1} sodium thioglycolate. Once active growth was observed, fermentations and MFCs were inoculated to a final protein concentration of 0.1mgL^{-1} . Due to the fact that the ratio of the different bacterial species in the mixed culture can change with time, it was not deemed worthwhile to relate the OD of the culture to protein concentration. Instead, an average OD_{600} to protein ratio, based on the values observed for each of the strains tested by Reiche (2012), was calculated and used to estimate the volume of inoculum that would be required, based on the OD_{600} of the AR2 pre-culture. AR2 was grown at 30°C . Fermentations were performed in triplicate for each condition tested.

With mixed culture experiments, fermentations and MFCs were sampled in the same manner and for the same reasons as pure culture experiments. Fermentations and MFCs were

sampled two to three times daily because mixed culture AR2 consumed glycerol at a much greater rate than pure cultures.

During the day, the working potential of MFCs was measured every 1h to 4h across a $1k\Omega$ resistor. Polarization curves were generated once the potential observed reached a stable maximum.

3.7 Crude glycerol from biodiesel

3.7.1 Crude glycerol produced with minimal sodium methoxide catalyst

Glycerol waste, resulting from the use of 0.23wt% NaOCH_3 catalyst and a 5:1 molar ratio of alcohol (methanol) to oil (degummed, unbleached soybean oil) in the first stage of a two-stage continuous process, was obtained from Dr. André Tremblay (Department of Chemical and Biological Engineering, University of Ottawa). The waste glycerol was neutralized to a pH of 7 using concentrated HCl (pH of waste glycerol was monitored during HCl addition with pH strips (Fisher Scientific)). Once neutralized, methanol was stripped from the glycerol waste using a rotary evaporator. After the evaporation of methanol from the waste glycerol was no longer evident, the final product was sampled, and the concentration of methanol determined via HPLC analysis to ensure that the majority had, in fact, been removed.

The concentration of salt in the final product was calculated based on the amount of HCl added to neutralize the catalyst and the amount of glycerol recovered. Detailed calculations can be found in Appendix A.7.

3.7.2 Crude glycerol produced with 0.8wt% sodium methoxide and potassium hydroxide catalysts

3.7.2.1 System

Biodiesel was produced in batch using a 500mL three-neck round bottom flask reactor. The temperature (measured using a thermocouple) was controlled by a heated water bath, and the reactor contents were continuously mixed using a magnetic stir rod. The reactor

was equipped with a reflux condenser to cool methanol vapours. The reactor was sealed from the atmosphere with rubber stoppers to prevent the loss of methanol and the interaction of reactor contents with moisture in the atmosphere.

3.7.2.2 Sodium methoxide catalyst

A 5:1 molar ratio of alcohol (methanol) to oil (degummed, unbleached soybean oil) was selected (to maintain consistency in the production process). An amount of sodium methoxide equal to 0.8% of the mass of oil used, catalyzed the transesterification reaction.

A stock solution of sodium methoxide was produced by diluting 56g of a 25wt% sodium methoxide in methanol solution with 276g of methanol. Oil and sodium methoxide solution were measured so that the volume of the reaction mixture totalled 250mL.

Oil was the first component added to the reactor. The oil was heated, and once a temperature of 50°C was achieved, the catalyst-methanol solution was added to the reactor. The reaction mixture was then heated to a final temperature of 63°C, and maintained at this temperature for 60min. After 60min, the reaction mixture was allowed to cool, after which the glycerol phase was separated from the organic phase via separatory funnel. Once separated, the glycerol phase was neutralized and stripped of methanol by following the same procedures used with the crude glycerol produced using minimal sodium methoxide catalyst.

The concentration of salt in the glycerol waste was calculated based on the assumption that a conversion of 98% was achieved. This assumption was based on the conversions observed in literature studies where similar catalyst concentrations, reaction times, reaction temperatures, and oil to alcohol ratios were employed (Santori et al., 2012).

3.7.2.3 Potassium hydroxide catalyst

A 5:1 molar ratio of alcohol (methanol) to oil (degummed, unbleached soybean oil) was selected (to maintain consistency in the production process). An amount of potassium hydroxide equal to 0.8 % of the mass of oil (degummed, unbleached soybean oil) used, catalyzed the transesterification reaction.

A stock solution of methanol and potassium hydroxide was produced by diluting 10.6g of solid KOH in 206g of methanol. Oil and potassium hydroxide solution were measured so that the volume of the reaction mixture totalled 250mL.

The procedures followed regarding the production, separation and the refining of waste glycerol were identical to the procedure followed with the use of 0.8wt% sodium methoxide catalyst.

3.8 Analytical methods

Bacterial growth was monitored by measuring the OD₆₀₀ of samples using a spectrophotometer (Thermo Electron Corporation). For pure cultures, protein concentration was calculated from OD₆₀₀ using the correlations developed by Reiche (2012), as shown in Appendix A.

The consumption of glycerol and subsequent production of metabolites was monitored using an Agilent 1200 series High Performance Liquid Chromatograph (HPLC) equipped with an Aminex HPX-87H column and a Refractive Index Detector (RID). A 5mM sulfuric acid solution was used as the eluant, at a flow rate of 0.6mLmin⁻¹; the temperature of the column was maintained at 50°C. The mobile phase was prepared by diluting a 1N sulfuric acid solution 100x with deionized water, then filtering through a 0.45µm pore size MF-Millipore mixed cellulose ester membrane filter. Compounds that were detected and their approximate retention times are shown in

Table 5. Standard curves were created that relate peak area to concentration for each compound (see Appendix B).

Table 5: Retention time of glycerol and various fermentation products

Compound	Retention time (min)
1,2 - Propanediol	17.15
1,3-Propanediol	17.48
Acetic acid	15.43
Ethanol	21.96
Formic acid	14.14
Glycerol	13.48
Propionic acid	18.17
Succinic acid	11.93

3.8.1 Electrochemical analysis

Polarization curves were generated for MFCs using a VersaSTAT 3 potentiostat (Princeton Applied Research) once a stable maximum potential had been achieved. Fuel cells were left in open circuit for 30 min to allow the OCV to stabilize; polarization curves were generated using a scan rate of 1mVs^{-1} .

Chapter 4. Waste glycerol from biodiesel produced using minimal catalyst, supplemented with sodium chloride and potassium chloride

4.1 Introduction

The production of biodiesel on an industrial scale often relies on alkaline catalysts to improve the rate of the transesterification reaction. The subsequent neutralization of any alkaline catalyst with a strong acid produces salts which remain dissolved in the glycerol phase of the product stream (due to its polar nature). Sodium methoxide and potassium hydroxide are common catalysts in commercial applications; their neutralization with hydrochloric acid produces sodium chloride and potassium chloride, respectively.

The primary objective of the work in this chapter was to determine the effect of sodium chloride and potassium chloride, at concentrations typically observed in crude glycerol from biodiesel production, on the performance of *E. coli* W3110, *P. freudenreichii* ssp. *shermanii*, and mixed culture AR2 as anodic catalysts in an MFC.

Crude glycerol, obtained from biodiesel produced using 0.23wt% sodium methoxide catalyst (based on the mass of oil used in the transesterification reaction) was supplemented with NaCl and KCl to mimic the salt content of crude glycerol that would have resulted if sodium methoxide or potassium hydroxide were used at concentrations of 0.6wt%, 0.8wt%, 1.0wt% and 1.2wt%. In each study, analytical grade glycerol, and in some cases, analytical grade glycerol supplemented with NaCl or KCl, were used as controls.

Maximum power density was the major parameter by which the performance of each strain was evaluated. In addition to calculating maximum power density, change in working potential, electrochemical efficiency, change in glycerol concentration, and identification of the nature and magnitude of energy losses, provided additional information with which to better understand the phenomena occurring within the MFCs.

4.2 *E. coli* W3110

In the following two studies, the effect of salt concentration on the performance of *E. coli* 3110 as an anodic catalyst in a glycerol oxidizing MFC was investigated. In the first

study, M9 medium was supplemented with sodium chloride to concentrations of 0.42gL^{-1} , 0.52gL^{-1} and 0.62gL^{-1} , which correspond to the salt concentrations that would result if 0.8wt%, 1.0wt% and 1.2wt% sodium methoxide were used, respectively, in biodiesel production. In the second study, M9 medium was supplemented with potassium chloride to concentrations of 0.39gL^{-1} , 0.52gL^{-1} , 0.64gL^{-1} and 0.77gL^{-1} , which correspond to the salt concentrations that would result if 0.6wt%, 0.8wt%, 1.0wt% and 1.2wt% potassium hydroxide, were used, respectively. In both studies, M9 medium was supplemented with 5gL^{-1} glycerol, 10gL^{-1} tryptone and 0.5gL^{-1} sodium thioglycolate.

4.2.1 Supplemented with sodium chloride

Polarization and power density curves generated for MFCs with NaCl are shown in Figure 4.1. The maximum power density achieved by all MFCs in this study ranged from 21.8mWm^{-2} ; observed with the use of crude glycerol supplemented to a final NaCl concentration of 0.52gL^{-1} , to 9.5mWm^{-2} ; observed with the use of analytical glycerol. The maximum power density observed with the use of analytical glycerol was found to be similar to that observed by Reiche (2012) (9.9mWm^{-2}). Similar power densities, 17.2mWm^{-2} and 17.8mWm^{-2} , were observed with the use of analytical glycerol supplemented to a final NaCl concentration of 0.52gL^{-1} and with the use of crude glycerol supplemented to a final NaCl concentration of 0.42gL^{-1} , respectively. In summary, the maximum power density observed for all MFCs supplemented with NaCl was found to be greater than that observed with the use of analytical glycerol.

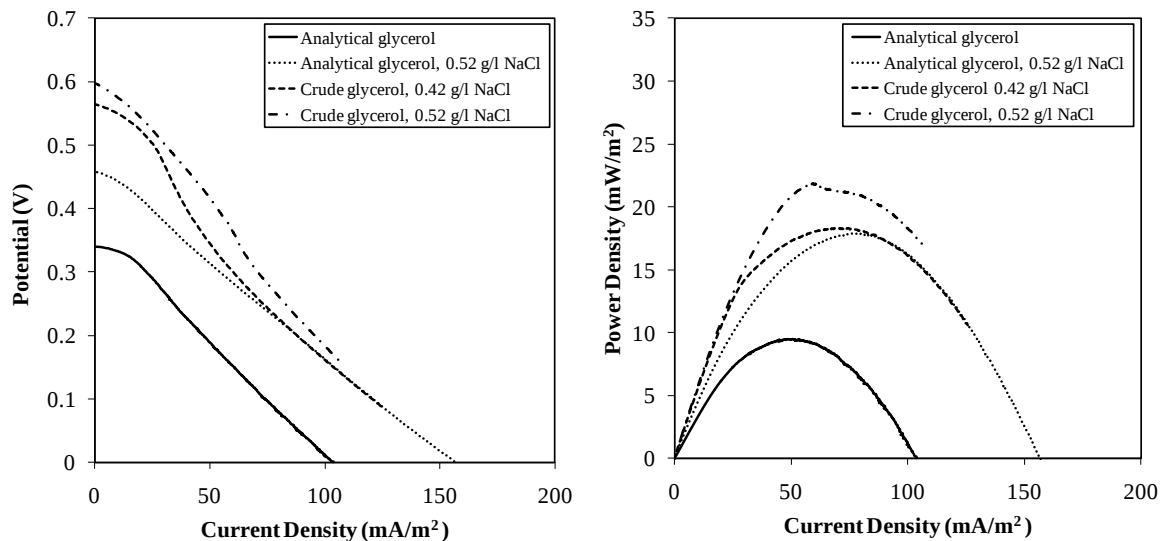


Figure 4.1: (Left) Polarization and (Right) power density curves observed with the use of *E. coli* W3110 as anodic catalyst grown on crude glycerol supplemented with NaCl. *Current density and power density were normalized to the anode surface area. Curves were generated for each condition tested after the observation of a stable potential; 105h: crude glycerol + 0.52gL⁻¹NaCl, analytical glycerol +0.52gL⁻¹NaCl; 121h: crude glycerol + 0.42gL⁻¹ NaCl, analytical glycerol.*

From the polarization curves shown in Figure 4.1, at low current densities, the potential observed decreased very little with each increase in current density. Current density eventually approached a value where resistive losses started to dominate, and the slope of the curve began to transition; a linear relationship was then observed between potential and current density. The only marginal decrease in potential observed at low current densities indicates that activation losses did not play a significant a role in the depletion of potential with increasing current density for *E. coli* W3110. In a system where ohmic losses are less significant, the use of a catalyst that can achieve efficient transfer of electrons from a mediating compound or terminal cytochrome to the anode will result in greater power generation than the use of a similar catalyst where the activation energy required for electron transfer to the anode is greater.

The internal resistance and electrochemical efficiency observed for MFCs in this study are shown in Table 6. The internal resistance was similar for all conditions tested. The

electrochemical efficiency observed when analytical glycerol was used as a carbon source was less than that observed when crude glycerol was used as a carbon source; additionally, electrochemical efficiency was enhanced through media supplementation with NaCl. The electrochemical efficiency observed with the use of analytical glycerol was similar to that observed by Reiche (2012) (0.31) under the same conditions.

Table 6: Internal resistance and electrochemical efficiency observed with the use of *E. coli* W3110 grown on crude glycerol supplemented with NaCl.

Quality of glycerol used, salt concentration	Internal resistance (Ω)	Electrochemical efficiency
Analytical	2586	0.28
Analytical, 0.52g L ⁻¹ NaCl	2328	0.37
Crude, 0.42g L ⁻¹ NaCl	2327	0.46
Crude, 0.52g L ⁻¹ NaCl	2892	0.48

Working potential and glycerol concentration in the anode chamber, at various points throughout the course of the study, are shown in Figure 4.2. The potential observed with the use of analytical glycerol supplemented to a final NaCl concentration of 0.52g L⁻¹ rose initially, stabilized at the maximum value, then decreased. The potential observed with all other fuel cells in this study rose initially then remained stable at the maximum value for the remainder of the study.

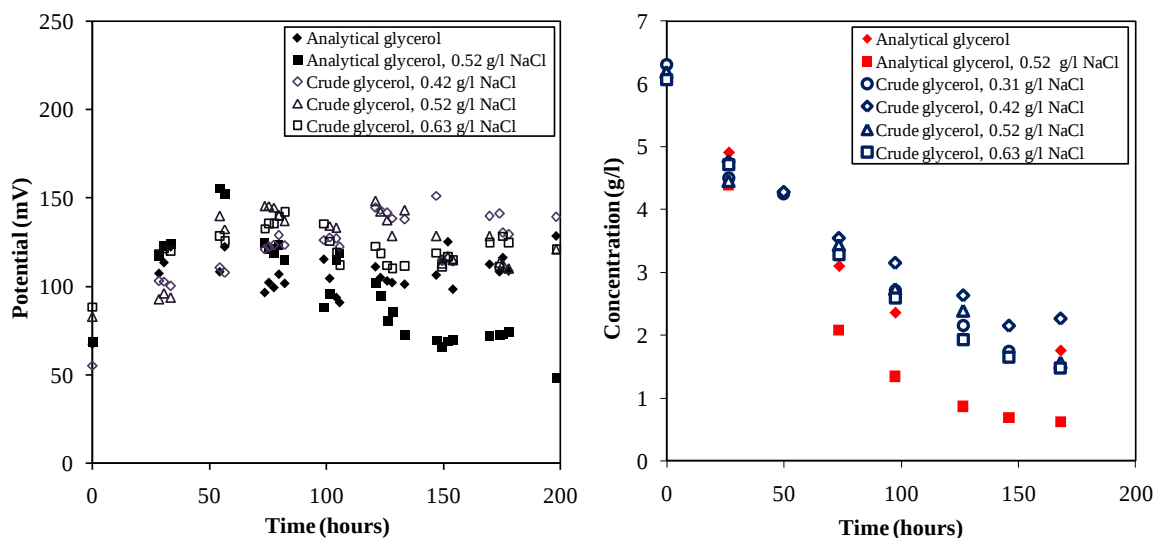


Figure 4.2: (Left) Potential achieved and (Right) glycerol concentration observed with the use of *E. coli* W3110 as anodic catalyst grown on crude glycerol supplemented with NaCl.

Potential was measured across a 1k Ω resistor.

Although a polarization curve was not generated for the fuel cell that used crude glycerol supplemented to a final NaCl concentration of 0.63gL⁻¹, working potential was still measured across a 1k Ω resistor at various points throughout the study. The maximum working potential observed with the use of crude glycerol supplemented to a final NaCl concentration of 0.63gL⁻¹ (142mV) was similar to that observed with the use of crude glycerol supplemented to a final NaCl concentration of 0.52gL⁻¹ (145mV) and to that observed with the use of crude glycerol supplemented to a final NaCl concentration of 0.42gL⁻¹ (151mV). Following the same trend as maximum power density, the maximum working potential achieved by all fuel cells that used anodic medium supplemented with NaCl were similar in magnitude.

For all fuel cells, the concentration of glycerol in the anode decreased throughout the duration of the study (see Figure 4.2). The rate and extent of glycerol consumption was similar for most of the fuel cells however, the extent of glycerol consumption observed with the use of analytical glycerol supplemented to a final NaCl concentration of 0.52gL⁻¹ was greater than that observed with all other fuel cells.

Seventy five hours after inoculation, the concentration of glycerol that remained in the anode of the fuel cell supplemented with analytical glycerol and NaCl to a concentration of 0.52gL^{-1} was approximately 0.5gL^{-1} ; additionally, the rate of glycerol consumption had decreased significantly. At this point in the study, the working potential observed began to decrease. Depletion of nutrients essential for glycerol metabolism could have caused a decrease in the rate of glycerol consumption and hence working potential however, a decrease in working potential was not obvious with any of the other fuel cells even though a significant decrease in the rate of glycerol consumption was observed.

4.2.2 Supplemented with potassium chloride

Polarization and power density curves generated for MFCs in this study are shown in Figure 4.3. The maximum power density achieved by all MFCs in this study ranged from 41.4mWm^{-2} ; observed with the use of crude glycerol supplemented to a final KCl concentration of 0.39gL^{-1} , to 17.3mWm^{-2} ; observed with the use of analytical glycerol. The maximum power density observed with the use of crude glycerol supplemented to a final KCl concentration of 0.52gL^{-1} and with the use of crude glycerol supplemented to a final KCl concentration of 0.77gL^{-1} was 18.4mWm^{-2} and 28.6mWm^{-2} , respectively. Similar to the previous study using NaCl, the potential observed with the use of medium supplemented with KCl was greater than that observed with the use of analytical glycerol, however; the maximum power densities observed in this study were generally greater than the maximum power densities observed in the previous study. The shapes of the polarization curves generated in this study were similar to the shape of polarization curves generated in the previous study (Figure 4.1).

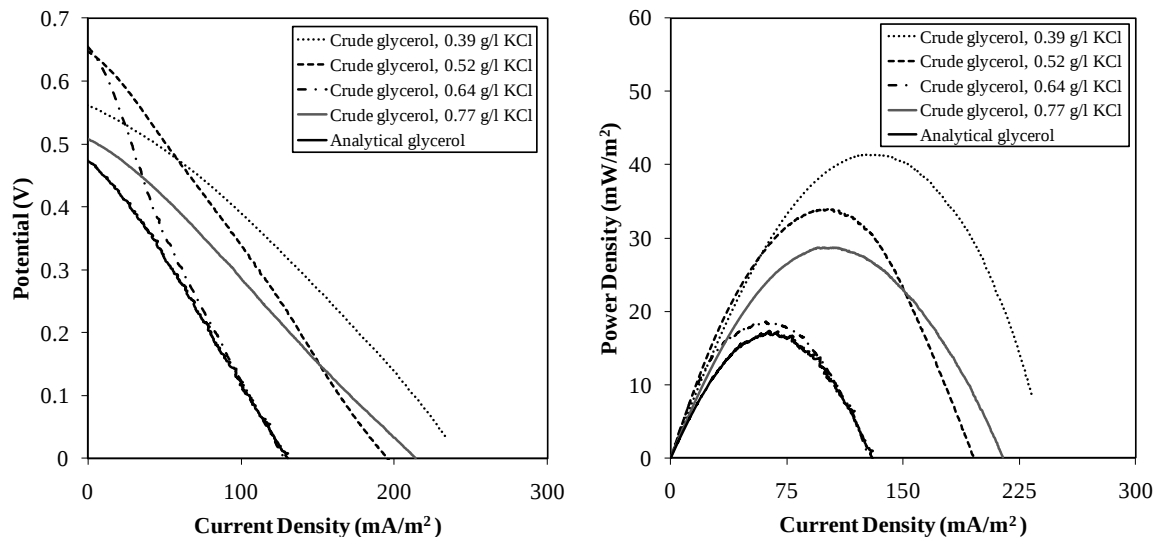


Figure 4.3: (Left) Polarization and (Right) Power density curves observed with the use of *E. coli* W3110 as anodic catalyst grown on crude glycerol supplemented with KCl. *Current density and power density were normalized to the anode surface area. Curves were generated for each condition tested after the observation of a stable potential; 95h: crude glycerol + 0.64gL⁻¹KCl; 140h: crude glycerol + 0.39gL⁻¹KCl, crude glycerol + 0.77gL⁻¹KCl, crude glycerol + 0.52gL⁻¹KCl, analytical glycerol.*

The internal resistance and electrochemical efficiency observed for MFCs in this study are shown in Table 7. Although the average maximum power density achieved by fuel cells in this study was greater than the average maximum power density achieved by fuel cells in the NaCl study, the range of internal resistances observed with fuel cells in this study overlapped with the range observed in the previous study (2327Ω to 2892Ω). Similar to the maximum power density achieved by fuel cells in this study, the electrochemical efficiency observed was greater than that observed in the previous study.

Table 7: Internal resistance and electrochemical efficiency observed with the use of *E. coli* W3110 as anodic catalyst grown on crude glycerol supplemented with KCl.

Quality of glycerol used, salt concentration	Internal resistance (Ω)	Electrochemical efficiency
Analytical glycerol	2923	0.39
Crude, 0.39gL ⁻¹ KCl	1869	0.46
Crude, 0.52gL ⁻¹ KCl	2205	0.53
Crude, 0.64gL ⁻¹ KCl	3356	0.52
Crude, 0.77gL ⁻¹ KCl	2095	0.41

Working potential and glycerol concentration in the anode at various points throughout the course of the study are shown in Figure 4.4. The trend observed with regard to the change in working potential over the course of this study differed with each MFC. The working potential observed with the use of crude glycerol supplemented to a final KCl concentration of 0.52gL⁻¹, crude glycerol supplemented to a final KCl concentration of 0.77gL⁻¹, and crude glycerol supplemented to a final KCl concentration of 0.39gL⁻¹, rose initially to a maximum value, then began to decrease.

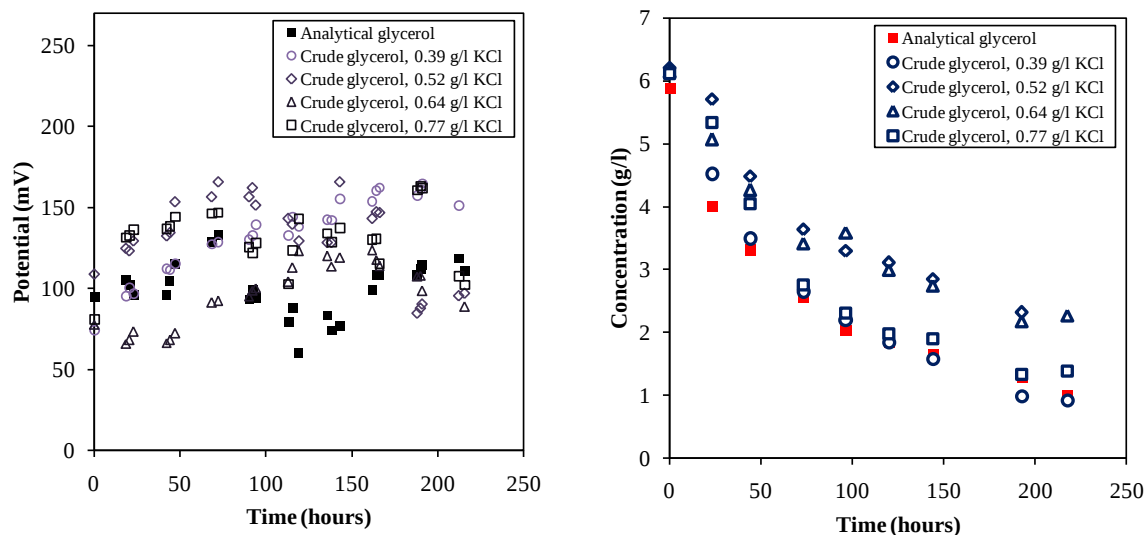


Figure 4.4: (Left) Potential achieved and (Right) glycerol concentration observed with the use of *E. coli* W3110 as anodic catalyst grown on crude glycerol supplemented with KCl.

Potential was measured across a 1kΩ resistor.

The potential observed with the use of analytical glycerol rose initially, decreased to 60mV, 119h after inoculation, and later increased to a potential of 118mV, 212h after inoculation. The potential observed with the use of crude glycerol supplemented to a final KCl concentration of 0.64g⁻¹ decreased initially, after which it started to rise, reaching a maximum of 125mV 140h after inoculation before decreasing once again.

The polarization curve for the fuel cell that used analytical glycerol was generated 140h after inoculation, just as the working potential observed was starting to increase. Therefore, if a polarization curve were obtained later in the study, the maximum power density observed with the use of analytical glycerol might have been greater. However, the working potential observed with the use of analytical glycerol was typically lower than that observed with all of the other fuel cells, indicating that the maximum power density that could have been achieved with the use of analytical glycerol would have still been less than that observed with all other fuel cells in the study.

For all MFCs, the concentration of glycerol in the anode decreased throughout the duration of the study (Figure 4.4). The rate of glycerol consumption was similar for all fuel cells. A decrease in the rate of glycerol consumption 150h after inoculation was

accompanied by a decline in the working potential observed for four of the five MFCs. Similar to the results of the previous experiment where medium was supplemented with NaCl, it can be hypothesized that the depletion of valuable nutrients has had an effect on glycerol metabolism. In a previous study, anaerobic fermentation of glycerol by *E. coli* W3110 could only be achieved in M9 medium through the addition of nutrient rich additives like tryptone and yeast extract (Reiche, 2012). In medium optimization experiments, Reiche (2012) found that when the concentration of tryptone was reduced from 10gL^{-1} to 5gL^{-1} , *E. coli* W3110 was not able to consume as much glycerol within a given time frame.

4.2.3 Summary

It was found that increasing the concentration of salt in the medium resulted in an increase in the maximum power density achieved by *E. coli* W3110. The use of crude glycerol from biodiesel production in place of analytical grade glycerol did not affect the rate of glycerol metabolism, however the depletion of valuable nutrients required for glycerol metabolism is likely responsible for the decrease in glycerol consumption observed at the end of both studies.

4.3 *P. freudenreichii* ssp. *shermanii*

In the following two studies, the effect of salt concentration on the performance of *P. freudenreichii* ssp. *shermanii* as anodic catalyst in a glycerol oxidizing MFC was investigated. In the first study, M9 medium was supplemented with sodium chloride to concentrations of 0.85gL^{-1} , 1.06gL^{-1} and 1.25gL^{-1} , which correspond to the salt concentrations that would result if 0.8wt%, 1.0wt% and 1.2wt% sodium methoxide were used, respectively, in biodiesel production. In the second study, M9 medium was supplemented with potassium chloride to concentrations of 0.77gL^{-1} , 1.03gL^{-1} , 1.29gL^{-1} and 1.55gL^{-1} , which correspond to the salt concentrations that would result if 0.6wt%, 0.8wt%, 1.0wt% and 1.2wt% potassium hydroxide, were used, respectively. In both studies, M9 medium was supplemented with 10gL^{-1} glycerol and 5gL^{-1} yeast extract. In the second study, medium was supplemented with sodium thioglycolate to a final concentration of 0.5gL^{-1} .

4.3.1 Supplemented with sodium chloride

The polarization and power density curves generated for MFCs in this study are shown in Figure 4.5. The maximum power density achieved by MFCs in this study ranged from 2.9mWm^{-2} ; observed with the use of analytical glycerol, to 28.4mWm^{-2} ; observed with the use of analytical glycerol supplemented to a final NaCl concentration of 1.25gL^{-1} . The maximum power density observed with the use of analytical glycerol in this study was less than that observed by Reiche (2012) (14.9mWm^{-2}). The maximum power density observed with the use of analytical glycerol supplemented to a final NaCl concentration of 1.25gL^{-1} and crude glycerol supplemented to a final NaCl concentration of 1.25gL^{-1} , was similar.

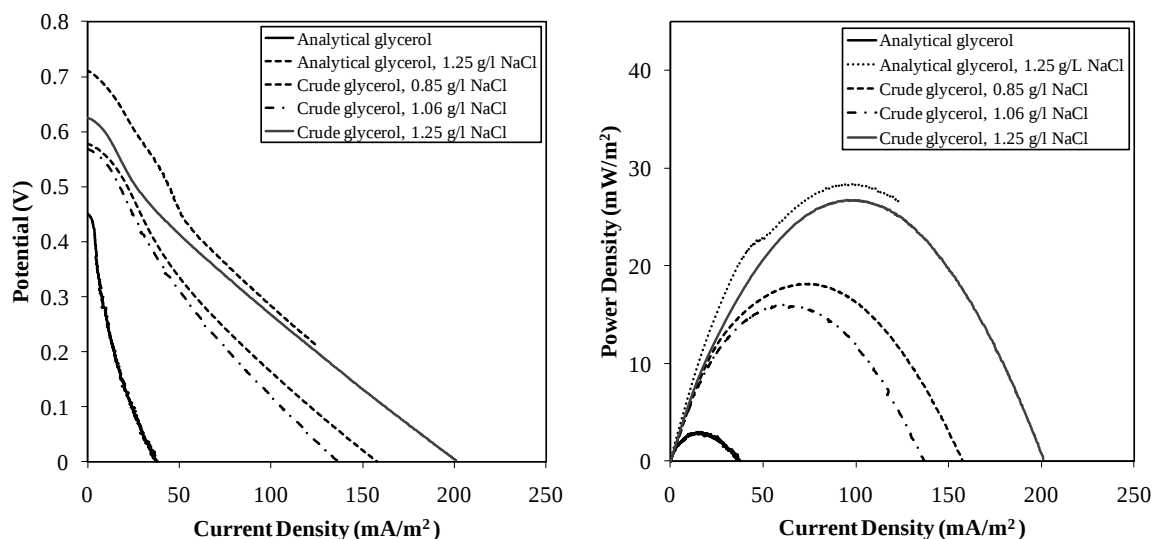


Figure 4.5: (Left) Polarization and (Right) power density curves observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst grown on crude glycerol supplemented with NaCl. Current density and power density were normalized to the anode surface area. Curves were generated for each condition tested after the observation of a stable potential; 125h: crude glycerol + 1.25gL^{-1} NaCl, analytical glycerol + 1.25gL^{-1} NaCl and analytical glycerol; 172h: crude glycerol + 0.85gL^{-1} NaCl and crude glycerol + 1.06gL^{-1} NaCl.

The polarization curves generated in this study were similar in shape. The polarization curves generated in this study differed in shape from the polarization curves generated when *E. coli* W3110 was used as the anodic catalyst; activation losses, which are dominant at low current densities, were an important source of energy loss. Activation losses

refer to any decrease in potential that is due to the activation energy required for electron transfer at the anode and at the cathode. Activation losses are more complex in MFCs than in conventional fuel cells, as electron transfer at the anode is dependent on the biochemical reactions occurring within the bacterial catalyst. The magnitude of activation losses observed by a system is influenced by electrode size, catalyst used, and the temperature at which the study is conducted. Ohmic losses, which result from the resistance to electron flow and the conduction of ions, were another source of energy loss. Large ohmic losses are characteristic of MFCs, especially in H-type dual chambered systems (Logan et al., 2006).

The electrochemical efficiency and the internal resistance observed with each MFC in this study are shown in Table 8. In this study, electrochemical efficiency was enhanced through media supplementation with NaCl. The electrochemical efficiency observed with the use of analytical glycerol was similar to that observed by Reiche (2012) (0.40) under the same conditions. Following the same trend observed with the maximum power density, the electrochemical efficiencies observed with the use of crude glycerol supplemented to a final NaCl concentration of 1.25gL^{-1} and observed with the use of analytical glycerol supplemented to a final NaCl concentration of 1.25gL^{-1} were similar. Electrochemical efficiencies observed in this study were greater on average than those observed when *E. coli* W3110 was used as the anodic catalyst. However, because activation losses were not as significant with the use of *E. coli* W3110 as they were with the use of *P. freudenreichii* ssp. *shermanii*, the maximum power density observed with both strains was similar.

Table 8: Internal resistance and electrochemical efficiency observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst grown on crude glycerol supplemented with NaCl.

Quality of glycerol used, salt concentration	Internal resistance (Ω)	Electrochemical efficiency
Analytical	7929	0.37
Analytical, 1.25gL ⁻¹ NaCl	2358	0.58
Crude, 0.85gL ⁻¹ NaCl	2528	0.47
Crude, 1.06gL ⁻¹ NaCl	2646	0.47
Crude, 1.25gL ⁻¹ NaCl	2135	0.51

The internal resistance observed with the use of analytical glycerol (7929 Ω) was found to be significantly greater than the internal resistance observed with all the other MFCs in this study, which ranged from 2135 Ω to 2646 Ω . Except for the MFC that used analytical glycerol, the internal resistance observed with all other MFCs in this study was similar to those observed when *E. coli* W3110 was used as the anodic catalyst.

Although the architecture and materials of construction were identical for each fuel cell, subtle changes in the position of the anode relative to the cathode or subtle changes in electrode size have the potential to provide small contributions to the internal resistance observed. In a study by Liu et al. (2005) changing electrode spacing from 4cm to 2cm in a single chamber MFC resulted in a significant decrease in internal resistance (from 161 Ω to 77 Ω , respectively).

Increasing the conductivity of the electrolyte in the anode and cathode through supplementation with salt has been shown to reduce internal resistance, however larger concentrations than those used in this study are required. In a study by Oh et al. (2006) it was found that increasing the concentration of KCl in the anodic medium of an H-type MFC from 0M to 0.4M reduced the internal resistance observed in the fuel cell from 1087 Ω to 625 Ω (for KCl, 1M =74.6gL⁻¹). The most significant decrease in internal resistance was observed when the concentration of KCl in the anode was increased from 0M to 0.1M. In a similar study by Liu et al. (2005) the ionic strength of a single chamber MFC was changed through

supplementation with NaCl. Increasing the concentration of NaCl in the medium from 0M to 0.1M reduced the internal resistance of the system from 161 Ω to 91 Ω (for NaCl, 1M = 58.5gL⁻¹).

Working potential and glycerol concentration in the anode chamber, at various points throughout the course of the study, are shown in Figure 4.6. The potential observed with the use of crude glycerol rose initially, then stabilized at the maximum value achieved. A significant decrease in the concentration of glycerol in the anode chamber was not observed with the use of crude glycerol; however, the yeast extract with which the medium was supplemented provided an additional source of carbon which could be metabolized to generate reducing equivalents.

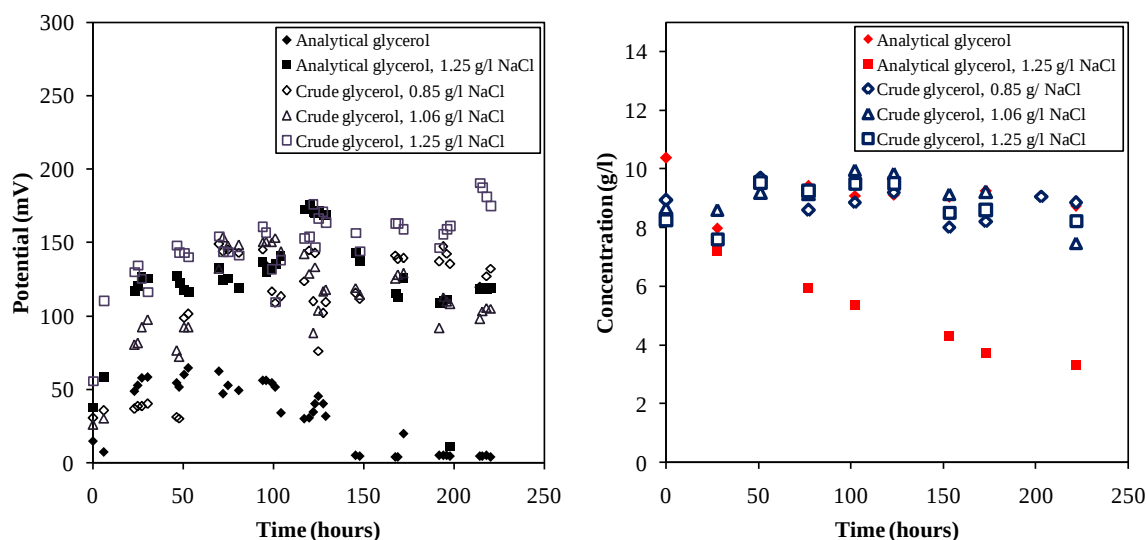


Figure 4.6: (Left) Potential achieved and (Right) glycerol concentration observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst grown on crude glycerol supplemented with NaCl. *Potential was measured across a 1k Ω resistor.*

The working potential observed with the use of analytical glycerol supplemented to a final NaCl concentration of 1.25gL⁻¹ rose initially, stabilized at the maximum value achieved, then decreased 150h after the fuel cell was inoculated. The decrease in potential observed with the use of analytical glycerol supplemented to a final NaCl concentration of 1.25gL⁻¹ coincides with, and was possibly the result of, a decrease in the rate of glycerol consumption.

The potential observed with the use of analytical glycerol rose initially, stabilized around 60mV, then decreased to 3mV 145h after inoculation. The change in working potential, the maximum power density and the internal resistance observed with the use of analytical glycerol over the course of this study, was highly uncharacteristic of what has been observed in previous studies with *P. freudenreichii* ssp. *shermanii*.

4.3.2 Supplemented with potassium chloride

Polarization and power density curves generated for MFCs in this study are shown in Figure 4.7. The maximum power density achieved by all MFCs in this study ranged from 6.6mWm^{-2} , observed with the use of crude glycerol supplemented to a final KCl concentration of 0.77gL^{-1} , to 3.1mWm^{-2} , observed with the use of crude glycerol supplemented to a final KCl concentration of 1.03 g L^{-1} . The maximum power densities achieved by the MFCs in this study were significantly less than what was observed in the previous study where medium was supplemented with NaCl as opposed to KCl.

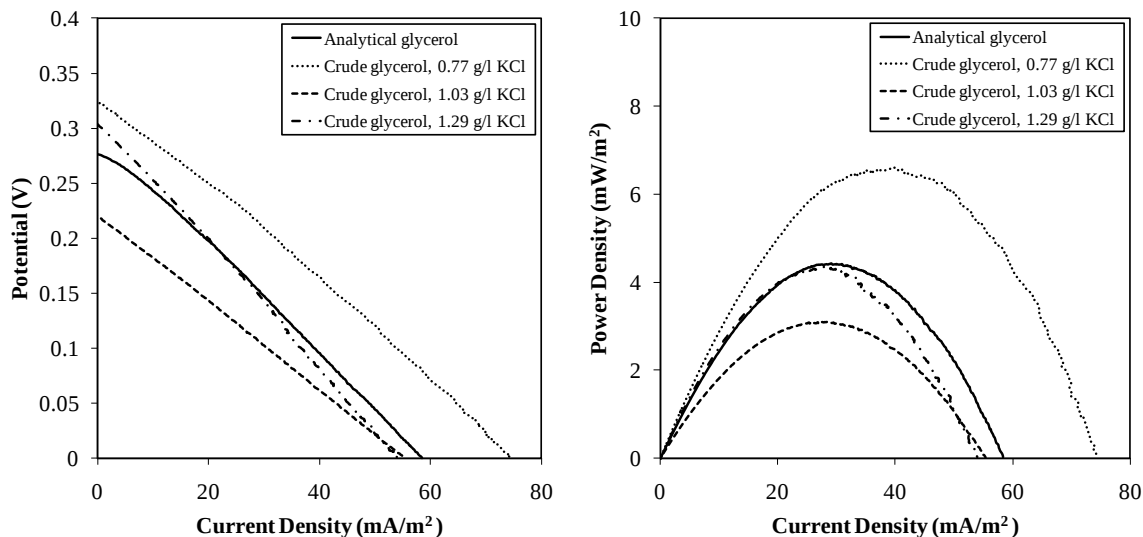


Figure 4.7: (Left) Polarization and (Right) power density curves observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst grown on crude glycerol supplemented with KCl. Current density and power density were normalized to the anode surface area. Curves were generated for each condition tested after the observation of a stable potential; 140h: analytical glycerol, crude glycerol + 0.77gL^{-1} KCl, analytical glycerol + 1.03gL^{-1} KCl and crude glycerol + 1.29gL^{-1} KCl.

From the shape of polarization curves generated in this study, it's evident that activation losses contributed less to the depletion of potential with increasing current density than in the previous study.

The internal resistance and electrochemical efficiency observed for all MFCs in this study are shown in Table 9. On average, the internal resistance observed with MFCs in this study was greater than that observed with MFCs in the previous study.

Table 9: Internal resistance and electrochemical efficiency observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst grown on crude glycerol supplemented with KCl.

Quality of glycerol used, salt concentration	Internal resistance (Ω)	Electrochemical efficiency
Analytical	4038	0.23
Crude, 0.77gL ⁻¹ KCl	3627	0.27
Crude, 1.03gL ⁻¹ KCl	3174	0.19
Crude, 1.29gL ⁻¹ KCl	4813	0.25
Crude, 1.55gL ⁻¹ KCl	Not available	Not available

As discussed in Section 2.2.2.2, ohmic losses, which result from the resistance to electron flow and conduction of ions, are dominant in the linear region of a polarization curve. Resistance to electron flow can result from poor connections between the anode, cathode, and elements of the external circuit; resistance to ion flow can result from reduced electrolyte conductivity or transport through the membrane used to separate the two chambers. Since ohmic losses are highly dependent on system architecture, the internal resistances observed for fuel cells in this study were expected to be similar to what was observed in the previous study.

Fouling of the membrane used to separate the two chambers with bacteria has been observed in MFCs, and would result in an increase in internal resistance. However, this was likely not the cause of the high internal resistance recorded in this study. Since the rate of glycerol consumption was low it can be assumed that the amount of biomass present was low as well. Although ohmic losses are dominant in the linear region of a polarization curve, electrode overpotentials vary with current density and are also accounted for in the internal resistance.

Electrochemical efficiencies observed for all MFCs in this study are shown in Table 9. Electrochemical efficiencies observed for MFCs in this study were much lower than the electrochemical efficiencies observed in the previous study with NaCl. Due to the reduced

environment created in the anode through the addition of sodium thioglycolate, a difference in potential between the anode and the cathode is apparent even before inoculation. In the absence of a bacterial catalyst, an open circuit potential of 0.27 V was achieved using an H-type fuel cell identical to that used in this study. The anolyte consisted of M9 medium, supplemented with 7gL^{-1} glycerol, 5gL^{-1} yeast extract, 2.8gL^{-1} NaCl and 0.5gL^{-1} sodium thioglycolate. The catholyte was a 10mM phosphate buffer (as was used in all other studies). Although there were small differences in the concentration of nutrients in the anolyte, because system architecture and the concentration of reducing agent sodium thioglycolate were identical in both cases, the OCP observed should be similar. By observing Figure 4.7, it becomes evident that the OCP observed for all MFCs in this study (0.23 to 0.33) barely exceeds, or is less than the OCP observed in the absence of a bacterial catalyst. The similarity between these values suggests that the anode may not have been used by *P. freudenreichii* ssp. *shermanii* as a terminal electron acceptor in these experiments, explaining the drop in electrochemical efficiency.

Working potential and glycerol concentration in the anode chamber, at various points throughout the course of the study, are shown in Figure 4.8. The working potential observed for MFCs in this study did not show an obvious increase from the values observed prior to inoculation. The concentration of glycerol in the anode also remained consistent over the course of the study apart from the small decrease observed 25h after inoculation. Both observations support the possibility that the anode was not used by *P. freudenreichii* ssp. *shermanii* as a terminal electron acceptor.

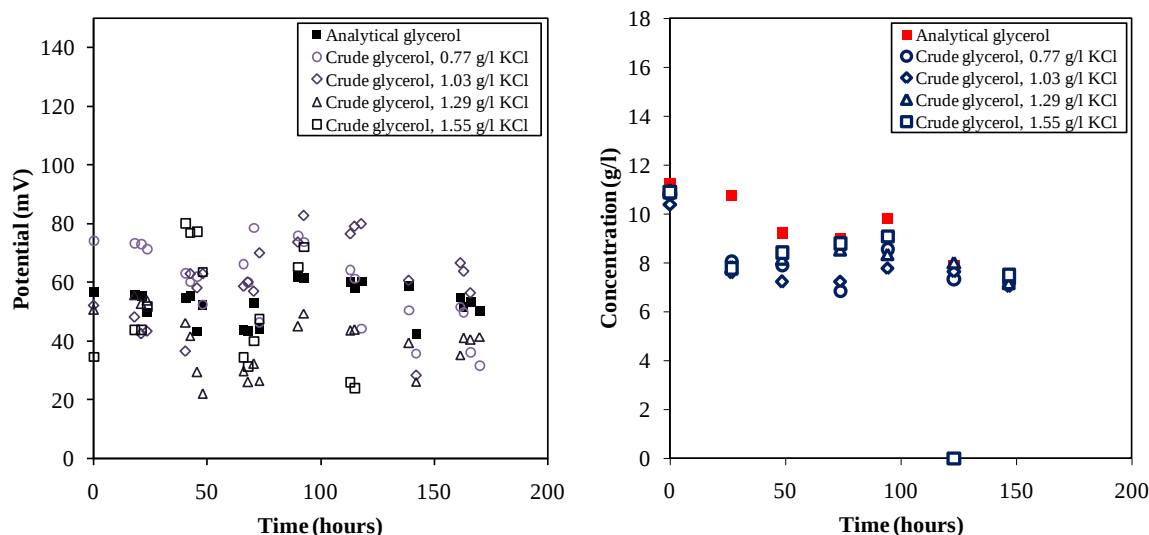


Figure 4.8: (Left) Potential achieved and (Right) glycerol concentration observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst grown on crude glycerol supplemented with KCl. *Potential was measured across a 1k Ω resistor.*

4.3.3 Summary

It was found that increasing the concentration of NaCl in the medium resulted in an increase in the maximum power density achieved by *P. freudenreichii* ssp. *shermanii*. *P. freudenreichii* ssp. *shermanii* did generate a current when crude glycerol supplemented with NaCl was used as fuel, however further experimentation will be necessary to determine whether other components in crude glycerol have a negative effect on cell growth or glycerol metabolism.

The results obtained in the experiments with KCl did not agree with any previous results obtained using analytical glycerol as the carbon source and *P. freudenreichii* ssp. *shermanii* as the anodic catalyst. It is possible that high concentrations of KCl have an inhibitory effect on *P. freudenreichii* ssp. *shermanii*, however this is difficult to confirm from the results of this study given that the results observed for the control (analytical glycerol) were atypical.

4.4 Mixed culture AR2

In the following two studies, the effect of salt concentration on the performance of mixed culture AR2 as an anodic catalyst in an MFC was investigated. In the first study, M9 medium was supplemented with sodium chloride to concentrations of 1.26gL^{-1} , 1.68gL^{-1} , 2.10gL^{-1} and 2.50gL^{-1} , which correspond to the salt concentrations that would result if 0.6wt%, 0.8wt%, 1.0wt% and 1.2wt% sodium methoxide were used, respectively, in biodiesel production. In the second study, M9 medium was supplemented with potassium chloride to concentrations of 1.55gL^{-1} , 2.58gL^{-1} and 3.10gL^{-1} , which correspond to the salt concentrations that would result if 0.6wt%, 1.0wt% and 1.2wt% potassium hydroxide, were used, respectively. In both studies, M9 medium was supplemented with 20gL^{-1} glycerol, 10gL^{-1} tryptone and 0.5gL^{-1} sodium thioglycolate.

4.4.1 Supplemented with sodium chloride

Polarization and power density curves generated for MFCs in this study are shown in Figure 4.9. The maximum power density achieved by all MFCs in this study ranged from 11.8mWm^{-2} , observed with the use of crude glycerol supplemented to a final NaCl concentration of 2.10gL^{-1} , to 16mWm^{-2} , observed with the use of crude glycerol supplemented to a final NaCl concentration of 1.26gL^{-1} . The range of maximum power densities observed by MFCs in this study was less extensive than the range observed in studies that used *E. coli* W3110 or *P. freudenreichii* ssp. *shermanii* as the anodic catalyst. A maximum power density of 14.8mWm^{-2} was observed with the use of analytical glycerol. The maximum power density observed in this study with the use of analytical glycerol was greater than the maximum power density observed by Reiche (2012) in a previous study (11.7mWm^{-2}).

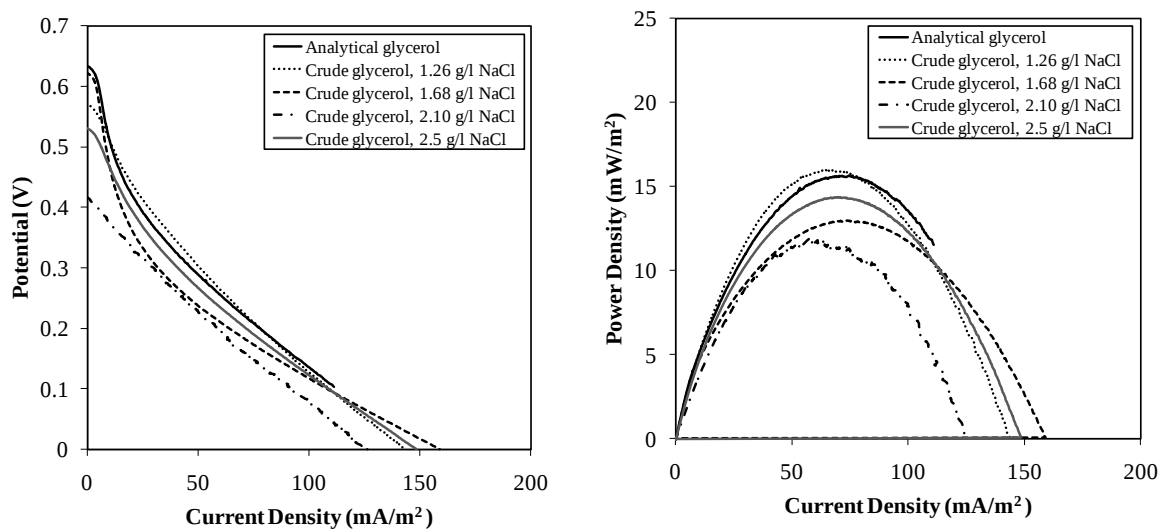


Figure 4.9: (Left) Polarization and (Right) power density curves observed with the use of mixed culture AR2 as anodic catalyst grown on crude glycerol supplemented with NaCl. Current density and power density were normalized to the anode surface area. Curves were generated for each condition tested after the observation of a stable potential, which in this case occurred 25h after inoculation.

The polarization curves generated using mixed culture AR2 as the anodic catalyst were similar in shape to those observed with the use of *P. freudenreichii* ssp. *shermanii*; the rapid decrease in potential observed at low current densities indicates that activation losses are significant.

The internal resistance and electrochemical efficiency observed with MFCs in this study are shown in

Table 10. The internal resistances observed in this study were similar to those observed in previous studies where *E. coli* W3110 and *P. freudenreichii* ssp. *shermanii* were used as anodic catalysts.

Table 10: Internal resistance and electrochemical efficiency observed with the use of mixed culture AR2 as anodic catalyst grown on crude glycerol supplemented with NaCl.

Quality of glycerol used, salt concentration	Internal resistance (Ω)	Electrochemical efficiency
Analytical	2220	0.51
Crude, 1.26g L^{-1} NaCl	2580	0.47
Crude, 1.68g L^{-1} NaCl	1748	0.50
Crude, 2.1g L^{-1} NaCl	2105	0.34
Crude, 2.5g L^{-1} NaCl	2117	0.43

Working potential and glycerol concentration in the anode at various points throughout the course of the study are shown in Figure 4.10. The working potential observed by all fuel cells in this study rose initially, stabilized at a maximum value, then declined 45 h after inoculation, coinciding with the complete consumption of glycerol. The rate of glycerol consumption was found to be similar for all conditions tested, and was the greatest of all catalysts tested. Although it may seem reasonable to expect a bacterial strain, capable of consuming glycerol at a greater rate, to also be capable of achieving a greater maximum power density, this is not necessarily the case. The fraction of glycerol used by the microorganism for the production of biomass, or fermentation products, as well as the method of electron transfer from the bacteria to the anode play a large role in the power that can be generated.

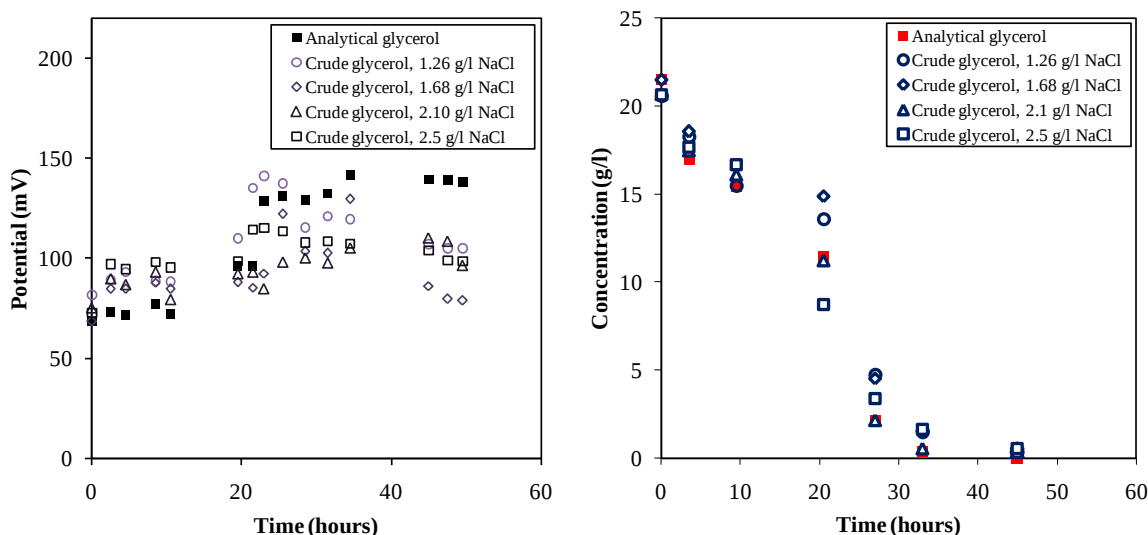


Figure 4.10:(Left) Potential achieved and (Right) glycerol concentration observed with the use of mixed culture AR2 as anodic catalyst grown on crude glycerol supplemented with NaCl. *Potential was measured across a $1k\Omega$ resistor.*

4.4.2 Supplemented with potassium chloride

Polarization and power density curves generated for MFCs in this study are shown in Figure 4.11. The maximum power density achieved by all MFCs in this study ranged from 6.4mWm^{-2} , observed with the use of crude glycerol supplemented to a final KCl concentration of 1.55gL^{-1} , to 20.7mWm^{-2} , observed with the use of crude glycerol supplemented to a final KCl to concentration of 3.10gL^{-1} . The maximum power density observed with the use of analytical glycerol, 19.8mWm^{-2} , was similar to that observed with the use of crude glycerol supplemented to a final KCl concentration of 3.10gL^{-1} .

If the maximum power density observed with the use of crude glycerol supplemented to a final KCl concentration of 1.55gL^{-1} were disregarded, the maximum power density achieved by fuel cells in this study would fall between 17.2mWm^{-2} and 20.7mWm^{-2} ; slightly greater than the values observed in the previous study with NaCl (11.8mWm^{-2} to 16mWm^{-2}). The MFC that used crude glycerol supplemented to a final KCl concentration of 1.55gL^{-1} did not produce an increase in potential over the course of the study, and will therefore not be included in further analysis.

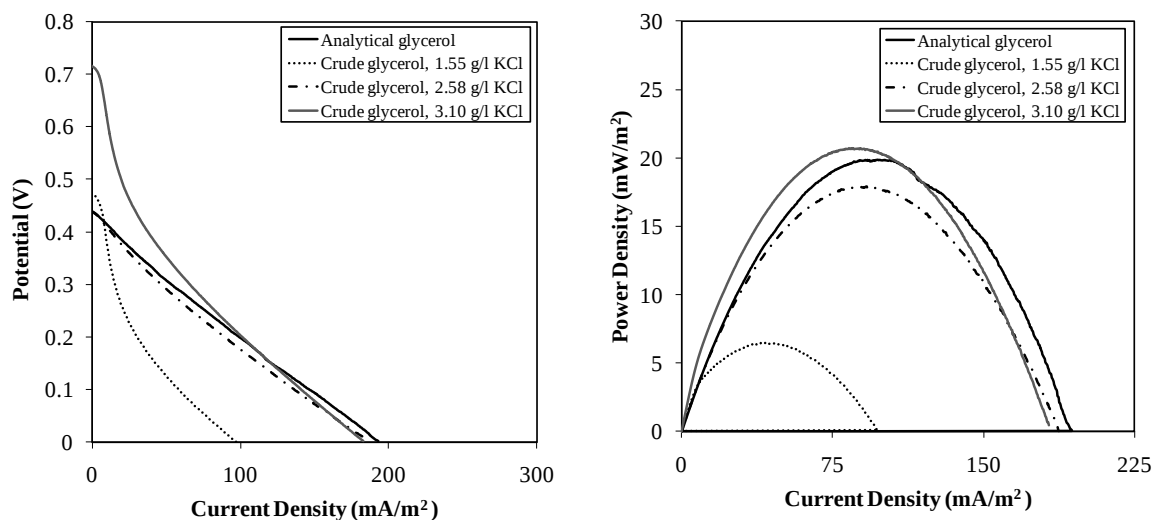


Figure 4.11: (Left) Polarization and (Right) power density curves observed with the use of mixed culture AR2 as anodic catalyst grown on crude glycerol supplemented with KCl. Current density and power density were normalized to the anode surface area. Curves were generated for each condition tested after the observation of a stable potential, which in this case occurred 22h after inoculation.

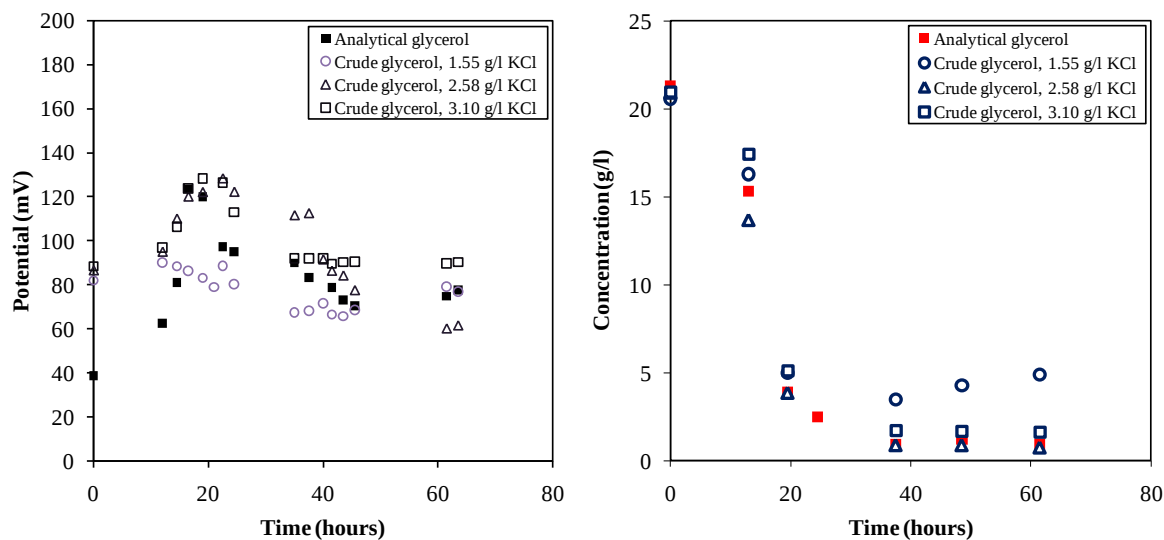


Figure 4.12: (Left) Potential achieved and (Right) glycerol concentration observed with the use of mixed culture AR2 as anodic catalyst grown on crude glycerol supplemented with KCl. Potential was measured across a $1k\Omega$ resistor.

The shape of the polarization curves generated in this study differed among fuel cells. Activation losses were more pronounced with the use of crude glycerol supplemented to a final KCl concentration of 3.10gL^{-1} and with the use of crude glycerol supplemented to a final KCl concentration of 1.55gL^{-1} . However, the internal resistance (see Table 11) was found to be similar for all the MFCs in this study. When a mixed culture is used as the anodic catalyst in an MFC, bacteria compete for use of the anode as a terminal electron acceptor. If different bacteria are dominant in each fuel cell (due to the different experimental conditions affecting growth), the differences observed in the metabolic pathways used for glycerol metabolism, and the differences observed in the method by which electrons are transferred to the anode, will result in polarization curves with different shapes.

Table 11: Internal resistance and electrochemical efficiency observed with the use of mixed culture AR2 as anodic catalyst grown on crude glycerol supplemented with KCl.

Quality of glycerol used, salt concentration	Internal resistance (Ω)	Electrochemical efficiency
Analytical	1525	0.36
Crude, $1.55\text{ g L}^{-1}\text{KCl}$	1923	0.39
Crude, $2.58\text{ g L}^{-1}\text{KCl}$	1508	0.37
Crude, $3.10\text{ g L}^{-1}\text{KCl}$	1889	0.58

The electrochemical efficiency observed for MFCs in this study are also shown in Table 11. The electrochemical efficiencies observed with the use of analytical glycerol, crude glycerol supplemented to a final KCl concentration of 1.55gL^{-1} , and crude glycerol supplemented to a final KCl concentration of 2.58gL^{-1} , were similar, ranging from 0.36 to 0.39; the electrochemical efficiency observed with the use of crude glycerol supplemented to a final KCl concentration of 3.10gL^{-1} was much greater.

Working potential and glycerol concentration in the anode at various points throughout the course of the study are shown in Figure 4.12. The working potential observed for all fuel cells in this study (crude glycerol supplemented with KCl to a final concentration of 1.55gL^{-1} as an exception) rose initially, then declined. Similar rates of glycerol

consumption were observed for all fuel cells. Initially, the concentration of glycerol in the anode decreased rapidly. Thirty eight hours after inoculation, the concentration of glycerol had stabilized between 5gL^{-1} and 0.75gL^{-1} in each MFC. For all MFCs, the decrease observed in the rate of glycerol consumption accompanies the decrease observed in the working potential.

4.4.3 Summary

It was found that increasing the concentration of salt in the medium did not affect the maximum power density achieved by the mixed culture AR2. The use of crude glycerol from biodiesel production in place of analytical grade glycerol did not affect the rate of glycerol metabolism.

Chapter 5. Waste glycerol from biodiesel produced using high catalyst concentrations

5.1 Introduction

Glycerol waste from biodiesel production can contain a number of different compounds. Compounds such as free fatty acids, biodiesel and salt result from the process and materials used in biodiesel production (Santori et al., 2012). Compounds such as tocopherols, sterols, triterpene alcohols, coloured bodies and a variety of rearrangement and decomposition products can result from the use of any partially refined vegetable oil as feed (Sleeter, 1981). Refining crude glycerol to meet USP grade standards for use in the food or pharmaceutical industries is expensive and not economically feasible for small to midsized operations (Pachauri and He, 2006).

The objective of the work in this chapter was to determine the effect of compounds (other than salt) in crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ as catalysts in biodiesel production, on the performance of *E. coli* W3110, *P. freudenreichii* ssp. *shermanii*, and mixed culture AR2 as catalysts in an MFC. The concentration of various compounds could have an effect on bacterial metabolism or electrolyte conductivity, which in turn could affect fuel cell performance.

The performance of fuel cells using crude glycerol produced using 0.8wt % NaOCH₃ and 0.8 wt % KOH was compared to fuel cells using crude glycerol produced using 0.23wt% NaOCH₃ catalyst and modified through the addition of NaCl and KCl (Chapter 4) to achieve the same salt concentration. The concentration of glycerol in the crude stream produced using 0.23wt% NaOCH₃ was 0.88gmL⁻¹, whereas the concentration of glycerol in the crude streams produced using 0.8wt% NaOCH₃ and 0.8wt% KOH was 0.79gmL⁻¹ and 0.65gmL⁻¹ respectively. The crude streams that contained lower concentrations of glycerol, contained greater concentrations of other compounds including water, methanol, free fatty acids and biodiesel.

Maximum power density was the major parameter by which the performance of each strain was evaluated. In addition to calculating maximum power density, change in working

potential, electrochemical efficiency, change in glycerol concentration, and identification of the nature and magnitude of energy losses, provided additional information with which to better understand the phenomena occurring within each fuel cell (further details provided in section 3.5).

5.2 *E. coli* W3110

The focus of this study was to determine the effect of compounds in crude glycerol, other than salt, on the performance of *E. coli* W3110 as anodic catalyst in an MFC.

The crude glycerol used throughout Chapter 4, produced using 0.23wt% NaOCH₃ in biodiesel production, was supplemented to final salt concentrations of 0.42g L⁻¹ NaCl and 0.52g L⁻¹ KCl, to mimic the concentration of salt found in crude glycerol produced using 0.8wt% NaOCH₃ and 0.8wt% KOH, respectively. M9 medium was supplemented with 5g L⁻¹ glycerol, 10 g L⁻¹ tryptone and 0.5g L⁻¹ sodium thioglycolate.

Polarization curves and power density curves are shown in Figure 5.1. The greatest maximum power density observed in this experiment (40.0mWm⁻²) was achieved with crude glycerol produced using 0.8wt% KOH catalyst. The greatest maximum power density observed in this study was similar to that observed in a previous experiment with *E. coli* W3110 as anodic catalyst: crude glycerol produced using 0.23wt% NaOCH₃ catalyst, supplemented to a final potassium chloride concentration of 0.39g L⁻¹ (to mimic the salt content of crude glycerol that would have resulted from using 0.6wt% KOH catalyst in biodiesel production), achieved a maximum power density of 41.4mWm⁻² (see Figure 4.4). There was little difference in the maximum power density achieved with crude glycerol produced using 0.8wt% NaOCH₃ catalyst, crude glycerol supplemented to a final potassium chloride concentration of 0.52g L⁻¹, and crude glycerol supplemented to a final NaCl concentration of 0.42g L⁻¹. The maximum power density observed with analytical glycerol (6.10mWm⁻²) was substantially less than that observed with crude glycerol. This trend was observed in all other studies that employed *E. coli* W3110 as anodic catalyst, with values ranging from 9.4mWm⁻² to 16.8mWm⁻².

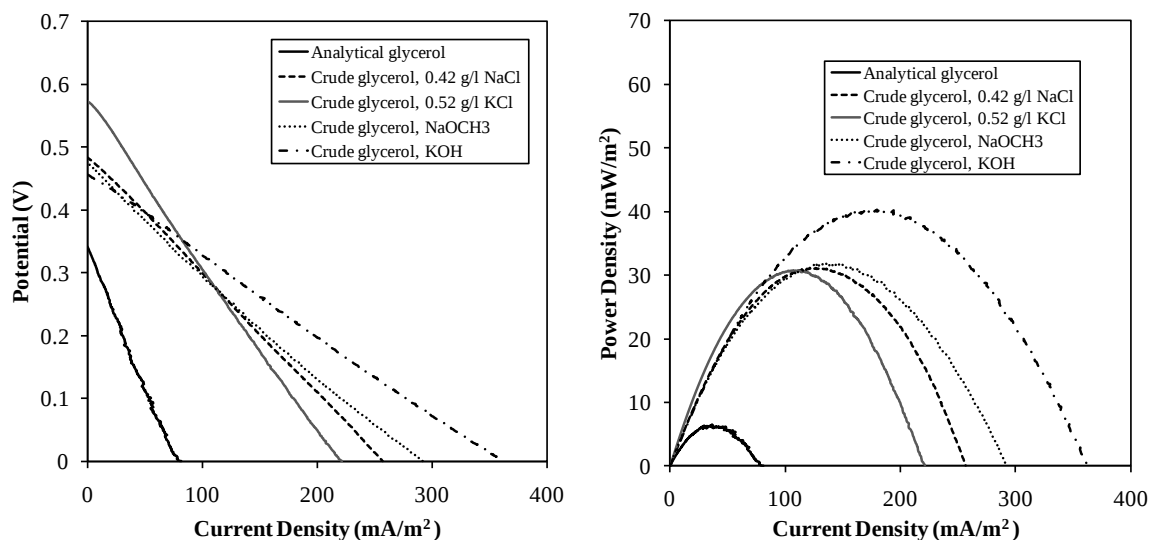


Figure 5.1: (Left) Polarization curves and (Right) power density curves observed with the use of *E. coli* W3110 as anodic catalyst grown on crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ in biodiesel production. *Current density and power density were normalized to the anode surface area. Curves were generated for each condition tested after the observation of a stable potential, which in this case occurred 95h after inoculation.*

Polarization curves (Figure 5.1) for all MFCs in this study showed nearly linear relationships between potential and current density, indicating that ohmic losses were dominant over the range where power generation was positive. The slope of polarization curves generated for MFCs in this study differed, indicating that the internal resistance recorded for each of the conditions tested differed.

The internal resistance and electrochemical efficiency observed with MFCs in this study are shown in Table 12. The internal resistance observed with the use of analytical glycerol was much greater than the internal resistance observed with the use of crude glycerol. The least amount of internal resistance was observed with crude glycerol produced using 0.8wt% KOH catalyst. Reducing ohmic losses in turn reduces internal resistance, thereby increasing the maximum power density that can be achieved. However, it isn't possible to differentiate between the contribution of ohmic losses and the contribution of electrode overpotentials to the internal resistance, making it difficult to quantify to

whatextent the system architecture and materials of construction were responsible for the loss in potential observed with increasing current density.

Table 12: Internal resistance and electrochemical efficiency observed with the use of *E. coli* W3110 as anodic catalyst grown on crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ in biodiesel production

Type of glycerol used	Internal resistance (Ω)	Electrochemical efficiency
Analytical glycerol	3283	0.278
Crude glycerol, 0.42 gL ⁻¹ NaCl	1544	0.393
Crude glycerol, 0.52 gL ⁻¹ KCl	1945	0.466
Crude glycerol, 0.8 wt% KOH	967	0.371
Crude glycerol, 0.8 wt% NaOCH ₃	1497	0.388

The electrochemical efficiency observed with crude glycerol supplemented to a final sodium chloride concentration of 0.42gL⁻¹, crude glycerol supplemented to a final potassium chloride concentration of 0.52gL⁻¹, and crude glycerol produced using 0.8wt% KOH catalyst, were similar (shown in Table 12). The electrochemical efficiency observed with the use of analytical glycerol was less than that observed with the use of crude glycerol.

Working potential and glycerol concentration in the anode at various points throughout the course of the study are shown in (Figure 5.2). Glycerol consumption was similar with each of the conditions tested. Glycerol consumption occurred at a fast rate during the first 74h of the study. At the end of the study, a small amount of glycerol remained unconsumed in all MFCs.

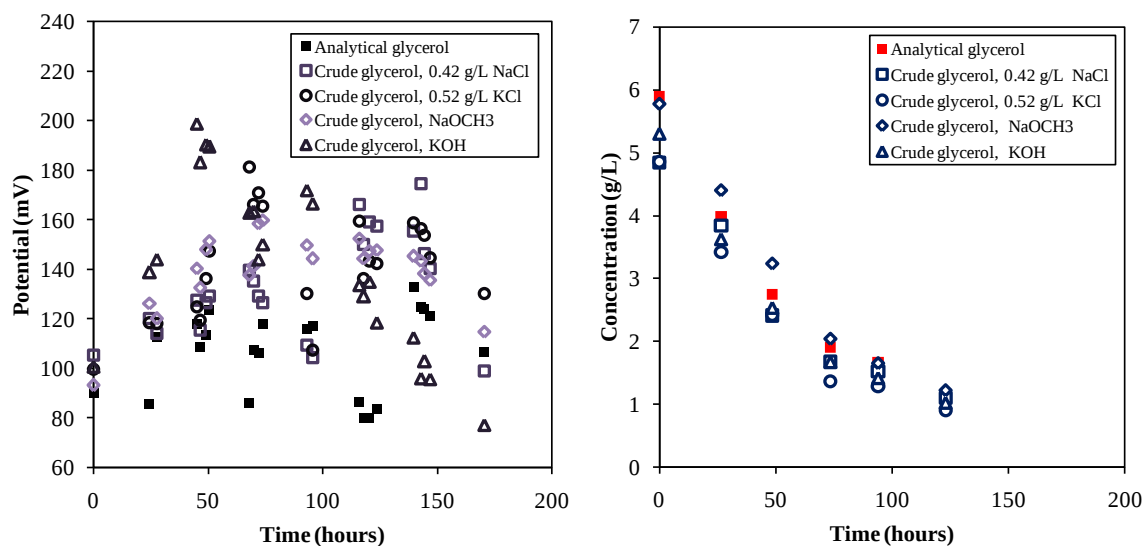


Figure 5.2: (Left) Potential achieved and (Right) glycerol concentration observed with the use of *E. coli* W3110 as anodic catalyst grown on crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ in biodiesel production. *Potential was measured across a 1k Ω resistor.*

An increase in working potential from the value recorded prior to inoculation was achieved by all MFCs that used crude glycerol. Between 100h and 150h after inoculation, the working potential observed for most of the MFCs that used crude glycerol decreased. The decrease in working potential observed did not occur within the same time frame as the decrease in the rate of glycerol consumption, which was observed much earlier in the study, only 74h after inoculation. This observation suggests that, although bacteria were no longer fermenting glycerol they must have been fermenting tryptone or other compounds in the medium to have been able to sustain the transfer of electrons to the anode.

The maximum working potential achieved by MFCs in this study ranged from 158mV, observed with crude glycerol produced using 0.8wt% NaOCH₃ catalyst, to 198mV, observed with crude glycerol produced using 0.8wt% KOH catalyst. The maximum working potential achieved by the fuel cell that used crude glycerol supplemented to a final sodium chloride concentration of 0.42g/L⁻¹, was 174mV. Given that the maximum working potential was recorded 150h after the start of the experiment, and power density curves were generated

95h after the start of the experiment, it's possible that the maximum power density that could have been achieved by this fuel cell is greater than the value currently recorded.

The working potential observed with the use of analytical glycerol did not show any significant increase over the course of the study. The stagnant nature of the working potential observed with the use of analytical glycerol combined with the large internal resistance displayed by this fuel cell provides reason to believe that poor connections between circuit elements may be responsible for the poor performance observed. In the studies documented in Chapter 4 of this thesis and in the study published by Reiche (2012), *E. coli* W3110 was successful as an anodic catalyst in MFCs that used analytical grade glycerol as fuel.

In this study, the performance observed with the use of crude glycerol was better than the performance observed with the use of analytical glycerol in other studies that employed *E. coli* W3110 as the anodic catalyst (see sections 4.2.1 and 4.2.2) (values obtained in this study with analytical glycerol are therefore misleading). A possible explanation for this observation is that compounds present in the unrefined oil or compounds that resulted from the biodiesel production process assisted the transport of electrons from bacteria to the anode. Given however, that the potential observed with the use of analytical glycerol supplemented to a final sodium chloride concentration of 0.52gL^{-1} was similar to that observed with the use of crude glycerol, it's more likely that the increase in performance is the result of an increase in electrolyte conductivity resulting from an increase in salt concentration (see section 4.2.1).

5.2.1 Summary

There was no significant difference in the performance observed with crude glycerol produced using 0.8wt% KOH catalyst, and crude glycerol produced using 0.23wt% NaOCH_3 catalyst and supplemented with potassium chloride. There was also no significant difference in the performance observed with crude glycerol produced using 0.8wt% NaOCH_3 catalyst, and crude glycerol produced using 0.23wt% NaOCH_3 catalyst and supplemented with sodium chloride. Therefore, it can be concluded that the compounds found in waste glycerol, at the concentrations found in the waste glycerol streams used in this study, did not affect the performance of *E. coli* W3110 as anodic catalyst.

5.3 *P. freudenreichii* ssp. *shermanii*

The focus of this study was to determine the effect of compounds in crude glycerol, disregarding salt content, on the performance of *P. freudenreichii* ssp. *shermanii* as anodic catalyst in an MFC.

The crude glycerol used throughout Chapter 4 that resulted from the use of 0.23wt% NaOCH₃ in biodiesel production, was supplemented to final salt concentrations of 0.59gL⁻¹ NaCl and 0.72gL⁻¹ KCl, to mimic the concentration of salt found in crude glycerol produced using 0.8wt% NaOCH₃, and 0.8wt% KOH, respectively. M9 medium was supplemented with 7gL⁻¹ glycerol, 5gL⁻¹ yeast extract and 0.5gL⁻¹ sodium thioglycolate.

Polarization curves and power density curves are shown in Figure 5.3. The greatest maximum power density achieved in this study was observed with the use of analytical glycerol. The maximum power densities observed with crude glycerol supplemented with sodium chloride, and with crude glycerol supplemented with potassium chloride, respectively, were next in magnitude, followed by crude glycerol produced using 0.8wt% NaOCH₃ catalyst, and crude glycerol produced using 0.8wt% KOH catalyst, respectively. The maximum power densities observed in this study (13.8mWm⁻² to 20.2mWm⁻²) were similar in value to those achieved by *P. freudenreichii* ssp. *shermanii* in a previous experiment (16.0mWm⁻² to 28.4mWm⁻²) where the effect of sodium chloride concentration on fuel cell performance was tested (further detail provided in section 4.3.1). Similar to previous studies, the maximum power density observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst, on average, was less than that observed with the use of *E. coli* W3110 as anodic catalyst.

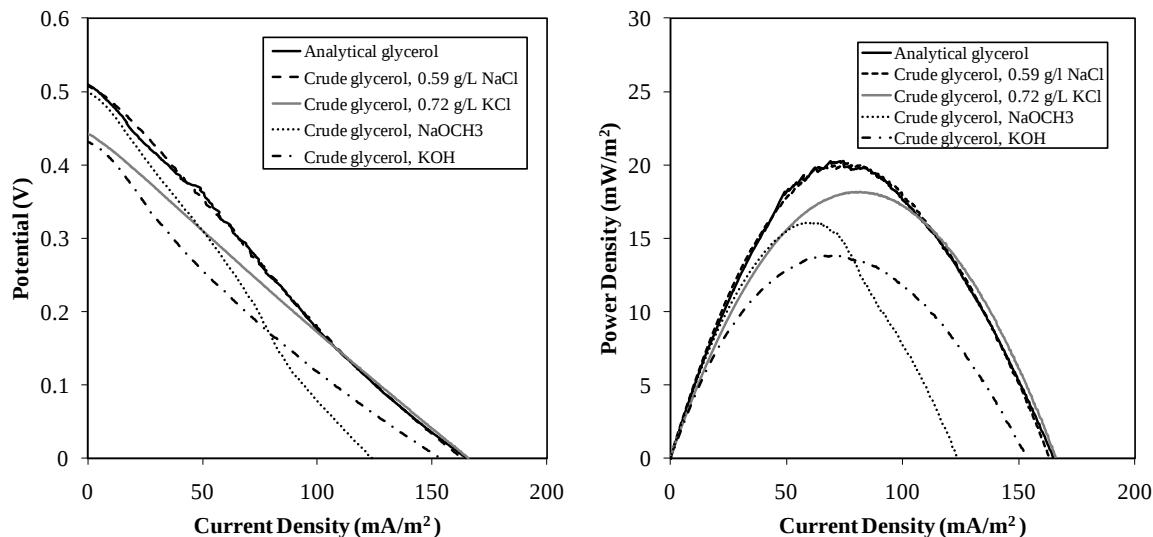


Figure 5.3: (Left) Polarization and (Right) power density curves observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst grown on crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ in biodiesel production. Current density and power density were normalized to the anode surface area. Curves were generated for each condition tested after the observation of a stable potential, which in this case occurred 145h after inoculation.

Polarization curves generated for MFCs in this study (Figure 5.3) were of similar shape. The contribution of activation losses to the total loss of potential observed with increasing current density was less significant in this study than in the previous study where the effect of sodium chloride concentration on fuel cell performance was investigated. Ohmic losses were a major source of loss of potential for MFCs in this study.

The internal resistance and electrochemical efficiency observed with MFCs in this study are shown in Table 13. The internal resistance and the electrochemical efficiency were similar among MFCs in this study; a relationship between the type of glycerol used, the internal resistance observed and the electrochemical efficiency achieved could not be identified.

Table 13: Internal resistance and electrochemical efficiency observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst grown on crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ in biodiesel production.

Type of glycerol used	Internal resistance (Ω)	Electrochemical efficiency
Analytical glycerol	2393	0.415
Crude glycerol, 0.59 gL ⁻¹ NaCl	2496	0.414
Crude glycerol, 0.72 gL ⁻¹ KCl	2076	0.361
Crude glycerol, 0.8 wt% KOH	2586	0.406
Crude glycerol, 0.8 wt% NaOCH ₃	1810	0.352

Working potential and glycerol concentration in the anode at various points throughout the course of the study are shown in Figure 5.4. Glycerol consumption was observed with all MFCs in this study. The rate of glycerol consumption observed with the use of analytical glycerol was greater than the rate of glycerol consumption observed with the use of crude glycerol; a total of 1.87gL⁻¹ was consumed within 174h. With crude glycerol, patterns could not be identified that would suggest that fuel cell performance was linked in any way to the concentration or type of catalyst used in biodiesel production. However, the yield of propionic acid, the major product of glycerol fermentation by *P. freudenreichii*, suggests otherwise (data is presented and discussed further in Chapter 6, Table 15 and Figures 6.11 and 6.12). A greater yield of propionic acid was observed with waste glycerol produced using less catalyst, supporting the hypothesis that glycerol metabolism is dependent on the concentration of various compounds found in waste glycerol (glycerol concentration was lower in the waste streams that resulted from the use of greater amounts of catalyst). This is further supported by the rate of glycerol consumption, the rate of growth, and the propionic acid yield observed with *P. freudenreichii* ssp. *shermanii* in fermentations.

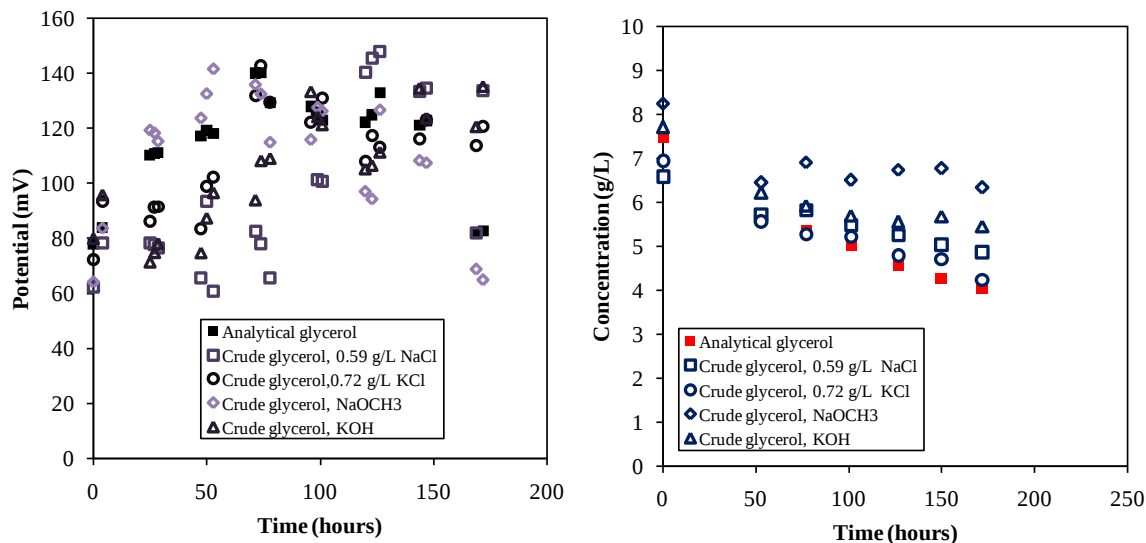


Figure 5.4: (Left) Potential achieved and (Right) glycerol concentration observed with the use of *P. freudenreichii* ssp. *shermanii* as anodic catalyst grown on crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ in biodiesel production. *Potential was measured across a 1k Ω resistor.*

The maximum working potential achieved by MFCs in this study ranged from 134mV to 145mV. The maximum working potential and the maximum power density achieved by the fuel cell that used crude glycerol produced with 0.8wt% KOH catalyst were 135mV and 13.8mWm⁻², respectively. Given that the maximum working potential was recorded 172h after the start of the experiment, and the power density curve was generated 145h after the start of the experiment, it's possible that the maximum power density that could have been achieved by this fuel cell is greater than the value currently recorded.

An increase in working potential was observed for all MFCs in this study. With most fuel cells, the potential increased initially then remained constant throughout the duration of the study; however, the working potential observed with analytical glycerol, and with crude glycerol produced using 0.8wt% NaOCH₃ catalyst, decreased at the end of the study. Since a large amount of glycerol remained unconsumed, the decrease in working potential could have resulted from the accumulation of fermentation products or the depletion of essential micronutrients. Furthermore, it's possible that these final data points represent a small discrepancy in performance, and the working potential measured with both fuel cells would

have returned to its steady state value had the duration of the study been extended to the point of glycerol depletion.

5.3.1 Summary

In this study, the performance of fuel cells that used waste glycerol from biodiesel production was equivalent to the performance of fuel cells that used analytical glycerol. Although there was a difference in the maximum power density achieved with the use of crude glycerol produced with minimal catalyst and with the use of crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ catalysts, the maximum working potentials observed did not follow the same trend; therefore it can be concluded that the concentration of free fatty acids, FAME and other components (neglecting salt) in crude glycerol did not affect fuel cell performance.

5.4 Mixed culture AR2

The focus of this study was to determine the effect of compounds in crude glycerol, disregarding salt content, on the performance of mixed culture AR2 as anodic catalyst in an MFC.

The crude glycerol used throughout Chapter 4 that resulted from the use of 0.23wt% NaOCH₃ in biodiesel production, was supplemented to final salt concentrations of 1.26gL⁻¹ NaCl and 1.52gL⁻¹ KCl, to mimic the concentration of salt found in crude glycerol produced using 0.8wt% NaOCH₃, and 0.8wt% KOH, respectively. M9 medium was supplemented with 15gL⁻¹ glycerol, 10gL⁻¹ tryptone and 0.5gL⁻¹ sodium thioglycolate.

Polarization curves and power density curves are shown in Figure 5.5. The maximum power density observed with the use of analytical glycerol was greater than the maximum power density observed with the use of crude glycerol. The maximum power density observed with crude glycerol supplemented to a final sodium chloride concentration of 1.26gL⁻¹, and with crude glycerol supplemented to a final potassium chloride concentration of 1.52gL⁻¹, was similar to the maximum power density observed with the use of analytical glycerol. The maximum power density observed with crude glycerol produced using 0.8wt% NaOCH₃ catalyst, and the maximum power density observed with crude glycerol produced

using 0.8wt% KOH catalyst, was significantly less than that observed with the other fuel cells in this study.

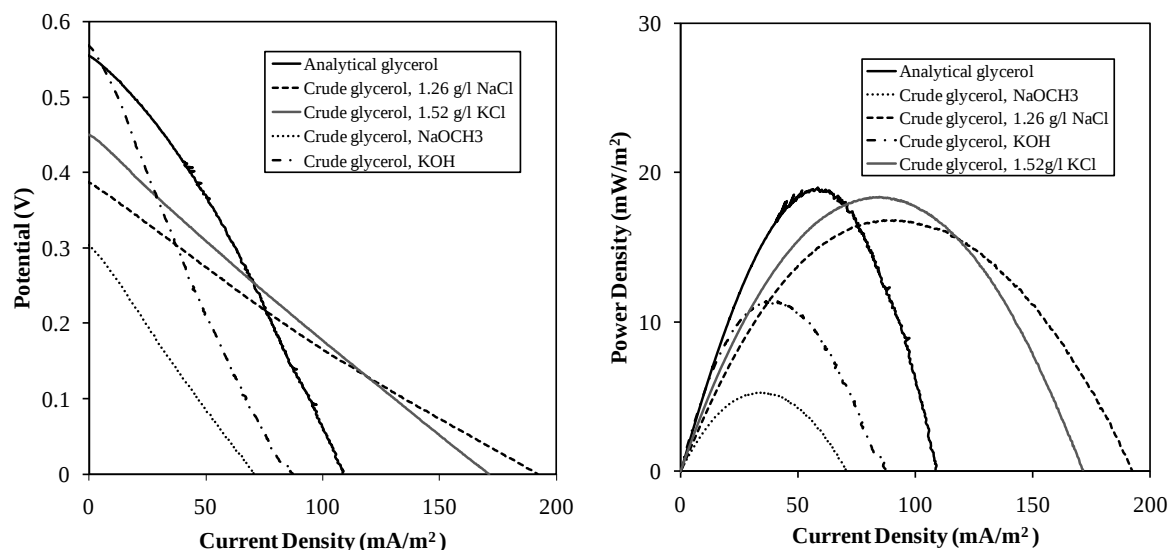


Figure 5.5: (Left) Polarization and (Right) power density curves observed with the use of AR2 as anodic catalyst grown on crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ in biodiesel production. *Current density and power density were normalized to the anode surface area. Curves were generated for each condition tested after the observation of a stable potential, which in this case occurred 145h after inoculation.*

The shape of polarization curves generated in this study (Figure 5.5) differed significantly among fuel cells. Similar to all other studies discussed in this thesis, ohmic losses were a major source of loss in potential.

The internal resistance and electrochemical efficiency observed with each MFC in this study is shown in Table 14. The internal resistance observed with the use of analytical glycerol was much greater than that observed in previous studies under identical conditions (however the concentration of glycerol used in previous studies was 20gL⁻¹ as opposed to 15gL⁻¹). The internal resistance observed with crude glycerol produced using 0.8wt% NaOCH₃ catalyst, and the internal resistance observed with crude glycerol produced using 0.8wt% KOH catalyst, were also large. Although internal resistance is calculated from the slope of the linear region of a polarization curve, and ohmic losses are the dominant form of energy loss for MFCs operating within this region, other losses related to bacterial

metabolism and electron transfer also contribute to the internal resistance of a fuel cell. The internal resistance observed with crude glycerol supplemented to a final sodium chloride concentration of 1.26gL^{-1} and crude glycerol supplemented to a final potassium chloride concentration of 1.52gL^{-1} were similar to that observed in previous studies where AR2 was employed as anodic catalyst.

Table 14: Internal resistance and electrochemical efficiency observed with the use of mixed culture AR2 as anodic catalyst grown on crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH_3 in biodiesel production.

Type of glycerol used	Internal resistance (Ω)	Electrochemical efficiency
Analytical glycerol	4987	0.451
Crude glycerol, $1.26\text{ gL}^{-1}\text{NaCl}$	1717	0.315
Crude glycerol, $1.52\text{ gL}^{-1}\text{KCl}$	2108	0.366
Crude glycerol, 0.8 wt% KOH	5183	0.462
Crude glycerol, 0.8 wt% NaOCH_3	3128	0.246

The electrochemical efficiencies achieved by MFCs in this study (except that which used crude glycerol produced with 0.8wt% KOH catalyst), were similar in value to those observed in the other studies that employed mixed culture AR2.

The working potential achieved by MFCs at various points throughout the course of the study, are shown in Figure 5.6. The working potential observed with crude glycerol produced using 0.23wt% NaOCH_3 catalyst, and supplemented with sodium chloride and potassium chloride, increased throughout the duration of the study. The working potential observed with the use of analytical glycerol remained constant from the time of inoculation to the end of the study. Despite having generated the greatest maximum power density of all MFCs in this study, the large internal resistance and the lack of increase in working potential observed with the MFC that used analytical glycerol, provide reasons why its performance should be neglected from further discussion. It's possible that the large internal resistance

observed with this MFC was caused by poor connections between the elements of the circuit, resulting in inconsistency between this fuel cell and the others included in the study.

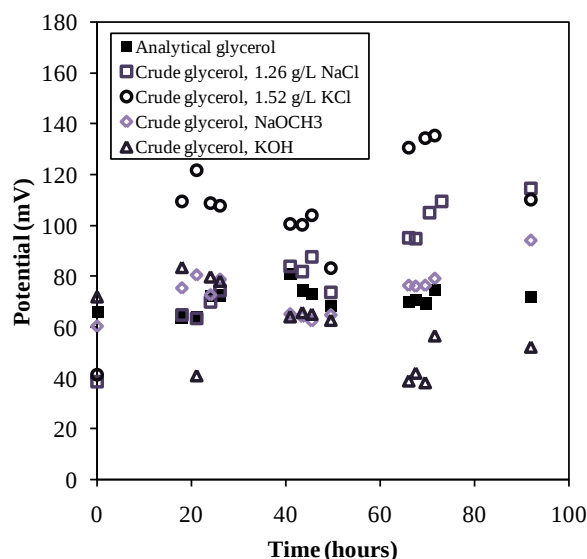


Figure 5.6: Potential achieved by mixed culture AR2 growing on crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ in biodiesel production. *Potential was measured across a 1k Ω resistor.*

Over the course of the study, the working potential observed with crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃ catalyst did not show any considerable increase from the time of inoculation. This agrees with the maximum power density achieved by each of these fuel cells, which was small. The working potential observed with crude glycerol produced using 0.8wt% KOH catalyst, decreased throughout the duration of the study. The working potential observed with crude glycerol produced using 0.8wt% NaOCH₃ catalyst, increased only a marginal amount throughout the duration of the study.

The samples extracted from the anode each fuel cells on several occasions throughout the study, were compromised, therefore glycerol consumption could not be documented. However, the growth of mixed culture AR2 in closed fermentation bottles on medium identical in composition to the medium used in the anode of MFCs was monitored, and the results are shown in Figure 5.7. Growth was observed in all fermentations, however there was significant variation in the growth observed with triplicate cultures growing on crude glycerol produced using 0.8wt% KOH catalyst. Since fermentations were inoculated with the same

pre-culture as MFCs, and were inoculated to the same protein concentration, it's likely that similar growth would have been observed in both. Given the large amount of variation observed with triplicate cultures, it's possible that there wasn't any significant growth observed with crude glycerol produced using 0.8wt% KOH catalyst, which would offer an explanation for the low power density observed.

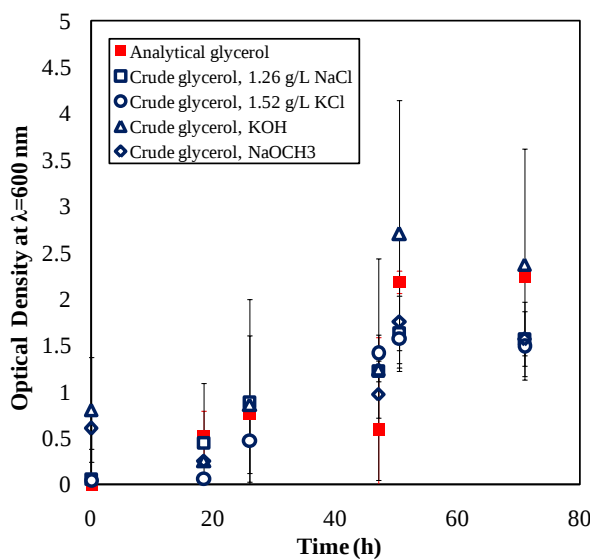


Figure 5.7: Growth observed with mixed culture AR2 growing on crude glycerol that resulted from the use of 0.8wt% KOH and 0.8wt% NaOCH₃ in biodiesel production (*average* $\pm$ *standard deviation*, $n=3$).

The concentration of glycerol in the medium used in this study was twice as large as the concentration used with *P. freudenreichii* ssp. *shermanii*, and three times as large as the concentration used with *E. coli* W3110; the concentration of other compounds in the medium will also follow this trend. The ability of a mixed culture to degrade waste glycerol and produce a current will depend on the bacteria present; in this case, it's likely that the exoelectrogenic bacteria that were dominant when waste glycerol produced using 0.23wt% NaOCH₃ or analytical glycerol was used as fuel, were inhibited by the high concentration of FAME and free fatty acids found in crude glycerol produced using 0.8wt% NaOCH₃ and 0.8wt% KOH catalyst.

5.4.1 Summary

For the mixed culture AR2, MFC performance was found to be related to the concentration of catalyst used in biodiesel production. This claim is strongly supported by the change in working potential observed over the course of the study and the maximum power density achieved. The working potential observed with crude glycerol produced using 0.8wt% NaOCH₃ and 0.8wt% KOH catalyst did not increase over the course of the study. The maximum power density achieved with crude glycerol produced using 0.23wt% NaOCH₃ catalyst and supplemented with either sodium chloride or potassium chloride was significantly greater than the maximum power density achieved with crude glycerol produced using 0.8wt% NaOCH₃ and 0.8wt% KOH catalyst.

Chapter 6. Metabolite analysis

6.1 Introduction

The fermentative metabolism of glycerol can yield a variety of products. The production and sale of value added products through the bioconversion of glycerol has been identified as a potential means of increasing the profitability of biodiesel operations; the market value of crude glycerol is predicted to continue declining as the biodiesel industry continues to grow thereby improving the profit margin (Yazdani and Gonzalez, 2007). Fermentation products such as 1,3-PDO and ethanol currently have a market value greater than that of refined glycerol (ICIS, 2011).

The oxidation of crude glycerol by bacteria in an MFC has the potential to yield valuable metabolites in addition to providing electrical power. However, the nature of power generation in an MFC is complex and highly dependent on the biochemistry of the organisms used and as a result the distribution of metabolites observed in the anode of an MFC versus a traditional fermentation may differ (Logan, 2008). Additionally, crude glycerol from biodiesel production can contain a variety of compounds such as free fatty acids, biodiesel, and high concentrations of salt, which result from the process and materials used in its production and may affect bacterial growth or glycerol metabolism.

The primary objective of the work in this chapter was to identify, quantify and compare the metabolites resulting from glycerol fermentation by *E. coli* W3110, *P. freudenreichii* ssp. *shermanii* and mixed culture AR2. For each culture, differences in the concentration of metabolites observed with each type of crude glycerol used, and differences in the type and concentration of metabolites observed for batch fermentations versus MFCs, were identified.

Batch fermentations were performed in triplicate alongside the fuel cell experiments discussed in Chapter 4 and Chapter 5. MFCs and batch fermentations employed the same medium composition (including salt and glycerol concentration) and used the same inoculum to allow for direct comparison.

6.2 *E. coli* W3110

In the following three studies, the effect of salt and other compounds in crude glycerol on the growth and glycerol metabolism of *E. coli* W3110 was investigated.

6.2.1 Waste glycerol from biodiesel production supplemented with sodium chloride

The growth and glycerol consumption observed with *E. coli* W3110 in fermentations is shown in Figure 6.1. The glycerol consumption observed with *E. coli* W3110 in MFCs is shown in Figure 4.2. Growth was monitored by measuring OD₆₀₀ which was then correlated to protein concentration. Active growth was observed for all fermentations during the first 100h of the study. There were no significant differences in cell growth observed in medium supplemented with analytical glycerol vs. medium supplemented with crude glycerol.

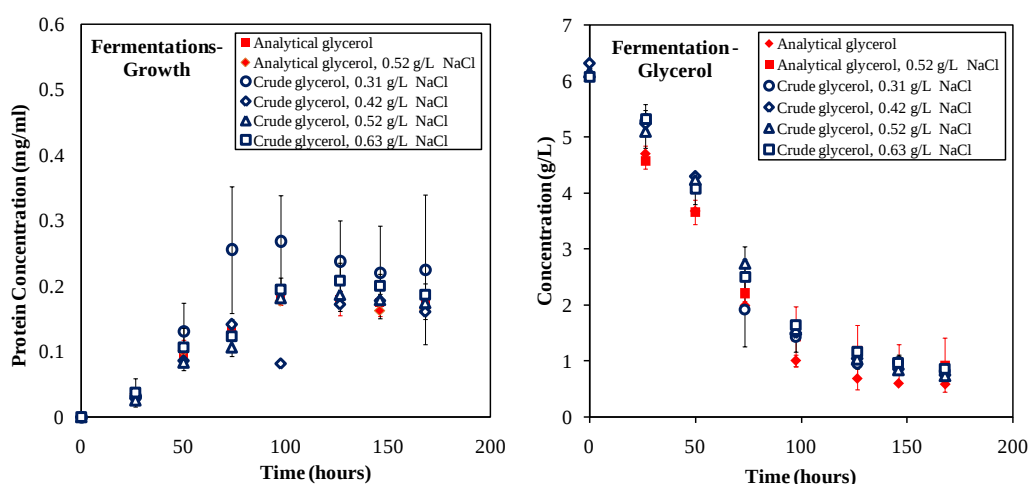


Figure 6.1: Glycerol concentration and growth observed with *E. coli* W3110 growing on crude glycerol supplemented with NaCl. (average $\pm$ standard deviation, $n=3$)

During active growth, the rate of glycerol consumption observed with fermentations was greater than the rate of glycerol consumption observed with MFCs. With fermentations, glycerol consumption was no longer observed 126h after inoculation, whereas with MFCs, glycerol consumption did not cease until 146h after inoculation. The rate of glycerol consumption observed with each of the salt concentrations tested was similar. For all conditions tested, a small amount of glycerol remained unconsumed at the end of the study.

The amount of glycerol that remained unconsumed was greater in MFCs than in fermentations.

The metabolites observed from glycerol fermentation in MFCs and in serum bottle fermentations include succinic acid, acetic acid, ethanol and formic acid. The concentration of succinic acid, ethanol and formic acid at various points throughout the course of the study are shown in Figure 6.2.

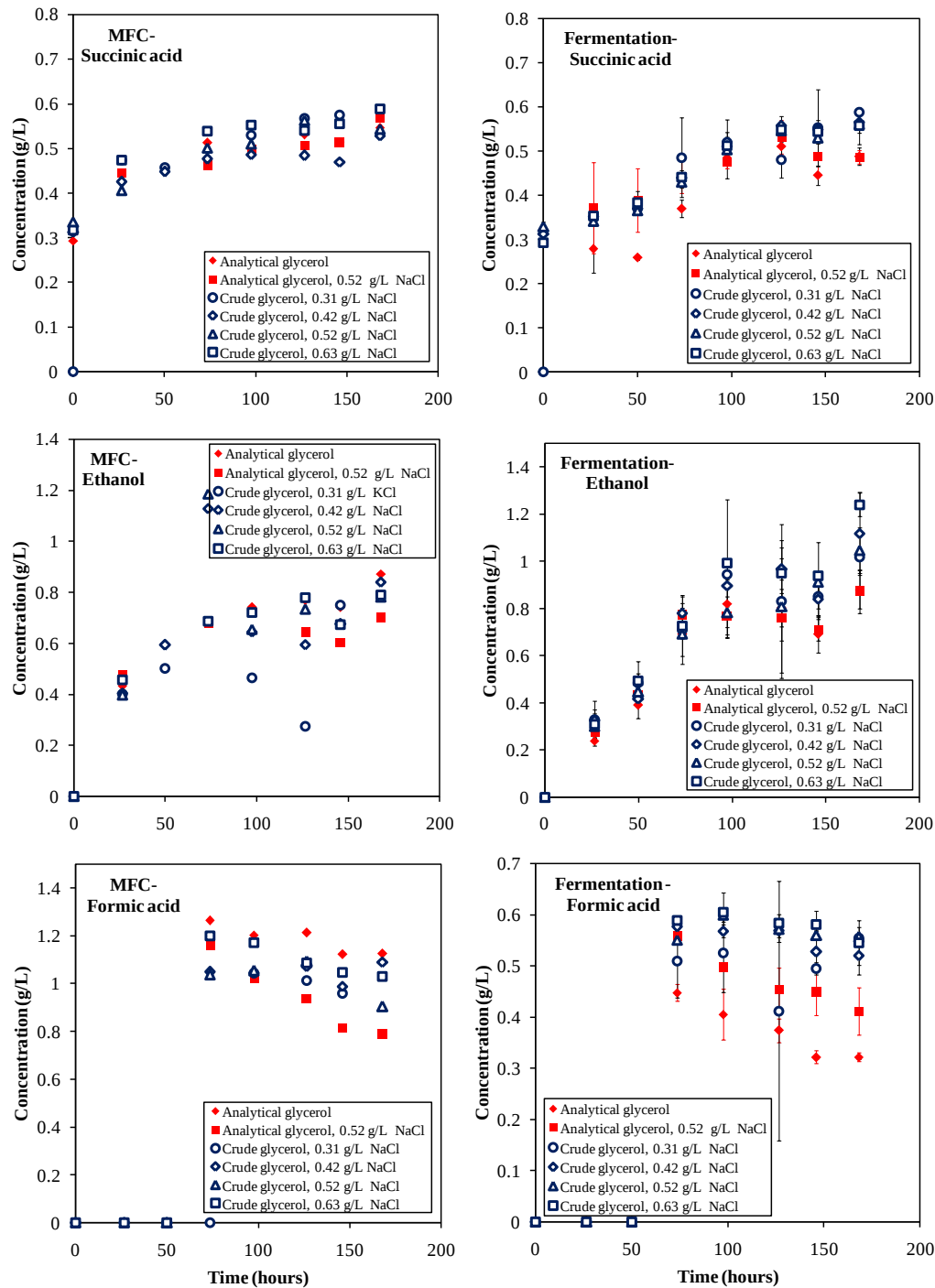


Figure 6.2: Major products of glycerol fermentation observed with *E. coli* W3110 growing on crude glycerol supplemented with NaCl. (average $\pm$ standard deviation, $n=3$).

Succinic acid is a component of tryptone, and was therefore present in the medium prepared for this study. The concentration of succinic acid observed in MFCs and in

fermentations increased over the course of the study, and the change in succinic acid concentration from the beginning to the end of the study was similar in fermentations and MFCs. The concentration of salt in the medium did not affect the final concentration of succinic acid observed, nor did it affect the rate at which it was produced.

Similar to succinic acid, the concentration of acetic acid observed at the end of the study with fermentations and with MFCs was small (data not shown). The change in acetic acid concentration followed the same trend as succinic acid, observing a gradual increase throughout the course of the study; however, the yield of acetic acid per gram of glycerol consumed was smaller in the fermentations than in the MFCs. Yields in the fermentations ranged from $0.031 \pm 0\text{gg}^{-1}$ with the use of analytical glycerol, to $0.049 \pm 0.001\text{gg}^{-1}$ with the use of crude glycerol supplemented to a final NaCl concentration of 0.63gL^{-1} . Yields in the MFCs ranged from 0.069gg^{-1} with the use of crude glycerol supplemented to a final NaCl concentration of 0.63gL^{-1} , to 0.111gg^{-1} with the use of analytical glycerol.

Ethanol was the most prominent metabolite observed in both the MFCs and the fermentations. The concentration of ethanol in fermentations and in MFCs increased initially coinciding with the rapid consumption of glycerol. For all conditions tested, the concentration of glycerol stopped increasing 96h after inoculation, remaining relatively unchanged throughout the remainder of the study. The concentration of ethanol observed at the end of the study was greater in fermentations than in MFCs however this difference in concentration is less apparent when the yield of ethanol per gram glycerol fermented is considered. In the fermentations, the yield of ethanol per gram of glycerol fermented 168h into the study ranged from $0.162 \pm 0.015\text{gg}^{-1}$, observed with the use of analytical glycerol, to $0.233 \pm 0.010\text{gg}^{-1}$, observed with the use of crude glycerol supplemented to a final NaCl concentration of 0.63gL^{-1} . In the MFCs, the yield ranged from 0.168gg^{-1} , observed with the use of crude glycerol supplemented to a final NaCl concentration of 0.52gL^{-1} , to 0.207gg^{-1} , observed with the use of crude glycerol supplemented to a final NaCl concentration of 0.42gL^{-1} . With fermentations, the final yield of ethanol was greater with the use of crude glycerol than with the use of analytical glycerol. The concentration of salt did not affect the final yield of ethanol observed with MFCs or with fermentations.

With MFCs and fermentations, the change in concentration of formic acid over the course of the study did not follow the same trend as many of the other metabolites. Formic acid did not appear in any samples until late exponential phase (76h after inoculation), after which the concentration decreased at a constant rate for the remainder of the study. The concentration of formic acid observed throughout the duration of the study was significantly greater with MFCs than with fermentations. The concentration of sodium chloride in the medium did not have an effect on the rate of formic acid production throughout the course of the study.

The compounds observed in this study agree with those identified in literature as products of glycerol fermentation by *E. coli*. In the absence of external electron acceptors, glycerol is metabolized by *E. coli* via mixed acid fermentation (further detail provided in section 2.3 and Figure 2.7). This highly branched pathway allows for the production of a wide variety of compounds, including succinic acid, acetic acid, formic acid, H₂, CO₂, ethanol and 1,2- PDO.

6.2.2 Waste glycerol from biodiesel production supplemented with potassium chloride

The growth and glycerol consumption observed with *E. coli* W3110 in fermentations is shown in Figure 6.3. The glycerol consumption observed with *E. coli* W3110 in MFCs is shown in Figure 4.4. Active growth was observed for all fermentations during the first 100h. Although the final concentration of cells observed in medium supplemented with crude glycerol was greater than the final concentration of cells observed in medium supplemented with analytical glycerol, given the large degree of variation among triplicate cultures this conclusion is not statistically significant.

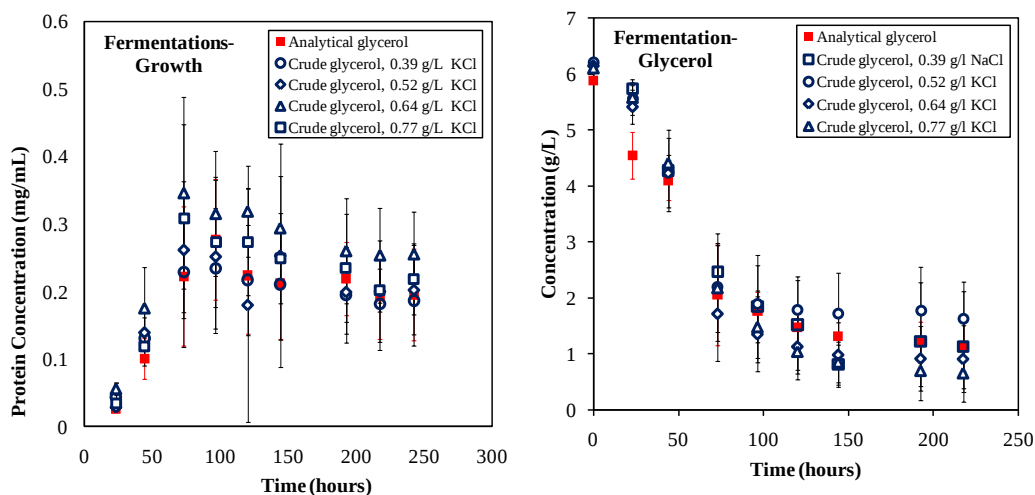


Figure 6.3: Glycerol concentration and growth observed with *E. coli* W3110 growing on crude glycerol supplemented with potassium chloride. (*average $\pm$ standard deviation, $n=3$*)

Similar to the previous study using NaCl, in experiments with KCl the rate of glycerol consumption observed with fermentations was greater than the rate of glycerol consumption observed with MFCs during active growth. For all conditions tested, a small amount of glycerol remained unconsumed at the end of the study; this amount was greater with MFCs than with fermentations. The concentration of salt in the medium did not have an effect on the rate of glycerol consumption observed.

As anticipated, the metabolites observed from glycerol fermentation in this study were identical to those observed in the previous study with NaCl. Metabolites observed from glycerol fermentation included succinic acid, acetic acid, ethanol and formic acid. The concentration of succinic acid, ethanol and formic acid at various points throughout the course of the study are shown in Figure 6.4.

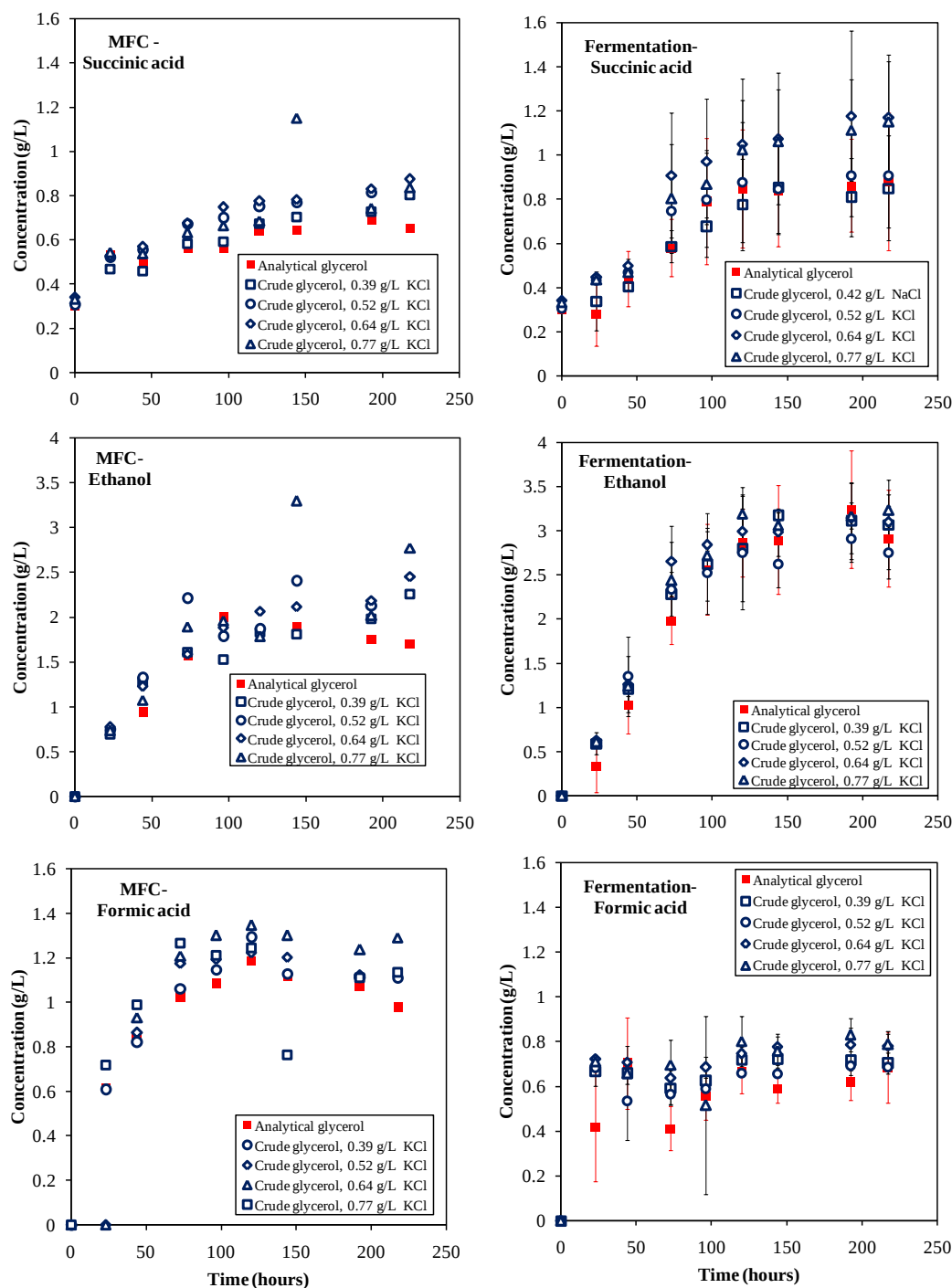


Figure 6.4: Major products of glycerol fermentation observed with *E. coli* W3110 grown on crude glycerol supplemented with KCl. (average $\pm$ standard deviation, $n=3$)

Many of the trends related to the shape of metabolite concentration profiles observed in the previous study with NaCl were also apparent in this study. The concentration profiles observed for succinic acid, acetic acid and ethanol over the duration of the study with MFCs

and fermentations were the same as the profiles observed for these compounds in the previous study. The concentration of succinic acid and acetic acid gradually increased throughout the course of the study whereas the concentration of ethanol increased during the initial stages of the study but remained unchanged for the remainder of the study as glycerol consumption slowed.

Similar to the previous study with NaCl, the concentration of salt in the medium did not have an effect on the maximum concentration of any of the metabolites observed nor on the change in metabolite concentration over the course of the study. However, the use of crude glycerol in place of analytical glycerol yielded, on average, greater concentrations of succinic acid, formic acid and acetic acid throughout the duration of the study.

Unlike the previous study with NaCl, the succinic acid yield per gram of glycerol fermented was marginally greater with fermentations than with MFCs, however the accuracy of this measurement is questionable as a large amount of variation was observed between triplicate cultures.

At the end of the study, the yield of acetic acid observed in the MFCs was slightly greater than the yield of acetic acid observed in the fermentations (data not shown). In the fermentations, the yield of acetic acid per gram of glycerol fermented ranged from $0.093 \pm 0.021 \text{ gg}^{-1}$, observed with the use of crude glycerol supplemented to a final KCl concentration of 0.77 gL^{-1} , to $0.110 \pm 0.027 \text{ gg}^{-1}$, observed with the use of crude glycerol supplemented to a final KCl concentration of 0.52 gL^{-1} . In the MFCs, the yield ranged from 0.106 gg^{-1} , observed with the use of analytical glycerol to 0.154 gg^{-1} observed with the use of crude glycerol supplemented to a final KCl concentration of 0.52 gL^{-1} .

Similar to the previous study with NaCl, the concentration of ethanol observed with fermentations was greater than the concentration of ethanol observed with MFCs, throughout the study. When considering the amount of ethanol produced per gram of glycerol fermented, the difference between the two conditions is still apparent, unlike the previous study. In fermentations the yield of ethanol per gram of glycerol fermented ranged from $0.587 \pm 0.040 \text{ gg}^{-1}$, observed with the use of crude glycerol supplemented to a final KCl concentration of 0.77 gL^{-1} , to $0.695 \pm 0.153 \text{ gg}^{-1}$, observed with the use of analytical glycerol.

The ethanol yield in MFCs ranged from 0.380gg^{-1} , observed with the use of analytical glycerol, to 0.550gg^{-1} , observed with the use of crude glycerol supplemented to a final KCl concentration of 0.64gL^{-1} .

The concentration profile observed for formic acid was different with MFCs than with fermentations. With fermentations, the concentration of formic acid remained constant throughout the duration of the study. With MFCs, the concentration of formic acid increased during the initial stages of the study then decreased after 120h. The maximum concentration of formic acid observed with MFCs was greater than the maximum concentration observed with fermentations.

6.2.3 Waste glycerol from biodiesel production at high catalyst concentrations

The growth and glycerol consumption observed with *E. coli* W3110 in fermentations is shown in Figure 6.5. The glycerol consumption observed with *E. coli* W3110 in MFCs is shown in Figure 5.2. Active growth was observed for all fermentations during the first 96h of the study. The biomass concentration achieved with the use of crude glycerol, produced using 0.8wt% KOH and 0.8wt% NaOCH_3 catalysts in biodiesel production, was greater than the concentration observed with the use of crude glycerol produced by employing 0.23wt% NaOCH_3 , and supplemented with 0.42gL^{-1} NaCl or 0.52gL^{-1} KCl.

A possible explanation for this phenomenon is that the free fatty acids found in the medium acted as an additional source of energy for bacteria, allowing a greater population density to be achieved. Further experimentation would be required to confirm the concentration of each compound in each type of crude glycerol, and to confirm whether *E. coli* W3110 is capable of metabolizing free fatty acids.

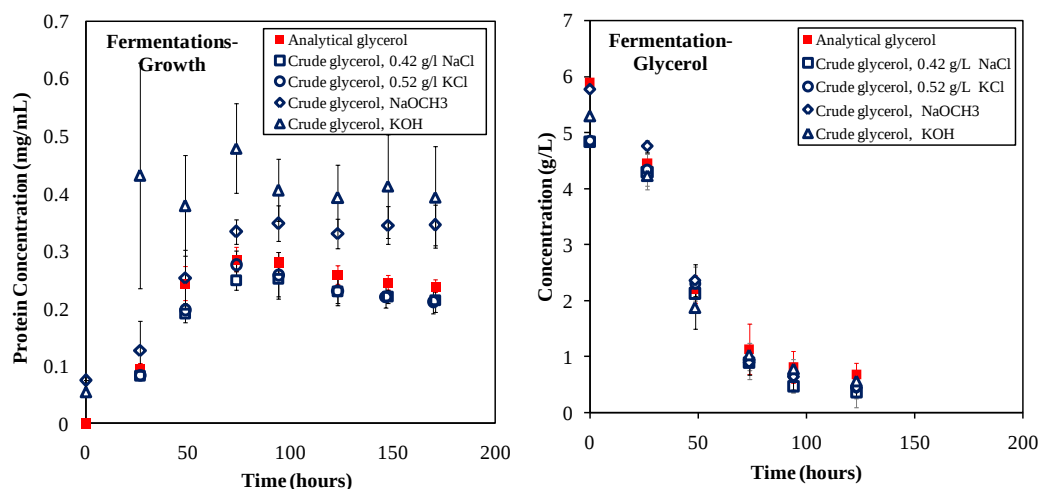


Figure 6.5: Glycerol concentration and growth observed with *E. coli* W3110 growing on crude glycerol from biodiesel produced using 0.8wt% KOH and 0.8wt% NaOCH₃ as catalysts. (average $\pm$ standard deviation, $n=3$)

Similar to the previous studies where the effects of NaCl and KCl on glycerol metabolism were investigated, the rate of glycerol consumption observed with fermentations was greater than the rate of glycerol consumption observed with MFCs during active growth. The rate of glycerol consumption was similar for each of the different glycerol species included in the study. For all conditions tested, a small amount of glycerol remained unconsumed at the end of the study (123h after the inoculation of both serum bottle fermentations and MFCs).

The concentration profiles observed for succinic acid, acetic acid, and ethanol over the duration of the study for fermentations and MFCs (shown in Figure 6.6) were the same as the profiles observed for these compounds in the previous two studies. The concentration profile observed for formic acid in this study was similar to the concentration profile observed for formic acid in the previous study with sodium chloride.

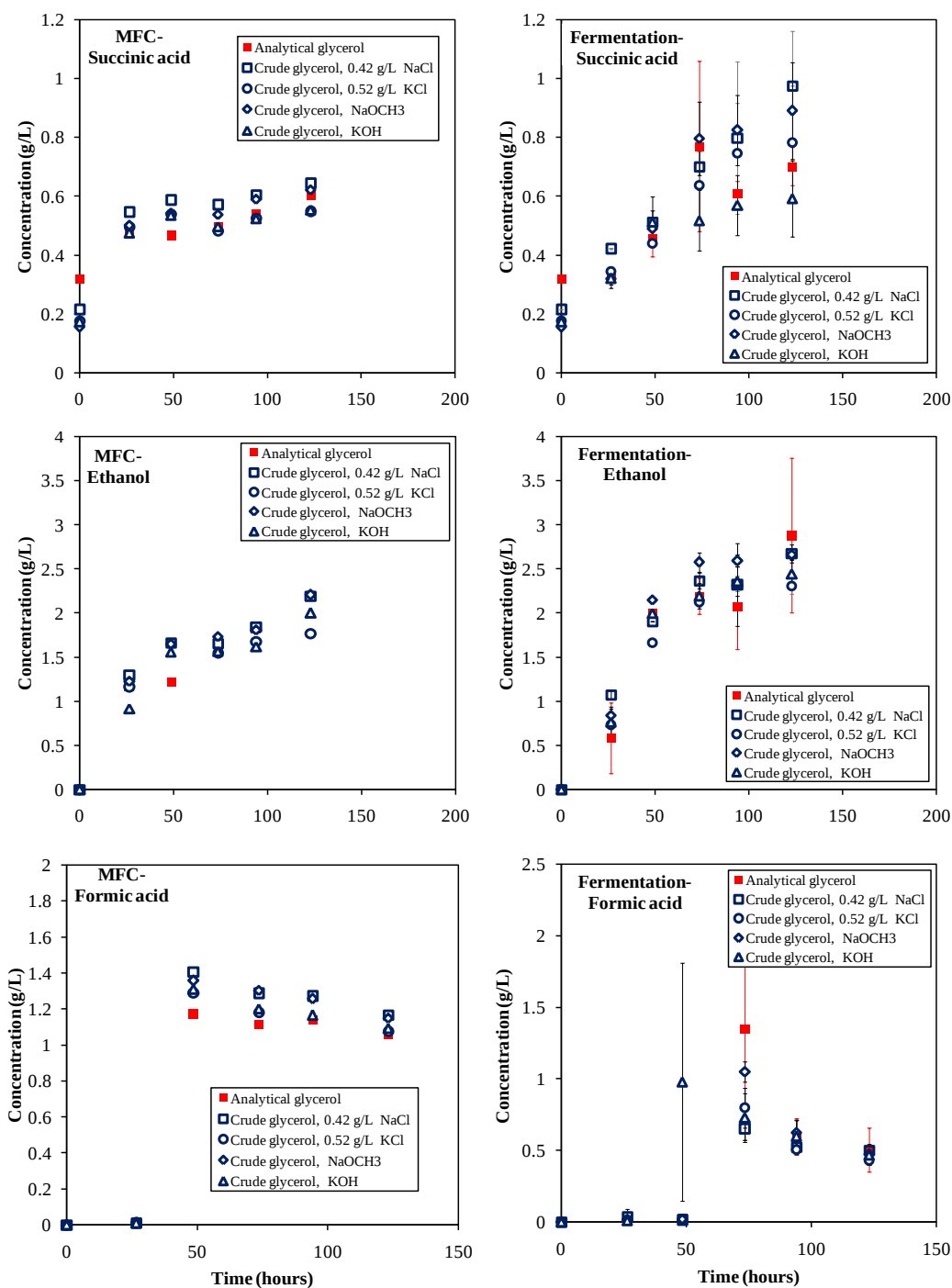


Figure 6.6: Major products of glycerol fermentation observed with *E. coli* W3110 growing on crude glycerol from biodiesel produced using 0.8wt% KOH and 0.8wt% NaOCH₃, as catalysts. (average $\pm$ standard deviation, $n=3$)

Following the same trend as the previous study with KCl, the final yield of succinic acid per gram of glycerol consumed was slightly greater with fermentations than MFCs. In

the first study, where the effect of NaCl concentration on glycerol metabolism was investigated, the concentration of succinic acid was found to be similar in fermentations and in MFCs.

The final yield of ethanol and the maximum concentration of formic acid observed was greater in fermentations than in MFCs, and the final yield of acetic acid was similar in MFCs and fermentations; these trends were also evident in both previous studies that used *E. coli* W3110.

Acetic acid yields in the fermentations ranged from $0.079 \pm 0.004 \text{ gg}^{-1}$ with the use of analytical glycerol, to $0.177 \pm 0.047 \text{ gg}^{-1}$ with the use of crude glycerol supplemented to a final NaCl concentration of 0.42 gL^{-1} . Acetic acid yields in the MFCs ranged from 0.099 gg^{-1} with the use of analytical glycerol, to 0.126 gg^{-1} with the use of crude glycerol supplemented to a final NaCl concentration of 0.52 gL^{-1} . Ethanol yields in the fermentations ranged from $0.510 \pm 0.035 \text{ gg}^{-1}$ with the use of crude glycerol, produced using 0.8wt% NaOCH_3 , to $0.601 \pm 0.044 \text{ gg}^{-1}$ with the use of crude glycerol supplemented to a final NaCl concentration of 0.42 gL^{-1} . Ethanol yields in the MFCs ranged from 0.458 gg^{-1} with the use of crude glycerol supplemented to a final KCl concentration of 0.52 gL^{-1} , to 0.588 gg^{-1} with the use of crude glycerol supplemented to a final NaCl concentration of 0.42 gL^{-1} .

The type of glycerol used did not have any effect on the concentration of metabolites observed with fermentations and with MFCs. Therefore, it can be concluded that at the concentrations observed in M9 medium, the components of crude glycerol obtained from biodiesel produced using 0.8wt% NaOCH_3 and 0.8wt% KOH as catalysts did not affect the concentration of metabolites observed at various points throughout the study. Since the concentration of compounds in the medium that originate from waste glycerol are dependent on the amount of glycerol added, it is possible that different results may be observed if the concentration of glycerol used is increased from 5 gL^{-1} .

6.2.4 Summary

In all three studies, several trends were apparent: the maximum concentration of formic acid observed in fermentations was greater than that observed in MFCs; the yield of ethanol per gram of glycerol consumed in fermentations was greater than that observed in

MFCs; the rate of glycerol consumption and total amount consumed was greater with fermentations than with MFCs; and the concentration (in the medium) of salt and other compounds found in waste glycerol did not have an effect on glycerol metabolism. In two of three studies, the yield of acetic acid per gram of glycerol consumed was greater with MFCs than with fermentations.

6.2.5 Discussion

In all three studies with *E. coli* W3110, the yield of various metabolites was different with MFCs and fermentations. Differences in the yield of metabolites observed with fermentations versus MFCs was expected; the transfer of electrons from bacteria to the anode of an MFC can be accomplished by two different mechanisms which can involve a wide range of redox-active compounds and the electron transfer mechanisms used by bacteria and the compounds involved will affect metabolite yield (Logan, 2008; Schröder, 2007).

The transfer of electrons from bacteria to the anode of an MFC can be accomplished by direct electron transfer or mediated electron transfer. Direct electron transfer occurs through the contact of a membrane bound protein or electronically conductive pili (nanowires) with the anode, whereas mediated electron transfer relies on redox species capable of diffusing into and out of the cell to accomplish the transfer of electrons to the anode surface (Logan, 2008; Logan et al., 2006; Schröder, 2007).

Mediated electron transfer can be accomplished via secondary metabolites or primary metabolites. Using secondary metabolic pathways, bacteria can produce low molecular weight compounds (such as pyocyanine) that are capable of shuttling electrons to the anode, or can use externally available compounds (such as humic acid) to achieve the same objective. Primary metabolites are directly associated with substrate oxidation and can result from anaerobic respiration or fermentation. For bacteria that oxidize substrates via anaerobic respiration, any terminal electron acceptor that has a reduction potential more negative than that of oxygen and is soluble in its reduced and oxidized forms has the potential to achieve electron transfer from the bacteria to the anode. For bacteria that oxidize substrates via fermentation, metabolites such as formic acid and ethanol can be further oxidized in the presence of an electrocatalytic anode (Schröder, 2007). Further experimentation would be

required to identify the nature of electron transfer occurring in the MFCs included in this study.

Formic acid can be used to as a substrate in the production of carbon dioxide and hydrogen gas in a reaction catalyzed by formate hydrogen lyase (FHL). In the absence of FHL activity, the molar amount of formic acid observed should equal the combined molar amounts of ethanol and acetic acid observed. The activity of FHL is regulated by several factors; it is not observed in the presence of the external electron acceptors nitrate and oxygen, and is reduced at an alkaline pH (Dharmadi et al., 2006). Dharmadi et al. (2006) found that the optimal pH for the conversion of formic acid to CO₂ was 6.3; and that conversion significantly decreased above a pH of 8 in *E. coli*. The activity of FHL was determined to be necessary for cell growth and glycerol fermentation to proceed. Carbon dioxide must be available to *E. coli* for use in the synthesis of small molecules, and for central metabolism (Lacoursiere et al., 1986; Repaske and Clayton, 1978).

pH was not monitored in any of the three studies; however, it is possible to hypothesize that the pH of the anodic medium in MFCs was, on average, more alkaline than the medium in serum bottle fermentations. The use of a NafionTM membrane to separate the two MFC chambers allows for the transport of positively charged ions from one chamber to the other. Although it has been shown by that the majority of positive species transported from the anode to the cathode to balance the charges in the two chambers are not protons, the transport of some protons still helps to main the pH of the anode closer to neutral than in serum bottle fermentations, where the accumulation of protons due to the production of organic acids cannot be alleviated (Rozendal et al., 2006).

The production of ethanol from glycerol is a redox balanced process; each mole of glycerol fermented yields two moles of reducing equivalents in the form of NADH (one from the reduction of glycerol to dihydroxyacetone and one from glycolysis) which are consumed to yield one mole of ethanol (Gadd, 2008). Since ethanol is a highly reduced compound, and current was observed with MFCs in all three studies, a logical conclusion is that use of the anode as a terminal electron acceptor has consumed some of the reducing equivalent that could have been otherwise used in the production of ethanol. However, if this were the case, acetyl-CoA would have been converted to acetic acid, which therefore would have been

observed at concentrations greater than what was observed in MFCs. This imbalance highlights the complex nature of the reactions occurring within the anode chamber of the MFC.

The amount of glycerol that remained unconsumed at the end of the study and the growth experienced was likely impacted by the pH of the medium and the concentration of salts in the medium. Murarka et al.(2008), found that the concentration of phosphate and potassium ions had an effect on the amount of glycerol *E. coli* MG1655 was capable of fermenting. At a pH of 7.5, approximately 6gL^{-1} of glycerol was consumed in a fermentation that used a minimal medium modified from the MOPS medium supplemented with 10gL^{-1} glycerol and 2gL^{-1} tryptone. When this medium was supplemented with 128mM KCl only 3gL^{-1} of glycerol was consumed. When this medium was supplemented with 34mM NaH_2PO_4 and 64mM K_2HPO_4 (same concentrations found in M9 medium), less than 2gL^{-1} of glycerol was fermented. The amount of glycerol that could have been consumed by *E. coli* W3110 in all three studies might have been greater if the concentration of potassium and phosphate were reduced. However, the amount of potassium chloride with which the medium was supplemented in the current work was minimal (0.39 gL^{-1} - 0.77gL^{-1}), and likely did not affect glycerol consumption. The reduced glycerol consumption that was observed with MFCs when compared to fermentations could have been the result of a more neutral pH.

As a species, *E. coli* is hardy and easy to grow, which supports the fact that there were no inhibitory effects associated with the use of crude glycerol in place of analytical grade glycerol. *E. coli* has been shown in previous studies to successfully ferment crude glycerol from biodiesel production (Shams Yazdani and Gonzalez, 2008).

1,2-PDO has been identified as a product of glycerol fermentation by *E. coli*; however, it was not observed in any of the samples collected. Found at a concentration of 0.0114gL^{-1} in a study by (Murarka et al., 2008), it is possible that 1,2- PDO was present in the samples but at a concentration below the detection limit of HPLC. Production of 1,2-PDO is hypothesised to be important for maintaining redox balance within the cell by consuming the reduction equivalents generated during cell growth.

6.3 *P. freudenreichii* ssp. *shermanii*

In the following three studies, the effect of salt and other compounds in crude glycerol on the growth and glycerol metabolism of *P. freudenreichii* ssp. *shermanii* was investigated.

6.3.1 Waste glycerol from biodiesel production supplemented with sodium chloride

The growth and glycerol consumption observed with *P. freudenreichii* ssp. *shermanii* in fermentations is shown in Figure 6.7. The glycerol consumption observed with *P. freudenreichii* ssp. *shermanii* in MFCs is shown in Figure 4.6.

Active growth was only observed with the use of analytical glycerol; however, large variation was observed between triplicate cultures. Similar growth was observed in two of the three serum bottle fermentations, yet growth in the third lagged behind. At the end of the study (222h after the inoculation of fermentations and MFCs), active growth was observed with the use of analytical glycerol supplemented with 1.27g/L^{-1} NaCl; however, similar to the results observed with the use of analytical glycerol, large variation between triplicate cultures was also observed.

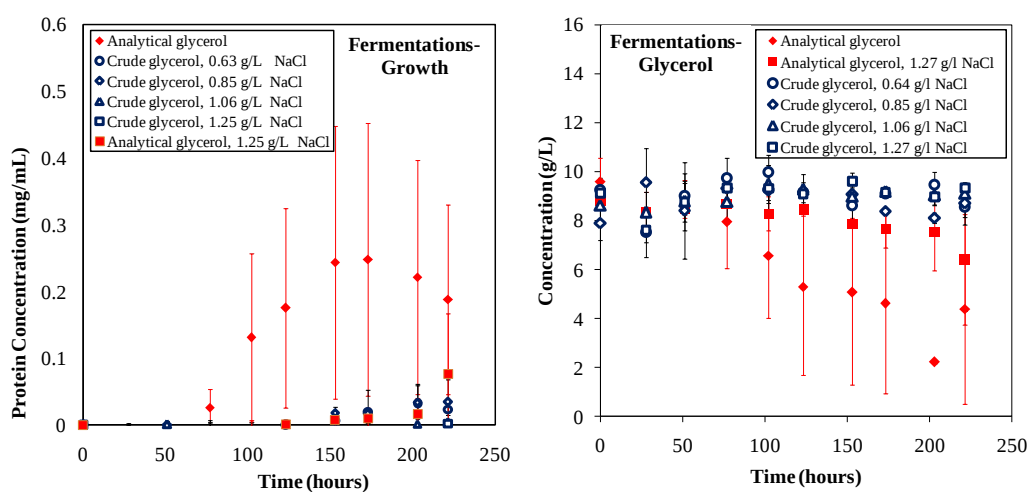


Figure 6.7: Glycerol concentration and growth observed with *P. freudenreichii* ssp. *shermanii* growing on crude glycerol supplemented with sodium chloride. (average $\pm$ standard deviation, $n=3$).

Glycerol consumption was only observed with the MFC that used analytical glycerol and with the fermentations that used analytical glycerol supplemented with 1.27gL^{-1} NaCl; however, with fermentations, a significant amount of variation was observed between triplicate cultures. The rate of glycerol consumption does support the observed lack of growth in the fermentations.

The metabolites observed with serum bottle fermentations and with MFCs include acetic acid and propionic acid. The concentration of acetic acid and propionic acid at various points throughout the course of the study are shown in Figure 6.8.

Due to the lack of growth and glycerol consumption observed in this study for most fermentations and MFCs, an increase in acetic acid and propionic acid concentration was only observed with the fermentation that used analytical glycerol and with the MFC that used analytical glycerol supplemented to a final NaCl concentration of 1.27gL^{-1} . With fermentations, a large degree of variation was observed between triplicate cultures.

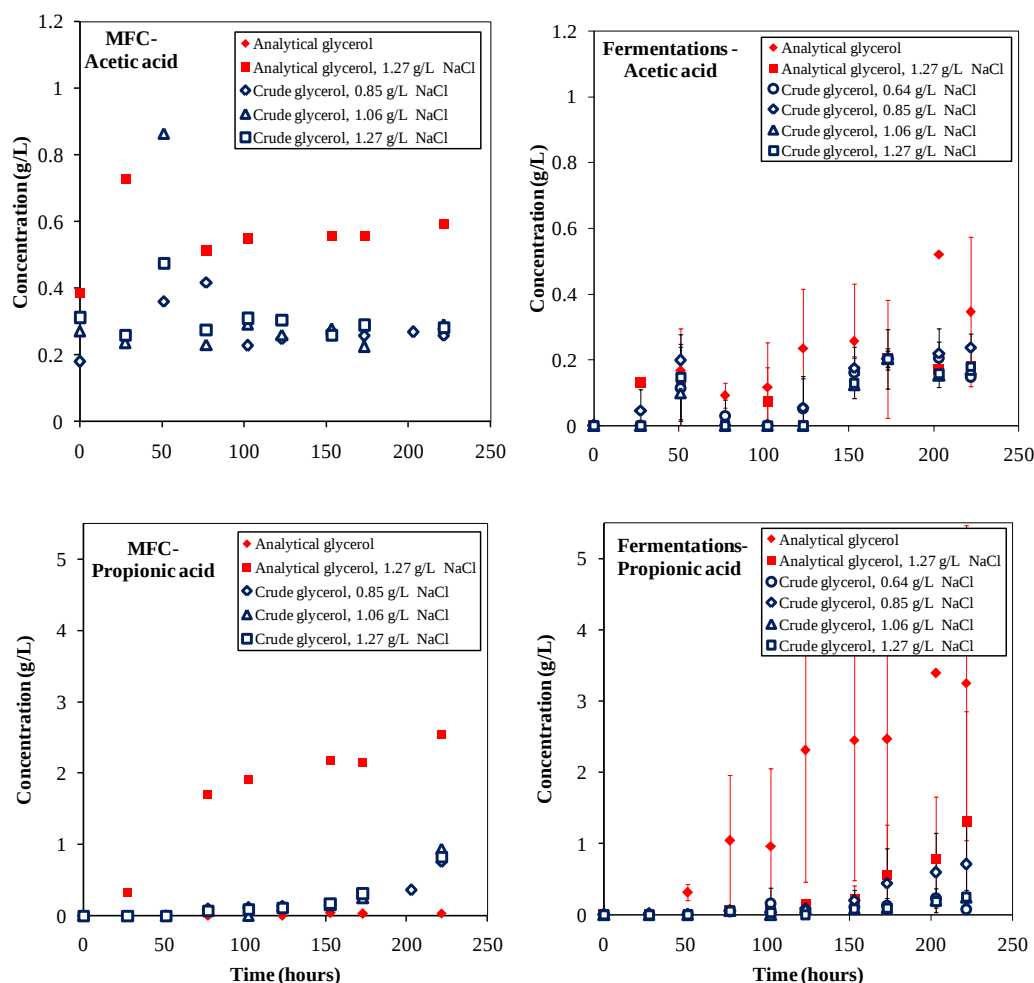


Figure 6.8: Major products of glycerol fermentation observed with *P. freudenreichii* ssp. *shermanii* growing on crude glycerol supplemented with NaCl. (average $\pm$ standard deviation, $n=3$)

The initial concentration of acetic acid observed in the anodic medium of MFCs ranged from 0.179g/L^{-1} to 0.388g/L^{-1} ; however, acetic acid was not observed in the medium used for fermentations. The presence of acetic acid in the anodic medium of MFCs could have resulted from aerobic bacteria that would have had the opportunity to have contaminated the medium prior to inoculation due to the procedures followed when preparing the MFCs and the absence of oxygen scavenger sodium thioglycolate. Propionic acid was the most abundant of the metabolites observed in this study.

6.3.2 Waste glycerol from biodiesel production supplemented with potassium chloride

For cultures growing on crude glycerol supplemented with KCl, the growth and glycerol consumption observed with *P. freudenreichii* ssp. *shermanii* in fermentations is shown in Figure 6.9. The glycerol consumption observed with *P. freudenreichii* ssp. *shermanii* in MFCs is shown in Figure 4.8.

Similar to the previous study with NaCl, glycerol consumption was negligible with most MFCs and fermentations. For most MFCs, a decrease in the concentration of glycerol in the anodic medium was observed from the time of inoculation until the first sample was taken 40h later, after which the concentration of glycerol remained constant throughout the remainder of the study. Little growth was observed in the fermentations, which was expected given the lack of glycerol consumption that was observed. In previous studies, under identical conditions, *P. freudenreichii* ssp. *shermanii* was able to completely consume 10gL^{-1} of glycerol within 200h (Reiche, 2012). Given that the behaviour of *P. freudenreichii* ssp. *shermanii*, when analytical glycerol is used as a carbon source, differs significantly from what was observed in previous studies, it's difficult to make any conclusions regarding the ability of *P. freudenreichii* ssp. *shermanii* to metabolise crude glycerol from biodiesel production. This statement also applies to the previous study, where growth was observed in MFCs with the use of analytical glycerol supplemented to a final NaCl concentration of 1.27gL^{-1} , and not with the use of analytical glycerol.

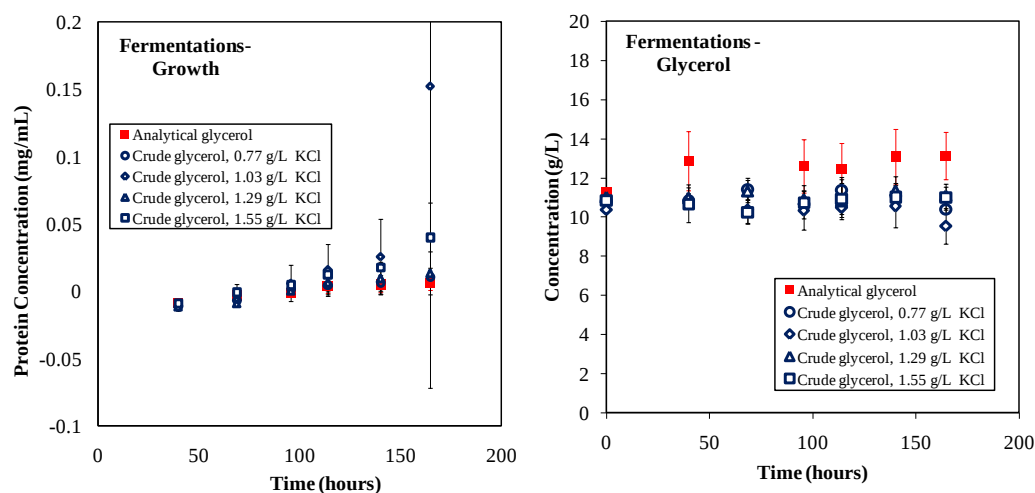


Figure 6.9: Glycerol concentration and growth observed with *P. freudenreichii* ssp. *shermanii* growing on crude glycerol supplemented with potassium chloride. (average $\pm$ standard deviation, $n=3$)

Since only a small amount of glycerol was consumed, the resulting concentration of metabolites observed with fermentations and MFCs at the end of the study was small. The concentration of acetic acid and propionic acid at various points throughout the course of the study are shown in Figure 6.10. After an initial increase in concentration that occurred in the hours after inoculation, the concentration of acetic acid observed with MFCs and fermentations remained constant throughout the course of the study. The concentration of propionic acid observed with MFCs and fermentations did increase over the course of the study however not enough to allow for easy comparison and identification of trends.

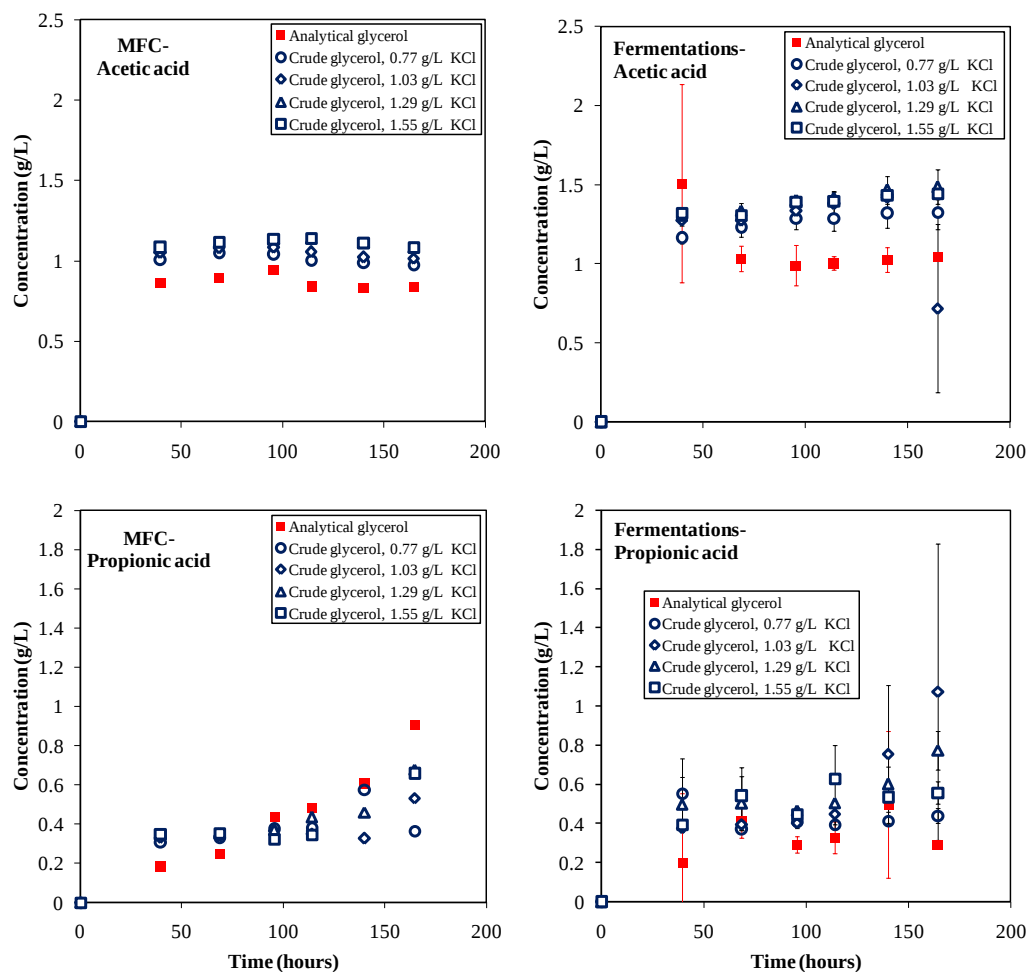


Figure 6.10: Major products of glycerol fermentation observed with *P. freudenreichii* ssp. *shermanii* growing on crude glycerol supplemented with potassium chloride. (average $\pm$ standard deviation, $n=3$)

6.3.3 Waste glycerol from biodiesel production at high catalyst concentrations

The growth and glycerol consumption observed with *P. freudenreichii* ssp. *shermanii* in fermentations is shown in Figure 6.11. The glycerol consumption observed with *P. freudenreichii* ssp. *shermanii* in MFCs is shown in Figure 5.4. Similar to the previous studies with NaCl and KCl, a large amount of variation was observed among triplicate cultures; however, unlike previous studies, trends can easily be identified.

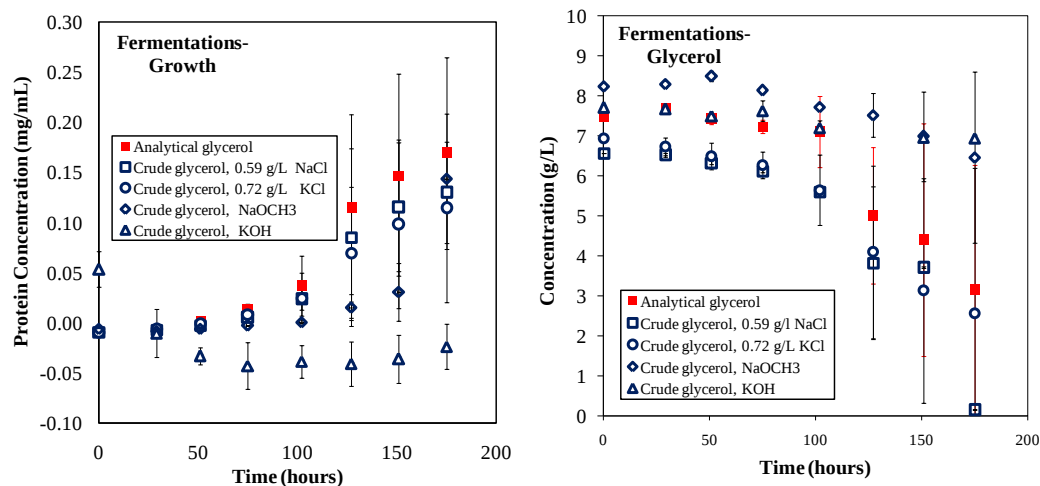


Figure 6.11: Glycerol concentration and growth observed with *P. freudenreichii* ssp. *shermanii* growing on crude glycerol from biodiesel produced using 0.8wt% KOH and 0.8wt% NaOCH₃ as catalysts. (average $\pm$ standard deviation, $n=3$)

A greater growth rate was observed with the use of analytical glycerol and with the use of crude glycerol supplemented to a final NaCl concentration of 0.59gL⁻¹ and KCl concentration of 0.72 gL⁻¹, compared to that observed with the use of crude glycerol obtained by employing 0.8wt% KOH or 0.8wt% NaOCH₃ in biodiesel production. As previously mentioned, the concentration of glycerol in the various types of crude glycerol used throughout the study differed, with the crude glycerol resulting from biodiesel produced using 0.8wt% KOH or 0.8wt% NaOCH₃ being the smallest. Therefore, the results of this study suggest that the concentration of compounds found in waste glycerol (other than salt) have a negative effect on the growth of *P. freudenreichii* ssp. *shermanii*.

With fermentations, the rate of glycerol consumption followed the same trend as the growth observed by each culture; the rate of glycerol consumption was greatest with fermentations that used either analytical glycerol or crude glycerol supplemented with either 0.59gL⁻¹ NaCl or 0.72gL⁻¹ KCl. The rate of glycerol consumption observed with MFCs followed the same trend but at the end of the study differences in the amount consumed were not as pronounced.

Overall, the amount of glycerol that remained in fermentations at the end of the study was greater than the amount that remained in MFCs. Because MFCs and fermentations were

inoculated with different pre-cultures and given the mixed behaviour displayed by *P. freudenreichii* ssp. *shermanii* in the previous two studies, it is difficult to identify to what extent the electrochemical environment affected the rate of glycerol consumption in MFCs.

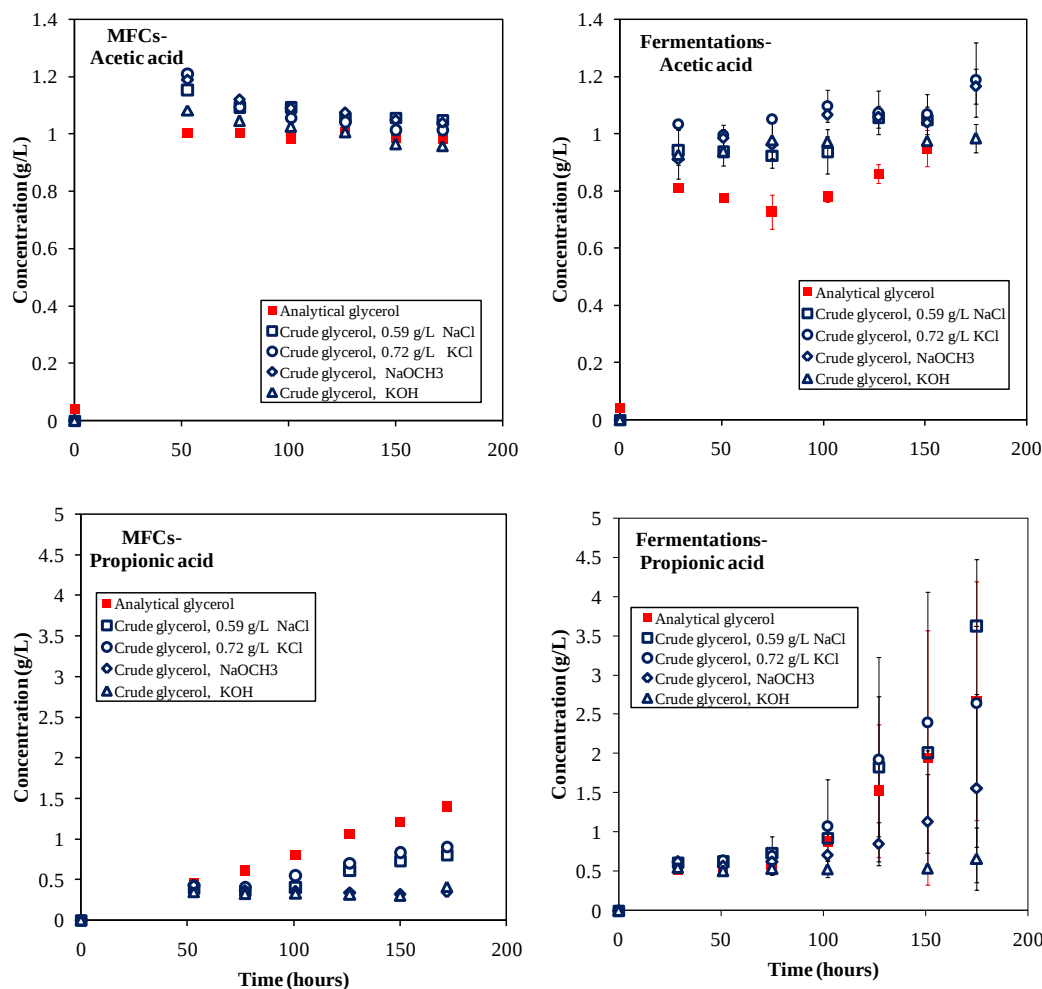


Figure 6.12: Major products of glycerol fermentation observed with *P. freudenreichii* ssp. *shermanii* growing on crude glycerol from biodiesel produced using 0.8wt% KOH and 0.8wt% NaOCH₃, as catalysts (average $\pm$ standard deviation, $n=3$).

Similar to the previous study, the concentration of acetic acid observed with MFCs increased during the initial hours of the study, then stabilized at a concentration of approximately 1g/L^{-1} for the remainder of the study. With MFCs and with fermentations, the concentration of acetic acid observed with the use of analytical glycerol was slightly less than the concentration of acetic acid observed with the use of crude glycerol. This trend was also observed in the previous study with KCl.

The concentration of propionic acid observed at the end of this study was the greatest with fermentations and MFCs that used analytical glycerol or crude glycerol supplemented with NaCl or KCl. With fermentations, the concentration of propionic acid observed with the use of analytical glycerol was $2.675 \pm 1.523 \text{ gL}^{-1}$ whereas the concentration of propionic acid observed with the use of crude glycerol produced using 0.8wt% KOH was only $0.659 \pm 0.39 \text{ gL}^{-1}$. Given that the amount of glycerol consumed differed significantly with MFCs and fermentations, the amount of propionic acid produced per gram of glycerol fermented (shown in Table 15) was calculated to provide a basis of comparison between the two. The amount of propionic acid produced per gram of glycerol fermented was fairly consistent among fermentations, but quite broad among MFCs. Therefore, a conclusion could not be made as to whether there exists any difference in the amount of glycerol converted to propionic acid in MFCs versus fermentations.

Table 15: Propionic acid yield observed with *P. freudenreichii* ssp. *shermanii* growing on medium supplemented with various types of glycerol

Type of glycerol used	Propionic acid yield per gram glycerol fermented(g)	
	MFC	Fermentation(n=3 $\pm$ Std)
Analytical glycerol	0.749	0.616 ± 0.351
Crude glycerol, 0.59 gL^{-1} NaCl	1.082	Not available
Crude glycerol, 0.72 gL^{-1} KCl	0.587	0.605 ± 0.421
Crude glycerol, 0.8 wt% NaOCH ₃	0.192	0.875 ± 0.676
Crude glycerol, 0.8 wt% KOH	0.279	0.860 ± 0.509

6.3.4 Summary

Although it was difficult to draw conclusions from the study where crude glycerol was supplemented with different concentrations of potassium chloride, it was possible to identify trends in both of the other studies. In both studies, propionic acid was the major metabolite observed, however given the quality of the results, whether a significant difference in the yield of propionic acid existed between fermentations and MFCs is unclear.

In the final study, where the effect of compounds in crude glycerol other than salt were investigated, the growth observed with the use of analytical glycerol and with the use of crude glycerol produced using 0.23wt% NaOCH₃ catalyst and supplemented with NaCl or KCl, was greater than the growth observed with the use of crude glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃.

6.3.5 Discussion

P. freudenreichii ssp. *shermanii* has been shown in previous studies to ferment analytical grade glycerol and crude glycerol.

Analytical glycerol was used in a study conducted by Himmi et al. (2000) using *P. freudenreichii* ssp. *shermanii* (ATCC 9614). Using a medium supplemented with 10gL⁻¹ yeast extract, 5gL⁻¹ tryptic soy broth and 20gL⁻¹ glycerol, the propionic acid yield per gram of glycerol fermented was 0.58g (Himmi et al., 2000).

In another study by, the ability of *P. freudenreichii* ssp. *shermanii* (NCIM 5137) to ferment crude glycerol and analytical glycerol was compared. The crude glycerol used in this study was obtained from biodiesel produced using soybean oil, and was partially refined (removal of methanol and salts) before use. The medium used in this study was complex, supplemented with 10gL⁻¹ tryptone, 10gL⁻¹ peptone, 1gL⁻¹ yeast extract and 20gL⁻¹ of either crude or analytical glycerol. In this study the yield of propionic acid per gram of substrate fermented was much greater with the use of crude glycerol (0.63g) than with the use of analytical glycerol (0.31g), however the growth rate observed with the use of analytical glycerol (1.4h⁻¹) was greater than that observed with the use of crude glycerol (0.69h⁻¹) (Ruhel et al., 2011).

Crude glycerol was also used in a literary study employing *P. freudenreichii* ssp. *shermanii* (ATCC 9614). The crude glycerol used in this study was partially refined through the removal of methanol, salts and lipids. The medium used was supplemented with 10gL⁻¹ yeast extract, 5gL⁻¹ trypticase soy broth and 30gL⁻¹ glycerol. The crude glycerol used was supplemented with corn steep liquor which provided a source of nitrogen in addition to trace amounts of lactic acid, acetic acid and xylose. Serum bottle fermentations were inoculated to

an OD₆₀₀ of 3.0 and complete consumption of glycerol was observed within 110h, yielding 0.64 ±0.013g of propionic acid per gram of glycerol fermented (Wang and Yang, 2013).

In the reports discussed above, the yields of propionic acid reported when *P. freudenreichii* ssp. *shermanii* was grown on crude glycerol versus analytical glycerol were inconsistent. In the study by Ruhai et al. (2011), the yield of propionic acid observed with the use of crude glycerol was double that of analytical glycerol; however, in the study by Himmi et al. (2000) where *P. freudenreichii* ssp. *shermanii* was grown on analytical glycerol, the yield of propionic acid was similar to that observed by both Wang and Yang (2013) and Ruhai et al. (2011) when grown on crude glycerol. These values are similar to those observed with the use of analytical glycerol and crude glycerol supplemented with 0.72gL⁻¹ KCl, shown in Table 15.

The rate of glycerol consumption was more rapid in literature than in the work presented in this thesis. Differences in inoculum size or medium composition likely played a role, as medium used in previous studies was not only enriched with yeast extract but with other additives such as peptone and trypticase soy broth, which is itself a complete growth medium. Similar to the study by Ruhai et al. (2011), the rate of growth observed with the use of crude glycerol produced using 0.8wt% NaOCH₃ and 0.8wt% KOH was lower than the rate of growth observed with the use of analytical glycerol.

A possible explanation for the difference in growth rate observed with the use of crude glycerol versus the use of analytical glycerol is that the presence of free fatty acids in the medium has an inhibitory effect on metabolism. In the study conducted by Ruhai et al. (2011), the effect of free fatty acids most abundant in soybean oil (oleic, linoleic, palmitic and stearic) at concentrations of 5gL⁻¹, was investigated. At a concentration of 5gL⁻¹, none of the free fatty acids tested had an effect on growth or product yield. However in a study conducted by (Boyaval et al., 1995), where YEL medium (which contained trypticase broth, yeast extract and sodium lactate at concentrations of 10gL⁻¹ (Malik et al., 1968)) was supplemented with 15v/v% glycerol and 100mgL⁻¹ linoleic acid, growth of *P. freudenreichii* ssp. *shermanii* (ATCC 9614) was mildly inhibited and growth of *P. freudenreichii* ssp. *shermanii* LRTL 30 was significantly inhibited. However, when *P. freudenreichii* ssp.

shermanii LRTL 30 was grown in milk supplemented with 5gL^{-1} linoleic acid, no inhibitory effects were observed due to the presence of lipids.

6.4 Mixed culture AR2

In the following three studies, the effect of salt and other compounds in crude glycerol on the growth and glycerol metabolism of mixed culture AR2 was investigated.

6.4.1 Waste glycerol from biodiesel production supplemented with sodium chloride

The growth and glycerol consumption observed with mixed culture AR2 in fermentations is shown in Figure 6.13. The glycerol consumption observed with AR2 in MFCs is shown in Figure 4.10. Growth was monitored by measuring OD_{600} . With all fermentations, active growth was observed during the first 27h of the study. The stationary phase population density observed was greater with the use of crude glycerol than with the use of analytical glycerol.

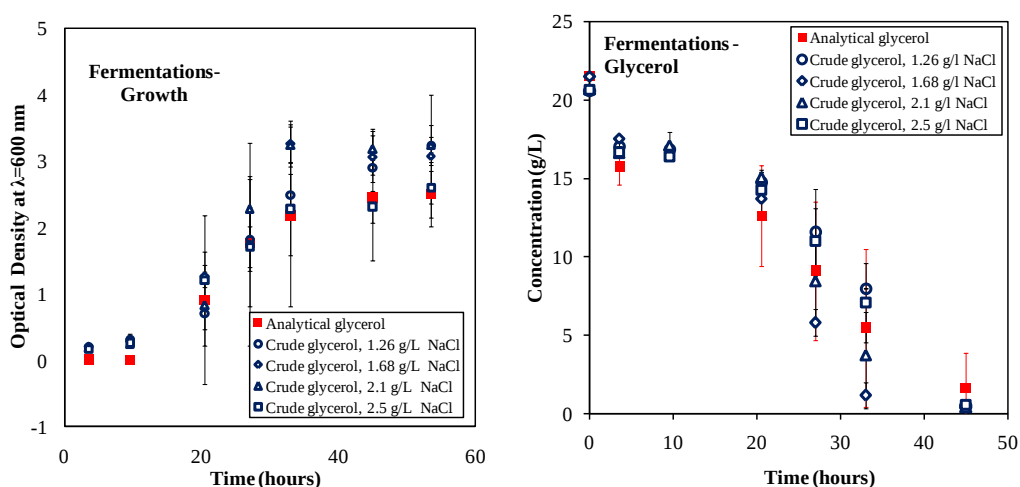


Figure 6.13: Glycerol concentration and growth observed with mixed culture AR2 growing on crude glycerol supplemented with NaCl. (*average $\pm$ standard deviation, $n=3$*)

AR2 showed the greatest rate of glycerol consumption of all the strains studied, leaving very little of the initial 20gL^{-1} unfermented in MFCs and fermentations 45h after inoculation. The concentration of salt in the medium did not have an effect on the rate of glycerol consumption or the amount consumed by the end of the study. The initial rate of

glycerol consumption observed with MFCs was greater than the initial rate of glycerol consumption observed with fermentations.

The metabolites observed from glycerol fermentation in MFCs and in serum bottle fermentations include succinic acid, acetic acid, ethanol, formic acid and 1,3-PDO. The concentration of succinic acid and ethanol at various points throughout the course of the study are shown in Figure 6.14.

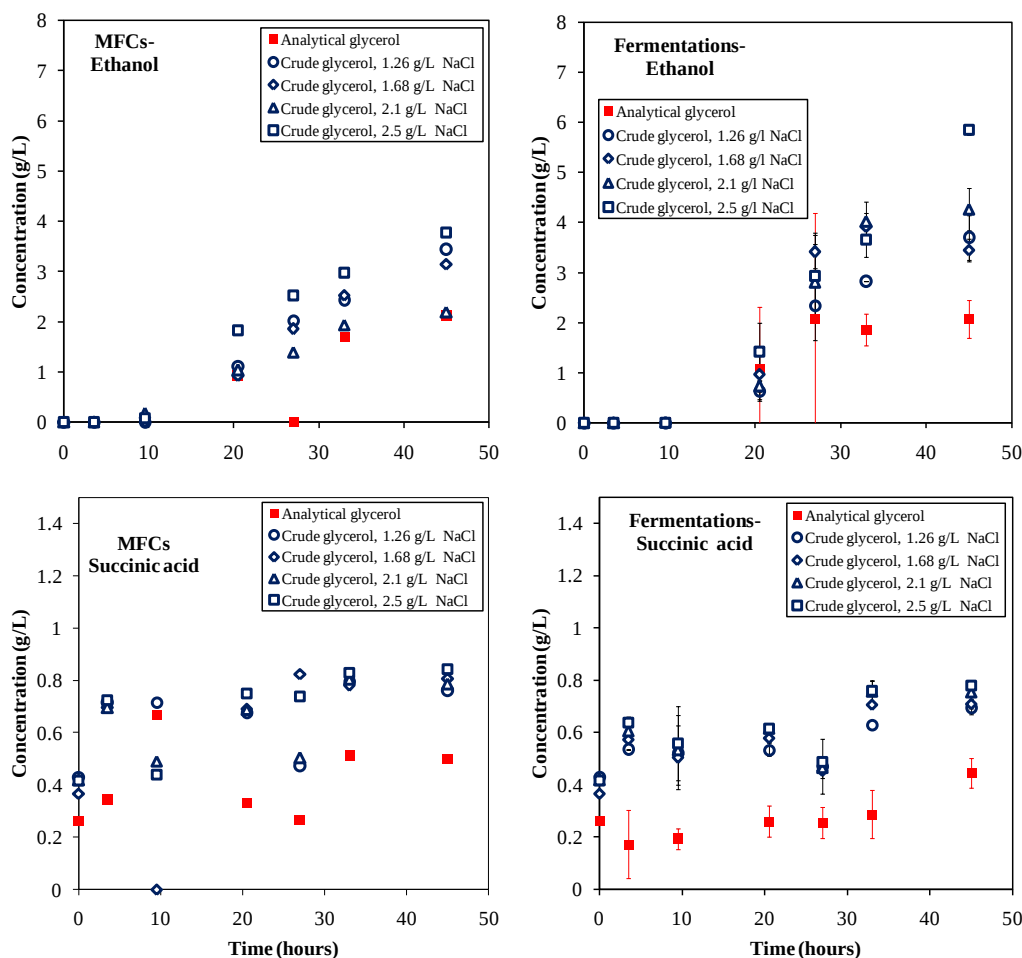


Figure 6.14: Ethanol and succinic acid concentration observed with mixed culture AR2 grown on crude glycerol supplemented with NaCl. (*average $\pm$ standard deviation, $n=3$*)

The concentration of ethanol observed at the end of the study was similar in MFCs and fermentations. In MFCs, the concentration of ethanol observed was dependent on the purity of the glycerol used. Greater amounts of ethanol were observed with the use of crude glycerol over the use of analytical grade glycerol. At the end of the study, with the use of

analytical glycerol, the amount of ethanol generated per gram of glycerol consumed was 0.098gg^{-1} , yet with the use of crude glycerol, the amount of ethanol generated per gram of glycerol consumed ranged from 0.098gg^{-1} to 0.188gg^{-1} . With fermentations, it was difficult to identify whether the differences observed in ethanol yield were a result of salt concentration, glycerol purity, or a combination of both; however, a strong correlation between salt content and ethanol yield was identified (see Figure 6.15). Similar correlations were also observed for succinic acid and acetic acid, but were not observed for 1,3- PDO.

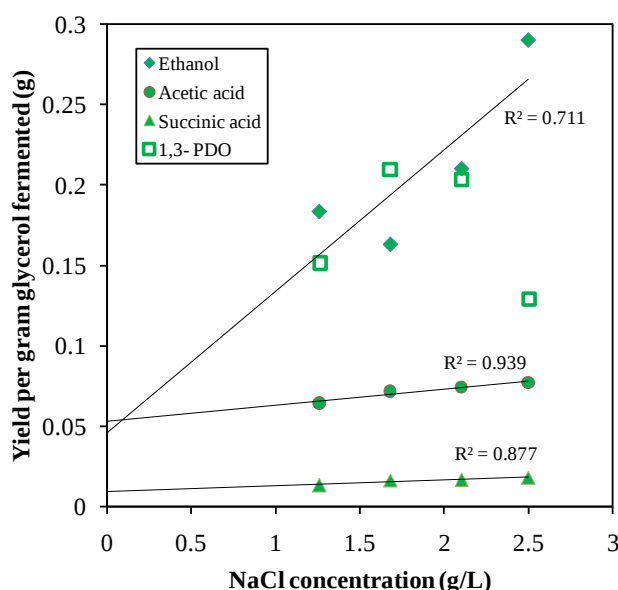


Figure 6.15: Effect of sodium chloride concentration on the yield of various metabolites observed with mixed culture AR2 grown on crude glycerol

The concentration of succinic acid observed at the end of this study was similar in MFCs and fermentations. Of all the metabolites quantified, succinic acid was the least abundant, and was observed at concentrations lower than 1gL^{-1} throughout the course of the study. Although the salt content of the medium appeared to play a role in the yield of succinic acid observed with fermentations, it did not appear to play a role in the yield of succinic acid observed with MFCs. In MFCs, the concentration of succinic acid observed was dependent on the purity of the glycerol used (similar to ethanol). At the end of the study, with the use of analytical glycerol the amount of ethanol generated per gram of glycerol consumed was 0.098gg^{-1} , yet with the use of crude glycerol, the amount of ethanol generated per gram of glycerol consumed ranged from 0.098gg^{-1} to 0.188gg^{-1} .

The concentration of 1,3-PDO and acetic acid at various points throughout the course of the study are shown in Figure 6.16. The concentration of acetic acid observed at the end of the study was greater with MFCs than with fermentations. Similar to the results observed with ethanol and succinic acid, a positive correlation between the concentration of acetic acid and salt in the medium was evident with fermentations, and greater amounts of acetic acid were observed with the use of crude glycerol over the use of analytical grade glycerol.

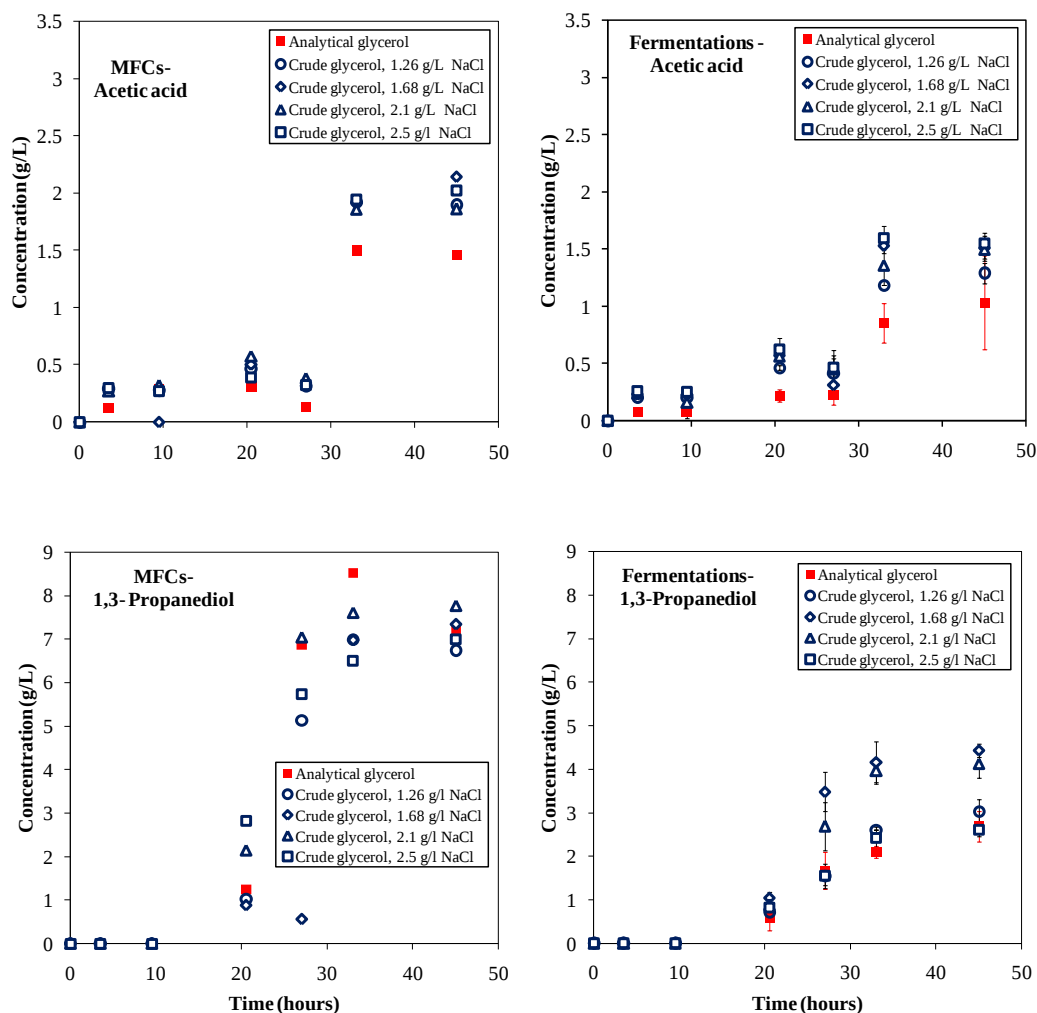


Figure 6.16: 1,3-PDO and acetic acid concentration observed with mixed culture AR2 grown on crude glycerol supplemented with NaCl. (average $\pm$ standard deviation, $n=3$)

The concentration of 1,3-PDO was significantly greater in MFCs than in fermentations. With fermentations, the concentration of 1,3-PDO observed was greater with the use of analytical glycerol; this trend, however, was not observed with MFCs.

The final yield of all products quantified was greater in MFCs than in fermentations at the end of the study. Citric acid, lactic acid, erythritol, butanol, and polyhydroxyalkanoates are examples of compounds that have been observed from glycerol fermentation (Yang et al., 2012), that were not tested for in this study. It is also possible that more of the glycerol was converted to biomass in fermentations than in MFCs.

6.4.2 Waste glycerol from biodiesel production supplemented with potassium chloride

The growth and glycerol consumption observed with mixed culture AR2 in fermentations is shown in Figure 6.17. The glycerol consumption observed with mixed culture AR2 in MFCs is shown in Figure 4.12.

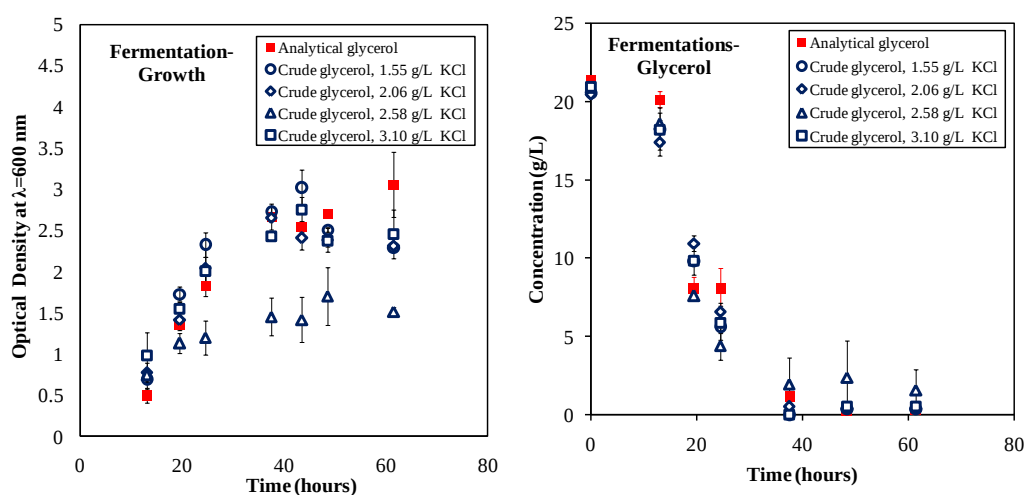


Figure 6.17: Glycerol concentration and growth observed with mixed culture AR2 growing on crude glycerol supplemented with KCl. (*average $\pm$ standard deviation $n=3$*)

With most fermentations, active growth was observed during the first 38 h of the study, and the average OD_{600} observed during stationary phase was slightly less than that observed in the previous study. The growth pattern observed with the use of crude glycerol supplemented to a final KCl concentration of 2.58g/L^{-1} differed from the growth pattern observed with the other fermentations. The growth rate observed with the use of crude glycerol supplemented to a final KCl concentration of 2.58g/L^{-1} was slower than the growth rate observed with all other fermentations.

Sixty two hours after inoculation, a small amount of glycerol remained unconsumed in MFCs and in fermentations. Most of the glycerol was consumed during active growth (the first 38h of the study), and little glycerol was consumed during stationary phase. Similar to the previous study, the initial rate of glycerol consumption was faster in MFCs than in fermentations.

Similar to the previous study, the metabolites observed from glycerol fermentation in MFCs and in serum bottle fermentations include succinic acid, acetic acid, ethanol, formic acid and 1,3-PDO. The concentration of succinic acid and ethanol at various points throughout the course of the study are shown in Figure 6.18.

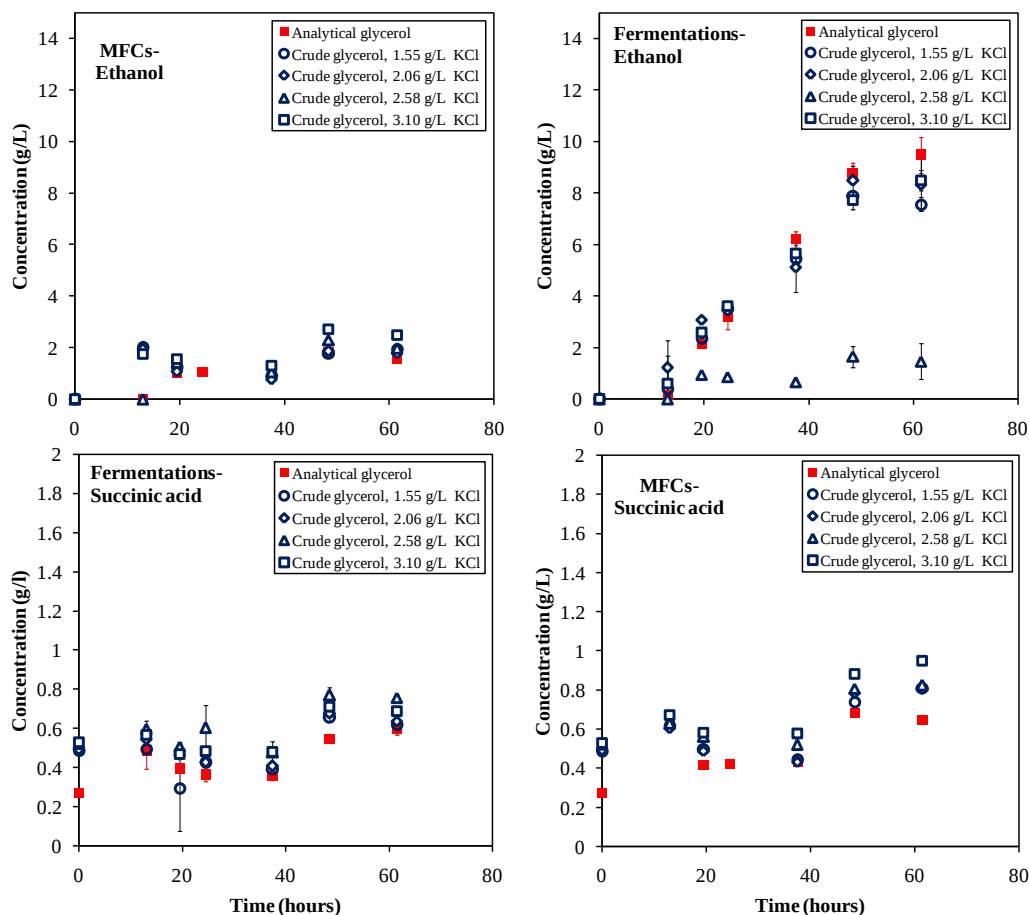


Figure 6.18: Ethanol and succinic acid concentration observed with mixed culture AR2 grown on crude glycerol supplemented with KCl. (average $\pm$ standard deviation, $n=3$)

In this study, there was a significant difference in the final yield of ethanol observed in fermentations and MFCs. With fermentations (excluding that which used crude glycerol

supplemented to a final KCl concentration of 2.58gL^{-1}), the ethanol yield per gram of glycerol fermented ranged from $0.375 \pm 0.013\text{gg}^{-1}$, observed with the use of crude glycerol supplemented to a final KCl concentration of 1.55gL^{-1} , to $0.451 \pm 0.032\text{gg}^{-1}$, observed with the use of analytical glycerol. With MFCs, the ethanol yield per gram of glycerol fermented ranged from 0.078gg^{-1} , observed with the use of analytical glycerol, to 0.128gg^{-1} , observed with the use of crude glycerol supplemented to a final KCl concentration of 3.10gL^{-1} . The ethanol yield observed with the fermentation that used crude glycerol supplemented to a final KCl concentration of 2.58gL^{-1} ($0.076 \pm 0.036\text{gg}^{-1}$) was similar to many of the ethanol yields observed in MFCs.

The concentration of succinic acid observed at the end of the study was similar in both MFCs and fermentations. The concentration of succinic acid in fermentations and in MFCs did not increase significantly over the course of the study and was not affected by the salt content or the purity of the glycerol used in the medium.

The concentration of acetic acid and 1,3-PDO at various points throughout the course of the study are shown in Figure 6.19.

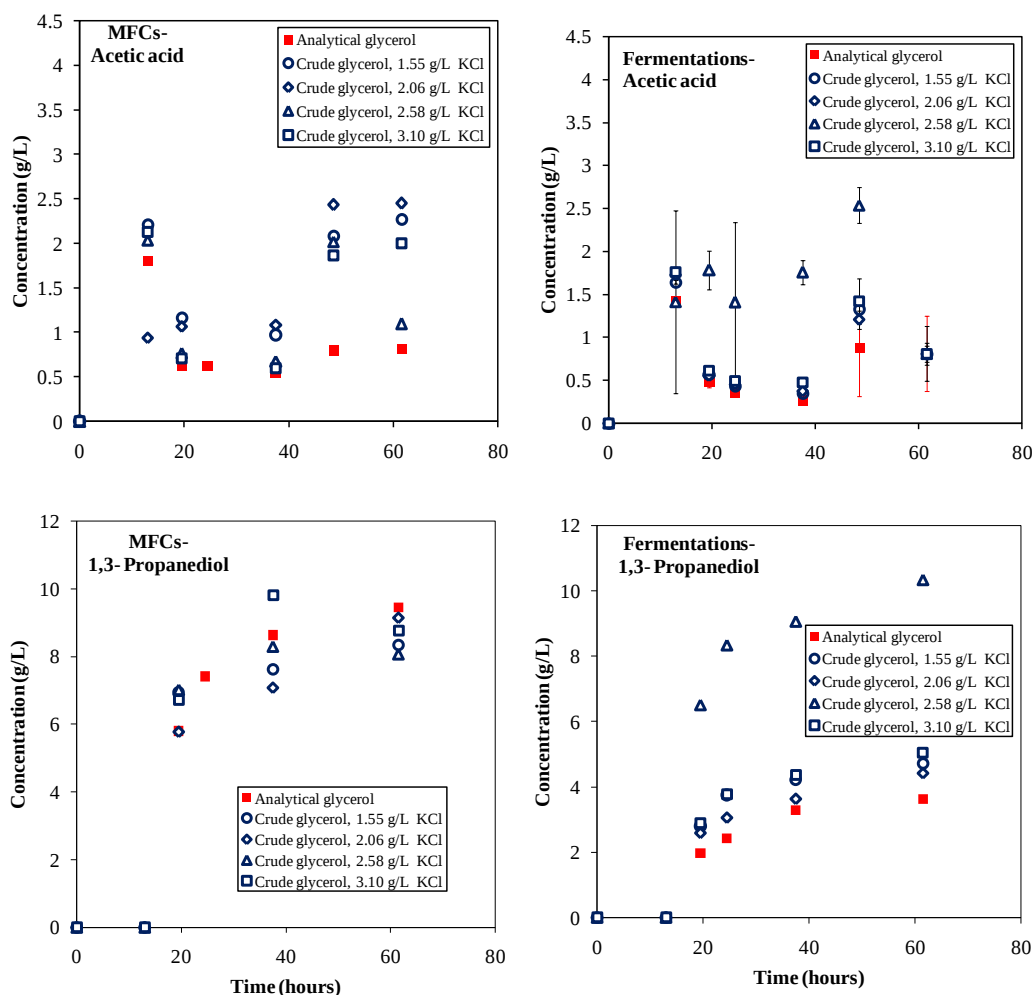


Figure 6.19: 1,3-PDO and acetic acid concentration observed with mixed culture AR2 grown on crude glycerol supplemented with KCl. (*average $\pm$ standard deviation, $n=3$*)

Unlike the previous study, where an increase in the concentration of acetic acid was observed in fermentations and in MFCs with time, in this study, the concentration of acetic acid observed in fermentations and MFCs fluctuated. However, it was apparent that the yield of acetic acid per gram of glycerol consumed was greater with the use of crude glycerol than with the use of analytical glycerol.

There was a significant difference in the yield of 1,3-PDO observed in fermentations and in MFCs. The final yield of 1,3-PDO per gram of glycerol consumed was significantly greater in MFCs than in fermentations.

With MFCs, the 1,3-PDO yield per gram of glycerol consumed ranged from 0.401gg^{-1} , observed with the use of crude glycerol supplemented to a final KCl concentration of 2.58gL^{-1} , to 0.539gg^{-1} , observed with the use of crude glycerol supplemented to a final KCl concentration of 2.06gL^{-1} . With fermentations (excluding that which used crude glycerol supplemented to a final KCl concentration of 2.58gL^{-1}), the 1,3-PDO yield per gram of glycerol consumed ranged from $0.173 \pm 0.012\text{gg}^{-1}$, observed with the use of analytical glycerol, to $0.247 \pm 0.031\text{gg}^{-1}$, observed with the use of crude glycerol supplemented to a final KCl concentration of 3.10gL^{-1} . With fermentations, the concentration of salt in the medium had a positive effect on the amount of 1,3-PDO observed throughout the course of the study. Similar to the yield of ethanol observed in the fermentation that used crude glycerol supplemented to a final KCl concentration of 2.58gL^{-1} , the yield of 1,3-PDO observed ($0.534 \pm 0.087\text{gg}^{-1}$) was similar to the yield of 1,3-PDO in MFCs.

Unlike the previous study, the salt content of the medium did not have an effect on the yield (per gram glycerol consumed) of ethanol, succinic acid or acetic acid.

6.4.3 Summary

In both studies, the yield of ethanol observed in fermentations was greater than the yield of ethanol observed in MFCs and the concentration of 1,3-PDO observed in MFCs was greater than the concentration of 1,3-PDO observed in fermentations.

6.4.4 Discussion

Bacterial species from the genera *Enterobacter*, *Clostridium*, *Lactobacillus*, *Bacillus*, *Citrobacter* and *Klebsiella* have been shown to ferment glycerol in a 1,3-PDO dependent manner (Yazdani and Gonzalez, 2007); however, the metabolites observed with mixed culture AR2 (with the exception of 1,3-PDO) are not unique to bacteria capable of fermenting glycerol in a 1,3-PDO dependent manner. *E. coli*, *Anaerobiospirillum succiniciproducens* and *Basfia succiniciproducens* have all been shown to ferment glycerol in a 1,3-PDO independent manner, yielding ethanol and succinic acid as the major fermentation products (Dharmadi et al., 2006; Lee et al., 2001; Murarka et al., 2008; Scholten et al., 2009). The appearance of 1,3-PDO at significantly greater concentrations in

MFCs than in fermentations could be indicative of a difference in the dominant bacterial species present, a difference in the metabolic pathways used, or a combination of both.

With mixed cultures, bacteria compete to use the anode as the terminal electron acceptor and, as a result, changes in bacterial populations can be observed with time. In the study conducted by Rabaey et al. (2004), DNA analysis of the bacteria present in the anode of an MFC at various points throughout the course of the study (155d) revealed that the dominant species present had changed multiple times. Population changes were observed with culture enrichment experiments (bacteria were removed from the anode and resuspended in a sterile MFC with fresh broth), and with continuous fuel cell operation (Rabaey et al., 2004). Similar results were observed in a study conducted by Futamata et al. (2012), where a clay and silt rich soil sample was used as inoculum in a fed-batch MFC with lactose as fuel. The change in bacterial species present coincided with increased oxygen concentrations, observed during the initial stages of the study, and decreasing lactose concentrations, observed as the study progressed (Futamata et al., 2012).

A difference in the yield of products generated by bacteria in a standard fermentation versus an MFC was found in a study conducted by Rabaey et al. (2004), in addition to the work presented in this thesis. Rabaey et al. (2004) observed that bacteria grown in an MFC then resuspended in a fermentor produced more hydrogen gas than they originally had. In a fuel cell environment where reducing equivalents are necessary for the generation of current, the concentration of reduced products would be expected to decrease; as was observed in previous studies with *E.coli* W3110. 1,3-PDO and ethanol are reduced products. One reduction equivalent is consumed in the production of 1,3-PDO and two reduction equivalents are consumed in the production of ethanol; however, fermentation of glycerol to ethanol is a redox balanced process (reduction equivalents are generated via glycolysis and via the metabolism of glycerol to DHAP). If 1,3-PDO were produced in parallel with acetate or another oxidized product, depending on the ratio of metabolites, a surplus of reduction equivalents would have been available for current generation. Additional data would be required to make further investigation of this hypothesis possible.

Chapter 7. Overall conclusions

7.1 Summary

In the work presented in this thesis, the effect of waste glycerol from biodiesel production on the metabolism and performance of three cultures (*E. coli* W3110, *P. freudenreichii* ssp. *shermanii* and mixed culture AR2), used as anodic catalysts in an MFC, was investigated. Significant conclusions for each organism are summarized below.

7.1.1 *E. coli* W3110

The performance of *E. coli* W3110 as anodic catalyst was found to be independent of the type of waste glycerol used, and therefore independent of the concentration of FAME, free fatty acids and other compounds found in crude glycerol (at the concentrations tested). Fuel cell performance was, however, enhanced through the supplementation of the medium with NaCl and KCl. Glycerol metabolism was not affected by the concentration of salt and other compounds found in waste glycerol. Glycerol metabolism was, however, affected by the MFC environment: the rate of glycerol fermentation was greater in serum bottle fermentations than in MFCs for all conditions tested and ethanol, the primary product of glycerol fermentation, was observed at greater yields in fermentations than in MFCs.

7.1.2 *P. freudenreichii* ssp. *shermanii*

The performance of *P. freudenreichii* ssp. *shermanii* as anodic catalyst was found to be independent of the type of waste glycerol used, and therefore independent of the concentration of FAME, free fatty acids and other compounds found in crude glycerol (at the concentrations tested). Fuel cell performance was, however, enhanced through the supplementation of the medium with NaCl. Glycerol metabolism was reduced when substituting analytical glycerol with waste glycerol from biodiesel produced using 0.8wt% NaOCH₃ and 0.8wt% KOH catalysts; these crudes contained the greatest concentration of additional components. Propionic acid was the primary product of glycerol fermentation.

7.1.3 Mixed culture AR2

The performance of mixed culture AR2 as anodic catalyst was reduced when analytical glycerol was substituted with waste glycerol produced using 0.8wt% KOH and 0.8wt% NaOCH₃; fuel cell performance was found to be inversely related to the concentration of compounds (other than salt) present in crude glycerol. Unlike the pure cultures, the concentration of salt in the anodic medium did not affect fuel cell performance. The MFC environment affected the metabolism of glycerol by AR2: the rate of glycerol metabolism was found to be greater in fermentations than in MFCs; the yield of ethanol observed with fermentations was greater than the yield of ethanol observed with MFCs; and the concentration of 1,3-PDO observed with MFCs was greater than the concentration of 1,3-PDO observed with fermentations. Since AR2 is a mixed culture, this may indicate a shift in the composition of the microbial community under MFC conditions.

7.2 Overall discussion

The ability of bacteria to oxidize waste glycerol from biodiesel production is important, as the end goal of this work is the development of an environmentally friendly technology that will use partially refined waste glycerol to generate electrical power and chemical products.

As catalysts in an MFC, all three cultures performed as well or better using crude glycerol, produced using minimal catalyst, as they did using analytical glycerol. The concentration of compounds, other than glycerol, in industrial biodiesel glycerol streams often range from 10 to 20 wt% (Fukuda et al., 2001). The concentration of compounds, other than glycerol and salt, found in each of the crude glycerol streams used in this thesis was greater than the concentration of impurities found in waste glycerol from commercial operations. This suggests that even though *E. coli* W3110 was the only culture that succeeded in metabolizing crude glycerol, produced using high catalyst concentrations, at the same rate as analytical glycerol, all three cultures would be capable of metabolizing waste glycerol produced by an industrial scale operation.

Although capacity to metabolize waste glycerol is important when selecting a catalyst for a glycerol oxidizing MFC, there are other additionally important factors to consider,

including: the ability of the catalyst to tolerate large concentrations of glycerol; the ability of the catalyst to consume glycerol at an efficient rate; the electrochemical efficiency observed with the catalyst; and the maximum power that can be generated. Of all the catalysts tested, the greatest maximum power density was achieved with *E. coli* W3110. Mixed culture AR2 consumed the greatest amount of glycerol (20gL^{-1}) of all the cultures studied, in the least amount of time (46h). Mixed culture AR2 was also shown to produce 1,3-PDO, which has a much greater market value than ethanol and propionic acid, the primary metabolites of glycerol fermentation by *E. coli* W3110 and *P. freudenreichii* ssp. *shermanii*, respectively. Because of their performance, *E. coli* W3110 and AR2 are good choices as the catalyst in a glycerol oxidizing fuel cell, and should be further studied.

7.3 Future directions

To continue investigating the prospect of a glycerol oxidizing microbial fuel as a means of reducing the amount of glycerol waste generated by the biodiesel industry, the fuel cell used in the experiments discussed in this work needs to be redesigned to allow for more efficient power production.

H-type fuel cells are known to have high internal resistance, which is mostly a result of large ohmic losses caused by use of a membrane to separate the anode chamber from the cathode chamber, use of a liquid catholyte as opposed to an air cathode, and use of unmodified carbon cloth at the cathode. Use of a single chamber fuel cell equipped with an air cathode has been shown to significantly increase power generation from that achieved with dual chambered systems (Logan, 2008). Power generation can also be increased by the incorporation of platinum on the cathodic electrode, and by increasing anode surface area, to encourage biofilm development. Increased power generation should magnify any differences observed in fuel cell performance, making comparisons between cells easier.

Additional fuel cell modifications might include having a continuous flow of electrolyte solution into and out of the anode chamber. This would be beneficial as it would simulate what might happen in an industrial process, allowing a more realistic perspective to be attained on how the cultures might behave over an extended period of time.

Mixed cultures have been widely studied in MFCs. The electrochemical environment within a fuel cell applies selective pressure, favouring bacteria that have the capacity to use the anode as a terminal electron acceptor. Although the power density observed with mixed culture AR2 was less than that observed with the pure cultures studied, it is likely that growing AR2 in the anode of a fuel cell for an extended period of time would enrich the concentration of exoelectrogenic bacteria at the anode, resulting in increased power production.

In literature, *E. coli* has been shown to ferment glycerol in amounts up to 10gL⁻¹. In the experiments conducted for this thesis project, *E. coli* W3110 was not able to metabolize the 5gL⁻¹ of the glycerol added to the medium. Further medium optimization experiments could be beneficial in helping to determine the optimal conditions for growth (Dharmadi et al., 2006; Murarka et al., 2008).

The mechanisms of electron transfer from bacteria to anode are not well understood, however understanding the redox reactions occurring in bacteria is important when looking for the best method to maximize power production. In several studies where glucose was used as fuel in an MFC, *E. coli*, which was used as the anodic catalyst, was shown to produce redox active mediators (Qiao et al., 2008; Wang et al., 2007; Zhang et al., 2008). However, because metabolic pathways in bacteria are complex, it is possible that the expression of various compounds might differ according to the carbon source used (i.e. with glycerol as opposed to glucose).

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Appendix A: Calculation procedures

A.1. Glycerol conversion

The concentration of glycerol in biodiesel waste, fermentations and in the anodic medium of MFCs was calculated by employing the standard curve shown in Figure B.. The concentration of metabolites in fermentations and in the anodic medium of MFCs was calculated by employing the standard curves shown in Figure B.. Samples of known concentration were prepared and analyzed by HPLC. Concentration, in gL^{-1} , was plotted against peak area, in refractive index units (nRIU). This data was fitted to a line, and the equation of this line (shown rearranged in Equation(10)) was used to calculate the concentration in samples.

$$x = \frac{y}{\text{slope}} \quad (10)$$

In Equation(10),

x, represents the concentration of the compound of interest in the sample (gL^{-1})

y, represents the peak area observed with HPLC analysis of the sample (nRIU)

A.2. Protein yield

Protein yield was calculated based on optical density values read at 600nm. Standard curves, that displayed the OD_{600} as a function of protein concentration, in mgL^{-1} , were created for each organism. This work was accomplished by Reiche (2012). Pure strains were grown anaerobically, and sampled at various stages of growth. Bovine serum albumin (BSA) standards and culture samples were used to perform the RC DCTM protein assay, as described by the manufacturer. A standard curve was created for the BSA standards and used to determine the protein concentration in culture samples. Equations (11) and (12) show the relationship between OD_{600} and protein concentration for *E. coli* W3110 and *P. freudenreichii* ssp. *shermanii*, respectively.

$$x_e = \frac{y_e + 0.0225}{0.3021} \quad (11)$$

$$x_p = \frac{y_p + 0.089}{0.0753} \quad (12)$$

In Equations(11) and(12),

x_e and x_p , represent the concentration of protein in a sample (mgL^{-1})

y_e and y_p , represent the optical density of the sample, read at 600nm

A.3. Product yield

The yield of certain fermentation products, based on the amount of glycerol consumed, was calculated to provide a means of comparing the metabolic behaviour of cultures at the conditions tested (Equation(13)).

$$Y_{p/g} = \frac{p_2 - p_1}{g_2 - g_1} \quad (13)$$

In Equation(13),

$Y_{p/g}$, represents the yield of product per gram of glycerol fermented (gg^{-1})

p_1 , represents the initial concentration of the fermentation product of interest (gL^{-1})

p_2 ,represents the final concentration of the fermentation product of interest (gL^{-1})

g_1 ,represents the initial concentration of glycerol (gL^{-1})

g_2 ,represents the final concentration of glycerol (gL^{-1})

A.4. Polarization and power density curves

Polarization curves were generated for each MFC. Theses polarization curves showed potential as a function of current. Polarization curves discussed in Chapters 4-6 show potential as a function of current density. Current was converted to current density using Equation (14)

$$I_{An} = \frac{I}{A_{An}} \quad (14)$$

where,

I_{An} , represents current density (mA m^{-2})

I , represents current (mA)

A_{An} , represents the area of the anode (m^2)

For each MFC, power density was calculated using each of the data points represented in the polarization curve. Power density was calculated using Equation (15)

$$P_{An} = \frac{IE}{A_{An}} 1000 \quad (15)$$

where,

P_{An} , represents power density (mW m^{-2})

E , represents the cell potential (V)

I , represents current (A)

A_{An} , represents the area of the anode (m^2)

A.5. Internal resistance

Internal resistance is equal to the slope of the linear region of a polarization curve (depicted in Figure A.). For each MFC, internal resistance was calculated by applying Equation (16) to the original, unmodified polarization curve.

$$R_{int} = \frac{E_1 - E_2}{I_1 - I_2} \quad (16)$$

In Equation(16),

R_{int} , represents the internal resistance of the MFC (Ω)

E_1 and I_1 , represent the potential (V) and current (A) respectively, of a first data point included in the linear region of the polarization curve

E_2 and I_2 , represent the potential (V) and current (A) respectively, of a second data point included in the linear region of the polarization curve

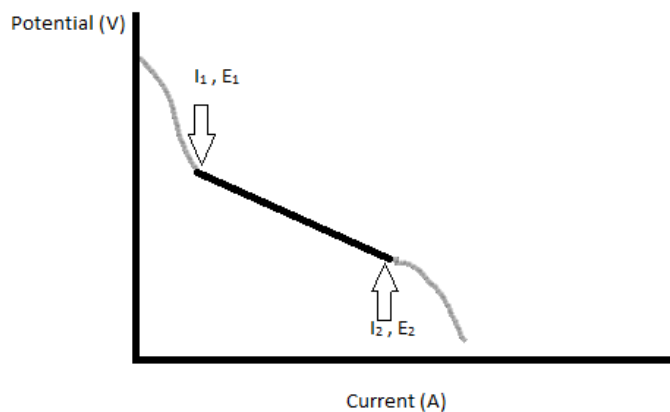


Figure A.1: Polarization curve with linear region identified

A.6. Electrochemical efficiency

Electrochemical efficiency was calculated using Equation (17)

$$\mu = \frac{OCV}{E^{\circ}} \quad (17)$$

where,

μ , represents the electrochemical efficiency

OCV, represents the equilibrium open circuit potential (V)

E° , represents the greatest open circuit potential that can be achieved at standard conditions according to thermodynamics (V)

The open circuit potential that can be achieved by a cell is limited by thermodynamics. This value, at standard conditions, can be calculated using Equation (18)

$$E^{\circ} = \frac{-\Delta G}{nF} \quad (18)$$

where,

n , represents the number of moles of electrons consumed per mole of glycerol consumed ($n=14$)

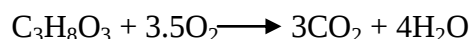
F , represents Faraday's constant (Cmol^{-1})

ΔG , represents the change in Gibbs free energy (Jmol^{-1})

The change in Gibbs free energy for the overall fuel cell reaction is equal to the Gibbs free energy of reactants subtracted from the Gibbs free energy of products (Equation(19))

$$\Delta G = \sum G_{\text{products}} - \sum G_{\text{reactants}} \quad (19)$$

The overall reaction, assuming that glycerol is completely oxidized to CO_2 is:



(It should be noted that this equation does not take into account the biological processes responsible for power generation (see section 2.2.2.1 for further detail).

Gibbs free energy of products and of reactants was calculated using Equation (20)

$$G = H_f^\circ - TS_f^\circ \quad (20)$$

where,

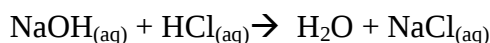
H_f° , represents the standard enthalpy of formation ($H_f^\circ = -665.9\text{kJmol}^{-1}$, 393.5kJmol^{-1} , and -285.84kJmol^{-1} for glycerol, carbon dioxide and water, respectively)

T, represents the temperature at which the fuel cell is operated (K)

S_f° , represents the standard entropy of formation ($S_f^\circ = -638.9\text{Jmol}^{-1}\text{K}^{-1}$, $-2.9\text{Jmol}^{-1}\text{K}^{-1}$, and $-163.3\text{Jmol}^{-1}\text{K}^{-1}$ for glycerol, carbon dioxide and water, respectively)

A.7. Salt added to crude glycerol

Industrial grade hydrochloric acid was used to neutralize any catalyst contained within the glycerol waste. The concentration of HCl in the industrial grade solution was unknown at the time of use but was determined after the fact through titration with a sodium hydroxide solution of known molarity. As HCl is a strong acid and NaOH a strong base, the amount of base required to neutralize a single mol of acid can be determined directly from the stoichiometric coefficients of the chemical equation.



Therefore, the molar amount of NaOH required for complete neutralization is equivalent to the molar amount of HCl found in the sample. Equation (21) was used to calculate the concentration of HCl in the industrial grade HCl solution. The titration was performed in triplicate.

$$M_{\text{HCl}} = \frac{V_{\text{NaOH}} 0.09 \rho_{\text{HCl}}}{m_{\text{HCl}}} \quad (21)$$

where,

M_{HCl} , represents the molarity of the industrial strength HCl solution (molL^{-1})

V_{NaOH} , represents the average volume of 0.09molL^{-1} NaOH used to neutralize samples (L)

m_{HCl} , represents the average mass of HCl samples (g)

ρ_{HCl} , represents the density of the industrial strength HCl solution (1200 gL^{-1})

The molarity of the HCl solution used in this study was found to equal 10.81 molL^{-1}

The molar amount of hydrochloric acid required to neutralized the sodium methoxide catalyst was calculated using Equation (22)

$$x_{\text{HCl}} = \frac{M_{\text{HCl}} m_{\text{HCl}}}{\rho_{\text{HCl}}} \quad (22)$$

where,

x_{HCl} , represents the amount of hydrochloric acid used (g)

m_{HCl} , represents the mass amount of industrial strength HCl used (62.8g)

The molar amount of HCl required to neutralize the waste glycerol was 0.565.

Given that one mol of sodium methoxide is required to neutralize one mole of hydrochloric acid, the mass amount of sodium contained in the glycerol waste, per gram glycerol, was calculated using Equation (23)

$$m_{\text{Na}1} = \frac{x_{\text{HCl}} \text{MW}_{\text{Na}}}{m_{\text{Gly}}} \quad (23)$$

where,

MW_{Na} , represents the molecular weight of sodium ($gmol^{-1}$)

m_{Gly} , represents the mass amount of glycerol in the waste glycerol (859g)

m_{Na1} , represents the mass amount of sodium contained in the glycerol waste, per gram glycerol (gg^{-1})

The mass amount of sodium per gram of glycerol in the glycerol phase was found to equal $0.015gg^{-1}$.

Assuming a conversion of 98%, The concentration of sodium (per gram glycerol) that would exist, given a particular catalyst concentration, was calculated using Equation (24)

$$m_{Na2} = \frac{c}{100} \left(\frac{1}{0.98} \right) \left(\frac{MW_{Na} MW_{Gly}}{MW_{NaOCH_3} MW_{oil}} \right) \quad (24)$$

where,

c , represents the catalyst concentration (wt%) based on the mass amount of oil used in the transesterification reaction

MW_{Gly} , represents the molecular weight of glycerol ($gmol^{-1}$)

MW_{oil} , represents the molecular weight of soybean oil ($gmol^{-1}$)

m_{Na2} , represents concentration of sodium per gram glycerol (dependent on concentration of catalyst used in biodiesel production) (gg^{-1})

MW_{NaOCH_3} , represents the molecular weight of sodium methoxide ($gmol^{-1}$)

The amount of sodium chloride (per gram of glycerol) that was added to M9 media to mimic each catalyst concentration was calculated using Equation (25):

$$m_{NaCl} = \left(\frac{MW_{NaCl}}{MW_{Na}} \right) (m_{Na2} - m_{Na1}) \quad (25)$$

where,

MW_{NaCl} , represents the molecular weight of sodium chloride ($gmol^{-1}$)

Appendix B: Standard curves

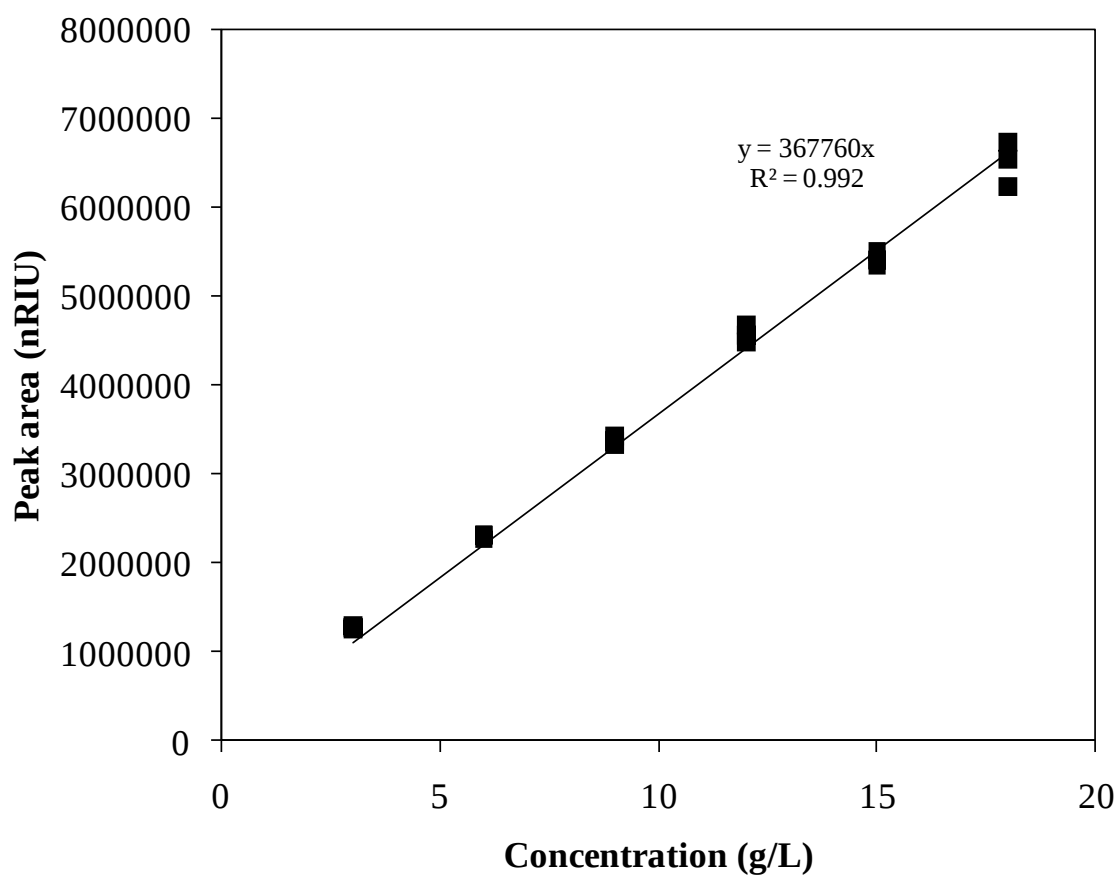


Figure B.1: Standard curve for the quantification of glycerol by HPLC

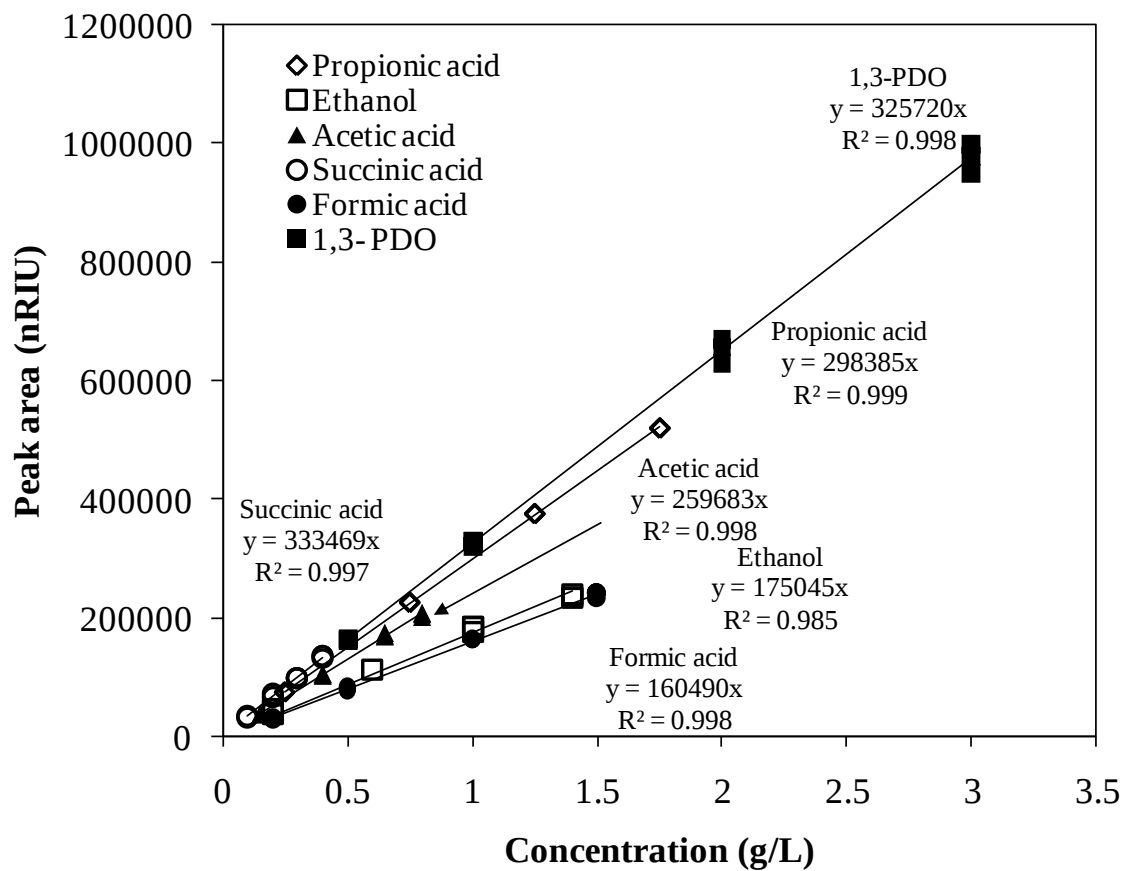


Figure B.2: Standard curves for the quantification of metabolites by HPLC