BIOCATALYST SELECTION FOR A GLYCEROL-OXIDIZING MICROBIAL FUEL CELL

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Abstract

Using glycerol from biodiesel production as a fuel in a microbial fuel cell (MFC) will generate electricity and valuable by-products from what is currently considered waste. This research aims to screen *E. coli* (W3110, TG1, DH5α, BL21), *P. freudenreichii* (subspecies *freudenreichii* and *shermanii*), and mixed cultures enriched from compost (AR1, AR2, AR3) as anodic biocatalysts in a glycerol-oxidizing MFC. Anaerobic fermentation experiments were performed to determine the oxidative capacity of each catalyst towards glycerol. Using an optimized medium for each strain, the highest anaerobic glycerol conversion from each group was achieved by *E. coli* W3110 (4.1 g/L), *P. freudenreichii* ssp. *shermanii* (10 g/L), and AR2 (20 g/L). These cultures were then tested in an MFC system. All three catalysts exhibited exoelectrogenicity. The highest power density was achieved using *P. freudenreichii* ssp. *shermanii* (14.9 mW m^{-2}), followed by AR2 (11.7 mW m^{-2}), and finally *E. coli* W3110 (9.8 mW m^{-2}).

Resumé

L'utilisation du glycérol, venant de la production du biodiésel, comme carburant dans une pile à combustible microbienne (PCM) générera de l'électricité et sous-produits utiles à partir de ce qui est présentement considéré un déchet. Cette recherche vise à investiguer *E. coli* (W3110, TG1, DH5α, BL21), *P. freudenreichii* (sous-espèce *freudenreichii* et *shermanii*), et des cultures mixtes enrichies de compost (AR1, AR2, AR3) en tant que biocatalyseurs anodiques dans une PCM oxydé de glycérol. Des expériences de fermentation anaérobique ont été effectuées pour déterminer la capacité d'oxydation de chaque catalyseur vers le glycérol. En utilisant un milieu de culture optimisé pour chaque souche, la conversion anaérobique la plus élevée du glycérol pour chaque groupe a été réalisé par *E. coli* W3110 (4.1 g/L), *P. freudenreichii* ssp. *shermanii* (10 g/L), et AR2 (20 g/L). Ces cultures ont ensuite été testées dans un système PCM. Les trois catalyseurs ont démontré l'exoélectrogénicité. La densité de puissance la plus élevée a été obtenue en utilisant *P. freudenreichii* ssp. *shermanii* (14.9 mW m^{-2}), suivie par AR2 (11.7 mW m^{-2}), et enfin *E. coli* W3110 (9.8 mW m^{-2}).

Statement of Contributions of Collaborators

I hereby declare that I am the sole author of this thesis. I performed all of the data analysis and wrote all the chapters presented in this work. I conducted all of the experimental work with the exception of the medium optimization experiments presented in Chapter 5, which were performed with the help of Bassam Javed.

Dr. Kathlyn Kirkwood supervised this thesis project and provided continual guidance throughout. She also made editorial contributions to the written work presented.

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Nomenclature

ε_c	Coulombic yield
η	Electrochemical efficiency
μ	Growth rate (h^{-1})
A_{An}	Anode surface area (m^2)
ABS_{750}	Absorbance read at 750 nm
$AREA_i$	Area under the HPLC curve for sample i
C_g	Glycerol conversion (g/L)
C_P	Coulombs produced (C)
C_t	Theoretical maximum Coulombs (C)
E_{cell}	Potential (V)
F	Faraday's constant (C/moles electrons)
f	Fermentation time (s)
G	Gibbs energy (J/mol)
G_i	Gibbs energy of formation for a specific species (J/mol)
g_i	Glycerol concentration of sample i (g/L)
H_f^o	Standard enthalpy formation (J/mol)
I	Current (A)
i_{opt}	Optimal current (A)
i	Integer
M	Molecular weight of the substrate (g/mol)
n	Moles of electrons produced per mole of substrate consumed
OCV	Open circuit voltage (V)
$OD_{600,i}$	Optical density read at 600 nm for sample i
P	Power (W)
P_{An}	Power density (W/m^2)
P_{max}	Maximum power density (W/m^2)
R_{ext}	External resistance (Ω)
R_{int}	Internal resistance (Ω)
S	Substrate concentration (g/L)
S_f^o	Standard entropy of formation (J/mol K)
T	Temperature (K)
t	Time (s)
v	Liquid volume (L)
Y	Protein yield (g protein/g substrate)
y_i	Protein concentration of sample i (g protein/L culture)
$y_{s,i}$	Protein concentration of bacterial standard i (g protein/L culture)

List of Abbreviations

Analytical techniques

BOD	Biological oxygen demand
BOD_5	Biological oxygen demand 5 day test
COD	Chemical oxygen demand
GC	Gas chromatography
HPLC	High performance liquid chromatography
OD_{600}	Optical density at 600 nm
OD_{750}	Optical density at 750 nm
RC DC™	Reducing agent detergent compatible
RID	Refractive index detector
TCD	Thermal conductivity detector

Growth Media

LB	Luria Bertani
RCM	Reinforced Clostridial Medium

Metabolites

1,3-PDO	1,3-propanediol
3-HPA	Hydroxypropionaldehyde
AcCoA	Acetyl Coenzyme A
BPG	1,3-Bisphosphoglycerate
CO_2	Carbon dioxide
DHAP	Dihydroxyacetone
G-3-P	Glyceraldehyde 3-phosphate
PEP	Phosphoenolpyruvate

Other

BSA	Bovine serum albumin
CEM	Cation exchange membrane
MFC	Microbial fuel cell
OCV	Open circuit voltage
SMFC	Sediment microbial fuel cell
SCMFC	Single-chamber microbial fuel cell
VSS	Volatile suspended solids

Chapter 1. Introduction

1.1 Background

Crude glycerol is generated as a high-volume co-product of biodiesel production. There has been a recent worldwide increase in the production of biodiesel which has resulted in an abundance of crude glycerol. This has turned a once valuable by-product for biodiesel producers into a waste stream and has negatively impacted the economic viability of current biodiesel production processes (Yazdani and Gonzalez, 2007). A market surplus of glycerol has necessitated the development of new platforms for these crude glycerol streams.

A microbial fuel cell (MFC) is an electrochemical device that can convert the chemical energy of a fuel to electrical energy. An MFC is similar to a conventional fuel cell except for the nature of the anodic catalyst which is biological as opposed to metallic. The microorganism in the anode chamber of the MFC oxidizes the fuel, resulting in the production of electrons. The electrons then travel through an external circuit from the anode to the cathode creating a current. Oxidation of the fuel occurs using the metabolic pathways of the microorganism and consequently can result in both the production of electrons and metabolic by-products. The anode chamber, which houses the microorganism catalyst, is kept anaerobic, so the microorganism must be able to anaerobically metabolize the fuel for the system to function. Pure or mixed cultures of bacteria can be used as the biocatalyst.

Biocatalysts used in MFCs are classified into either exoelectrogenic or non-exoelectrogenic strains. Non-exoelectrogenic strains require the addition of a chemical mediating compound to transport the electrons from inside the bacterial cells to the electrodes in order to complete the circuit. Exoelectrogenic bacteria are able to transfer their electrons to the electrode without the addition of chemical mediators. The use of exoelectrogenic bacteria is preferred in industry since chemical mediators are expensive and toxic compounds (Bullen et al., 2006). For this reason, researchers are focusing on the development of mediatorless MFCs using exoelectrogenic bacteria as catalysts.

Using crude glycerol from biodiesel production as a fuel in an MFC would allow for the generation of both electricity and value-added products from what is currently considered a waste stream. Implementing this type of process into current biodiesel production facilities would increase their economic viability thereby making the biodiesel initiative a more attractive and feasible option.

To develop an MFC for crude glycerol oxidation, a microbial catalyst must be selected that can anaerobically metabolize glycerol. While aerobic glycerol metabolism is common in bacteria, anaerobic conversion of glycerol has only been reported in limited genera (Yazdani and Gonzalez, 2007), and even fewer are appropriate for use in an industrial process. Therefore, the first major hurdle for the development of a glycerol-oxidizing MFC is to find an appropriate anodic catalyst that is ideally exoelectrogenic.

1.2 Objectives and thesis overview

The main objective of this research is to identify anodic biocatalysts (both pure and mixed cultures of bacteria) for use in a glycerol-oxidizing MFC and then to compare their performance for the ultimate selection of a catalyst to use in future MFC development. To this end, the research was split into three sections: biocatalyst selection, determination of the anaerobic oxidative capacity of potential biocatalysts towards glycerol, and analysis of biocatalyst performance in a glycerol-oxidizing MFC. The sub-objectives and experimental approach for each section are described below.

1.2.1 Biocatalyst selection (Chapter 4)

The main objective of this chapter was to find potential anodic catalysts (both pure and mixed cultures) for use in a glycerol-oxidizing MFC. Specific objectives include:

1. Find pure strains in literature that have been shown to anaerobically metabolize glycerol and that are appropriate for use on an industrial scale
2. Enrich mixed cultures derived from compost for bacterial communities with a high anaerobic oxidative capacity towards glycerol

For pure culture selection, a thorough literature review was performed to find strains that have previously been shown to anaerobically metabolize glycerol. Potential catalysts were selected from this group through applying the strict criteria of only choosing non-pathogenic and non-stringent anaerobes. The selected catalysts were therefore appropriate for any future scale-up applications.

1.2.2 Anaerobic glycerol conversion of strains (Chapter 5)

The main objective for this chapter was to screen the potential catalysts selected in Chapter 4 for the ability to anaerobically metabolize glycerol. Promising strains will be further studied in MFC systems (Chapter 6). Specific experimental objectives include:

1. Determine the anaerobic oxidative capacity of both pure and mixed cultures towards glycerol
2. Identify some of the conversion products of anaerobic glycerol metabolism in the pure and mixed cultures
3. Perform medium optimization to enhance anaerobic glycerol conversion in the pure cultures

In order to determine the oxidative capacity of strains towards glycerol, batch fermentation experiments were performed anaerobically using glycerol as the sole carbon source. Glycerol consumption was monitored through high performance liquid chromatography (HPLC), and glycerol conversion was used as a measure of oxidative capacity. The production of conversion products, 1,3-propanediol (1,3-PDO) and carbon dioxide (CO_2), was detected through HPLC and gas chromatography (GC), respectively.

The initial batch fermentation experiments were performed using M9 minimal medium with added tryptone as a rich nutrient source. Medium optimization experiments were then performed by varying the concentration of tryptone in the medium or supplementing the tryptone with yeast extract. Fermentation experiments were then repeated to see the effect of rich medium additives on anaerobic glycerol conversion.

Based on the glycerol conversion results of each strain after medium optimization, catalysts were selected for further screening in an MFC system (Chapter 6).

1.2.3 MFC performance (Chapter 6)

The main objective of this chapter was to take the most promising catalysts from Chapter 5 and test them in an MFC for power generation in order to select the best anodic catalyst(s) for future development. Specific experimental objectives described in this chapter were to:

1. Design a functional H-type MFC to test potential anodic catalysts
2. Perform proof-of-concept experiments for the use of each catalyst in an MFC
3. Compare catalyst performance in a mediatorless MFC

An H-type MFC was designed and inoculated with the promising catalysts identified in Chapter 5. Voltage and power generation were used as performance indicators. Polarization and power density curves were created and compared in order to select the most promising catalyst(s) for future development.

Chapter 2. Literature review

The following chapter provides a current review of the literature pertaining to this project and also summarizes necessary background information on relevant technologies and processes, such as MFCs and the anaerobic fermentation of glycerol. This literature review is split into three major sections: glycerol from biodiesel production, anaerobic bioconversion of glycerol, and microbial fuel cells. The first section describes the current state of the market for glycerol and the impact of the market on biodiesel production processes. The second section summarizes the bacteria shown in literature to anaerobically metabolize glycerol as a carbon source and the metabolic conversion pathways used. Finally, the last section introduces the concept of MFCs and pertinent information on various biocatalysts that have previously been described in literature. MFC performance measures and applications are also reviewed.

2.1 Glycerol from biodiesel production

The worldwide consumption of energy is projected to increase by 49% from 2007 to 2035 (U.S Energy Information Administration, 2010). Currently, fossil fuels are used for the majority of energy production worldwide. Coal, oil and natural gas provide over 85% of all energy consumed in the United States (U.S Department of Energy, 2010). The non-renewable nature of fossil fuels limits their use as a future energy resource. By 2025, the demand for oil is expected to exceed supply from all known and anticipated reservoirs (Logan, 2008). In addition to issues with supply and demand, the combustion of fossil fuels results in greenhouse gas production thereby contributing to global climate change. Due to the aforementioned limitations of fossil fuels as an energy resource, there has been a recent surge of interest in the development of renewable energy platforms such as biofuels.

Biofuels include liquid or gaseous based fuels derived mainly from biomass (Demirbas, 2007). The use of biofuels as energy resources offers several advantages, including the renewable nature of biomass as a feedstock and no net release in carbon dioxide. Biodiesel is a biodegradable, non-toxic alternative to petroleum-based fuels with low emission profiles (Zhang et al., 2003). The use of biodiesel is a promising alternative to

conventional diesel since 100% biodiesel can be used without any modifications to current engines (Johnson and Taconi, 2007). Biodiesel is produced through a transesterification reaction where the triglycerides in the feed oil react with an alcohol, usually methanol or ethanol, in the presence of a catalyst to produce esters (biodiesel) and glycerol (Fig. 2.1) (Marchetti et al., 2007).

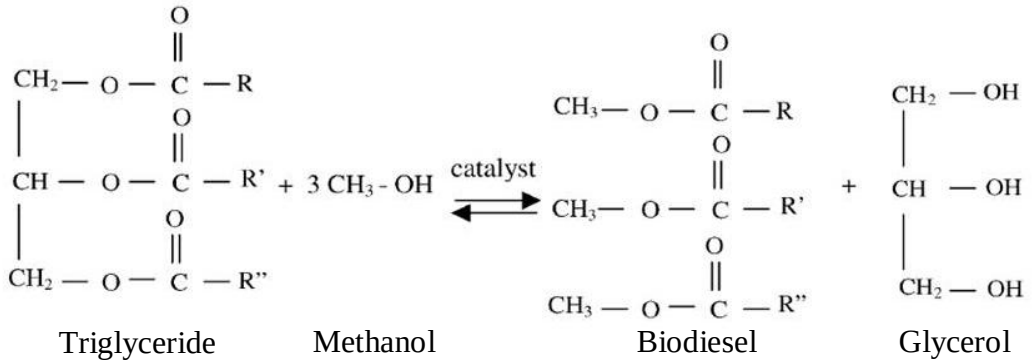

Figure 2.1. Transesterification reaction for the production of biodiesel

Biodiesel production generates 10% weight crude glycerol as a by-product (Johnson and Taconi, 2007). Recent increases in the worldwide production of biodiesel (Fig. 2.2) have resulted in a market surplus of glycerol which has caused the closure of several glycerol producing and refining operations, including Dow Chemicals and Proctor and Gamble Chemicals (Yazdani and Gonzalez, 2007). Pure glycerol is used in the production of pharmaceuticals, soaps, and cosmetics, as well as several food and beverage products. The U.S. market for glycerol is 600 million lbs per year; consequently, crude glycerol generated from biodiesel production processes was once viewed as a valuable by-product. The glycerol surplus has resulted in a drop in crude glycerol prices from 45 cents/lb to approximately 10 cents/lb turning this once marketable product into a waste stream with added disposal costs. Additionally, he glycerol streams generated from biodiesel production processes contain impurities such as alcohols, salts, and heavy metals (Johnson and Taconi, 2007). Refining operations cost an additional 15 cents/lb and are currently not an appealing option for biodiesel producers due to the current market situation. A process model was

developed by Haas et al. (2006) in order to estimate the factors contributing to biodiesel production costs. The production costs were found to vary inversely and linearly with the market prices of glycerol. In order to improve the economic viability of current biodiesel production processes, efficient ways to convert glycerol to value-added products or energy must be developed.

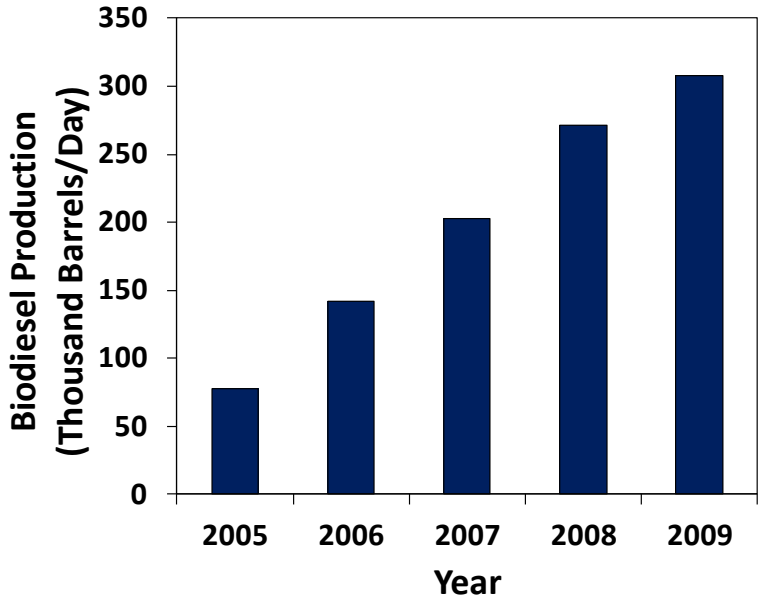

Figure 2.2. World biodiesel production per year (U.S. Energy Information Administration, 2010).

2.2 Anaerobic bioconversion of glycerol

2.2.1 Anaerobic bioconversion of glycerol in bacteria

Glycerol can be converted to value-added products through both chemical and biological methods. Chemical processes for glycerol conversion include pyrolysis, steam gasification, and catalytic treatment (Pathak et al., 2010). Initial purification steps and high operating temperatures and/or pressures result in high capital and operating expenses when implementing these processes into biodiesel production facilities. Biological conversion offers several advantages over traditional, chemical catalysis including milder operating conditions and high product specificity (Yazdani and Gonzalez, 2007). When considering fermentation of glycerol to value-added products, anaerobic processes offer advantages compared to aerobic conditions, such as eliminating the operational costs associated with aeration and reducing the biomass yields. Lower biomass yields result in a higher production of value-added products.

Bacteria are able to metabolize glycerol through incorporation into the glycolytic pathway (Fig. 2.3). Glycerol is converted to glycerol-3-phosphate and subsequently to dihydroxyacetone phosphate (DHAP) which is used in glycolysis for the formation of pyruvate (Fig. 2.4). Under aerobic conditions, pyruvate enters the citric acid cycle while under anaerobic conditions, fermentation to various by-products occurs depending on the species and growth conditions (Garrett and Grisham, 2005). While aerobic glycerol metabolism is common, anaerobic bioconversion of glycerol has only been documented in certain species of bacteria (Yazdani and Gonzalez, 2007).

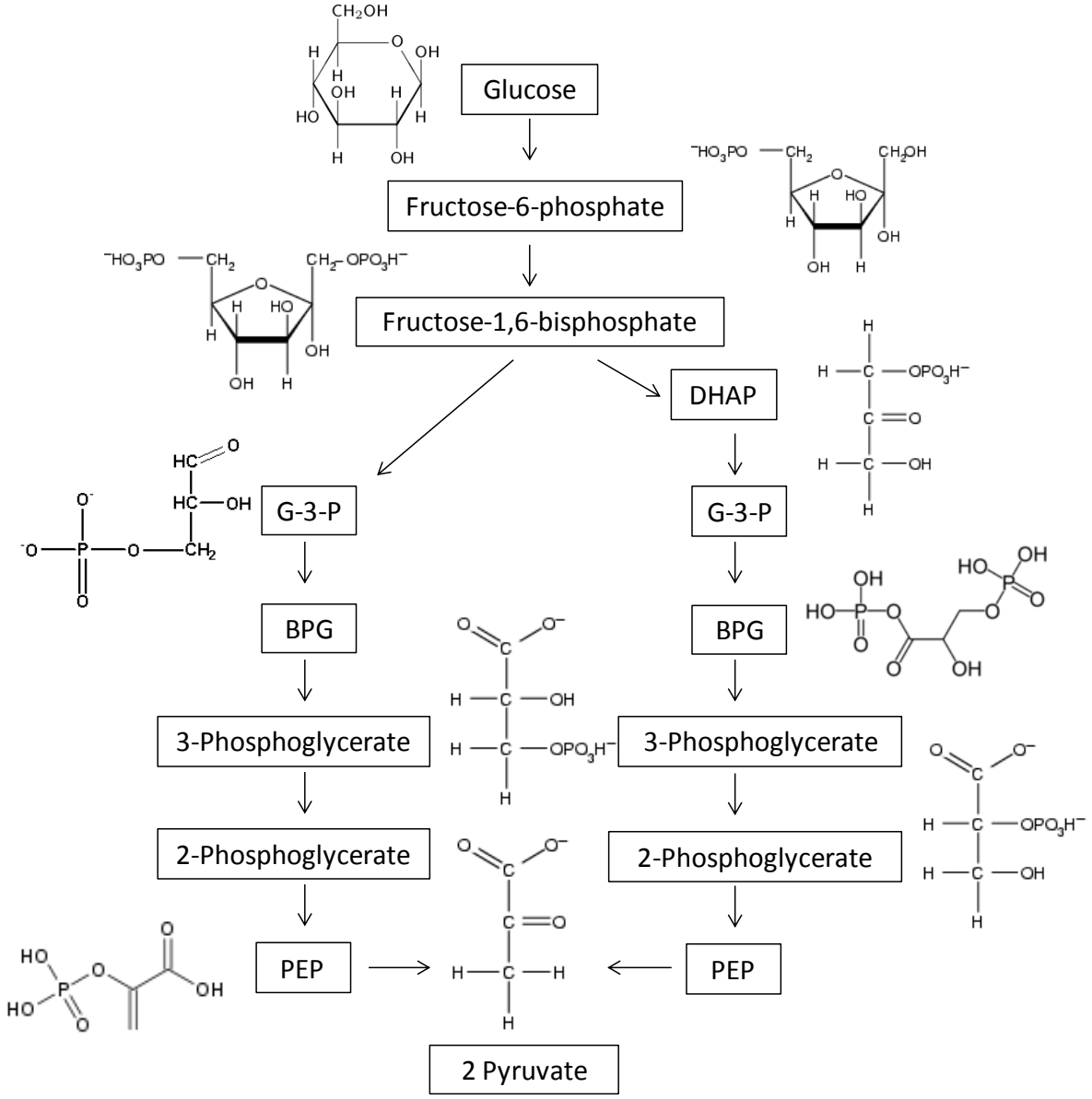

Figure 2.3. Glycolytic pathway in bacteria. Abbreviations are as follows: DHAP: Dihydroxyacetone phosphate; G-3-P: Glyceraldehyde 3-phosphate; BPG: 1,3-Bisphosphglycerate; PEP; Phosphoenolpyruvate

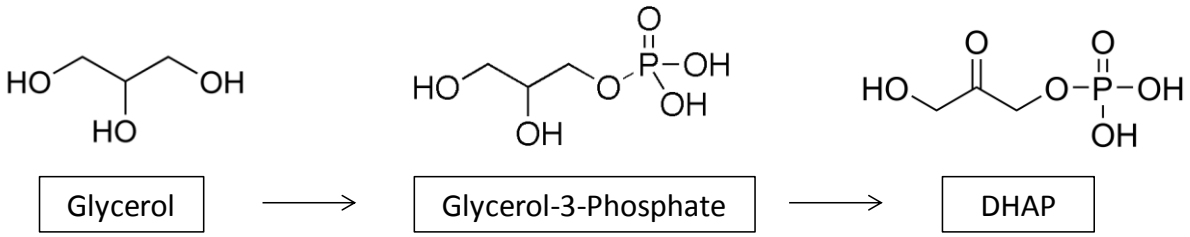

Figure 2.4. Conversion of glycerol to dihydroxyacetone phosphate (DHAP)

Anaerobic fermentation of glycerol in bacteria is generally categorized into two pathways: 1,3-propanediol (1,3-PDO) dependent conversion and 1,3-PDO independent conversion. For the biotransformation of glycerol into 1,3-PDO, a dismutation process occurs involving simultaneous oxidative and reductive pathways (Fig. 2.5) (Yazdani and Gonzalez, 2007). In the oxidative pathway, DHAP is produced, and in the reductive pathway, 1,3-PDO is produced via the formation of hydroxypropionaldehyde (3-HPA) (Willke and Vorlop, 2008). These pathways together are able to achieve a redox balance without the addition of external electron acceptors. Genera able to ferment glycerol in a 1,3-PDO dependent manner include, *Klebsiella*, *Citrobacter*, *Clostridium*, *Lactobacillus*, and *Bacillus* (Yazdani and Gonzalez, 2007). Bacterial genera reported to anaerobically ferment glycerol without the production of 1,3-PDO include *Propionibacterium*, *Escherichia*, and *Anaerobiospirillum*. In both the oxidative pathways of 1,3-PDO dependent and independent fermentations, a variety of products can be formed including succinic acid, propionic acid, butanol, ethanol, formic acid, hydrogen, and carbon dioxide (Fig. 2.6). The current market prices of these products are listed in Table 2.1.

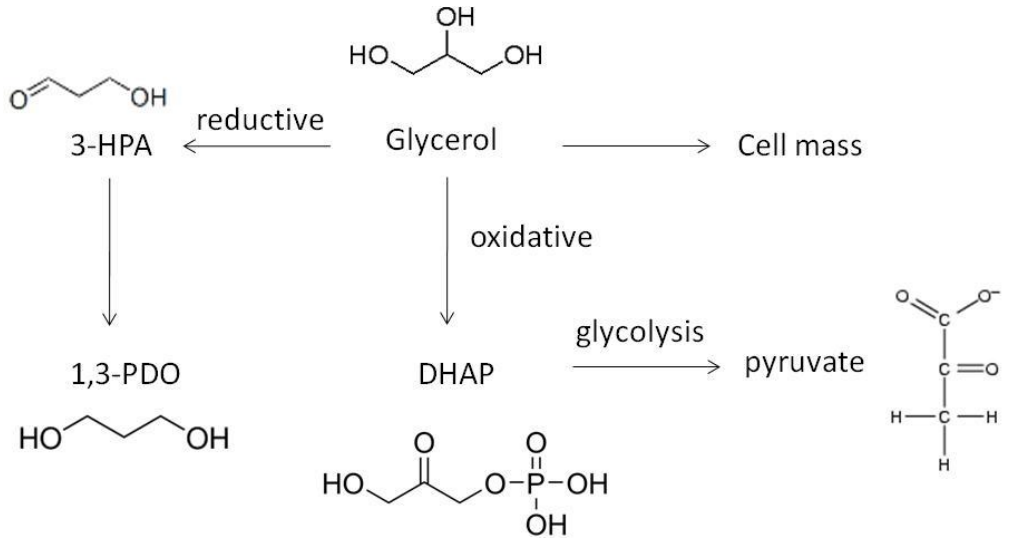

Figure 2.5. Oxidative and reductive pathways of glycerol dissimilation in 1,3-PDO dependent fermentation. Figure adapted from Yazdani and Gonzalez, 2007.

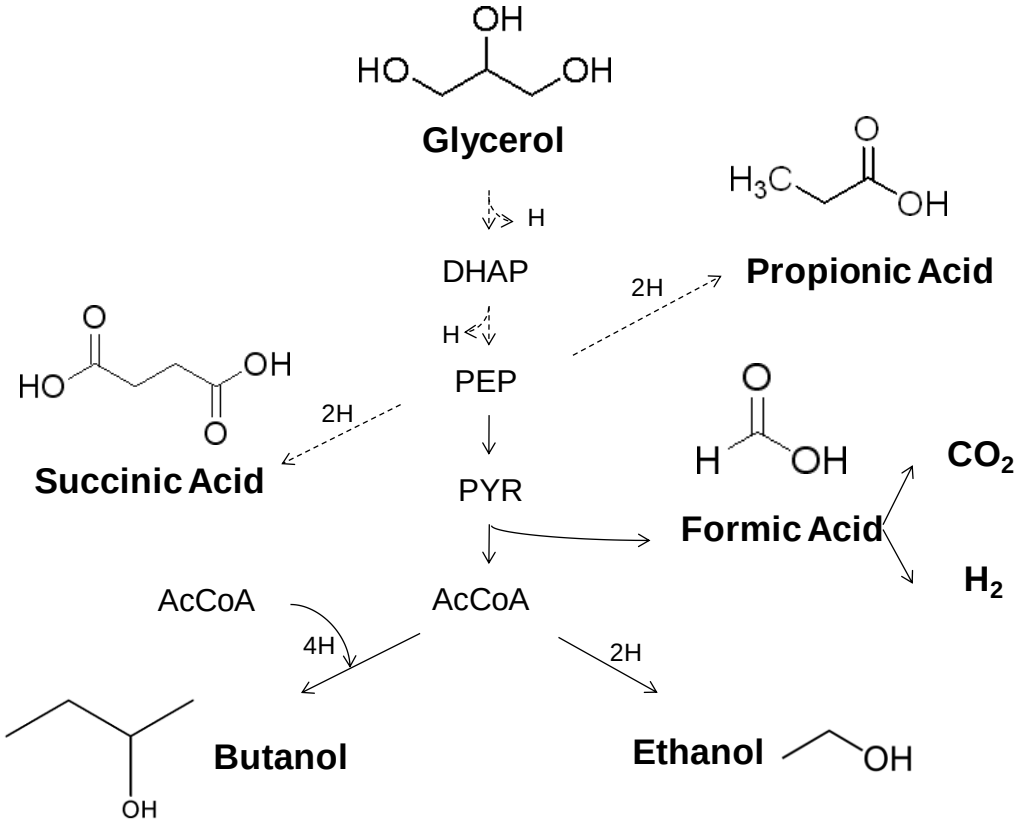

Figure 2.6. Oxidative pathways for anaerobic glycerol metabolism in bacteria. Figure adapted from Yazdani and Gonzalez, 2007. AcCoA refers to acetyl coenzyme A

Table 2.1. Current market prices of select glycerol fermentation products

Compound	**Price (US$/lb)**[1]
1,3-Propanediol	0.8
Butanol	0.57-0.58
Glycerol	0.45
Ethanol	0.55
Propionic acid	0.46-0.49

[1] Prices obtained from ICIS, 2011

The anaerobic, biotransformation of glycerol to 1,3-PDO has been extensively studied in the *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Clostridium* genera (Yazdani and Gonzalez, 2007). As a fermentation product, 1,3-PDO is valuable since it is a monomer for the synthesis of several polyesters (Witt et al., 1994). These bacterial families have also been used to convert glycerol to other products including butanol, hydrogen, and ethanol. Several glycerol conversion studies using these strains are summarized in Table 2.2.

Table 2.2. Summary of pure culture glycerol fermentation products

Genus	Species	Major Products	Reference
Citrobacter	*C. freundii*	1,3-propanediol	Boenigk et al., 1992 Deckwer, 1995
Clostridium	*C. butyricum*	1,3-propanediol, butyrate, hydrogen	Biebl et al., 1992 González-Pajuelo et al., 2005 Günzel et al., 1991 Heyndrickx et al., 1991 Reimann et al., 1998
	C. pasteurianum	1,3-propanediol, acetate, butanol, butyrate, ethanol, hydrogen	Biebl, 2001 Dabrock et al., 1992 Heyndricks et al., 1991
	C. propionicum	propionic acid	Barbirato et al., 1997
Klebsiella	*K. pneumonia*	1,3-propanediol, 2,3-butanediol, ethanol	Biebl et al., 1998 Deckwer, 1995 Menzel et al., 1997 Oh et al., 2011
Enterobacter	*E. aerogenes*	ethanol, hydrogen	Ito et al., 2005 Markov et al., 2011
	E. agglomerans	1,3 propanediol, acetate	Barbirato et al., 1995

In a study comparing the 1,3-PDO yields of *Citrobacter freundii* and *Klebsiella pneumoniae*, it was determined that the latter strain exhibited twice the glycerol tolerance compared to *C. freundii*. *K. pneumoniae* can achieve 99.6% conversion of 120 g/L glycerol in a batch fermentation with 56% recovery as 1,3-PDO (Deckwer, 1995). However, this high glycerol tolerance is of little use on an industrial scale since *K. pnuemoniae* is classified as an opportunistic pathogen and can infect the urinary tract, lungs, and central venous catheters in newborns and immunocompromised patients. *C. freundii* is also an opportunistic pathogen and is associated with gastroenteritis (Feigin et al., 2004).

High glycerol conversion has also been achieved using *Clostridium butyricum* in a low nutrient medium with the addition of biotin as a growth factor (Himmi et al, 1999).

Conversion at concentrations of 129 g/L pure glycerol and 121 g/L industrial glycerol were achieved with 1,3-PDO being the main metabolite in both cases. Industrial glycerol was produced through the transesterification of rapeseed oil and contained 67% glycerol. *Clostridium* species are obligate anaerobes and require stringent growth conditions that are difficult to achieve on an industrial scale. Glycerol conversion reported in the *Enterobacter* family has generally been lower compared to the aforementioned strains. Pure glycerol conversion on the order of 20 g/L has been demonstrated using *Enterobacter agglomerans* (Barbirato et al., 1995), and complete conversion of 10 g/L glycerol in biodiesel waste was achieved using *Enterobacter aerogenes* (Ito et al., 2005). *E. agglomerans* and *E. aerogenes* are both opportunistic pathogens, and therefore their use in industrial processes is limited.

Studies involving mixed culture anaerobic fermentation of glycerol have also been performed. Results of several investigations are summarized in Table 2.3. Generally less specific product formation is achieved with mixed cultures, and glycerol conversion levels are typically around the same level as pure cultures. The use of mixed cultures, however, is more appropriate for an industrial scale where contamination of pure strains can be difficult to avoid.

Table 2.3. Mixed culture fermentation of glycerol

Inoculum	Glycerol conversion (g/L)	Major products	Reference
Anaerobic digested sludge	17	1,3-propanediol, acetic acid, butyric acid, ethanol, lactic acid, hydrogen	Seifert et al., 2009
Tomato soil, wheat soil, compost, sludge	6	1,3-propanediol, acetate, ethanol, hydrogen[1]	Selembo et al., 2008
Distillery wastewater, potato starch processing sludge	25	1,3-propanediol, acetate, ethanol, formate	Temudo et al., 2008

[1]Hydrogen yields varied based on inoculum source

2.2.2 Anaerobic fermentation of glycerol in *E. coli* and *Propionibacteria*

E. coli and *P. freudenreichii* are ideal candidates for use in fermentation and other industrial processes since they are mostly non-pathogenic, and they are non-stringent anaerobes. Anaerobic fermentation of glycerol has previously been documented in both species. Conversion levels and by-product formation are dependent on the specific growth conditions and strains used.

E. coli is a gram-negative bacterium that is facultatively anaerobic. It was only recently determined that *E. coli* can ferment glycerol anaerobically (Dharmadi et al., 2006). It was found that glycerol fermentation in *E. coli* is pH-dependent and requires the supplementation of medium with rich nutrients such as tryptone and yeast extract. The most abundant metabolites produced from glycerol fermentation in *E. coli* were ethanol and succinate, accounting for 93% of the product mixture. Out of the initial 10 g/L glycerol in the medium, approximately 1.2 g/L remained unfermented. The need for supplementation was investigated by varying the tryptone concentration in the medium. Cell growth reached an OD_{550} of 1.25 ± 0.06 when using 10 g/L tryptone and an OD_{550} of 0.89 ± 0.05 when using 2 g/L tryptone. Also, the amount of fermented glycerol decreased from 8.42 ± 0.46 to 7.03 ± 0.56 upon decreasing tryptone concentrations from 10 g/L to 2 g/L.

Further experiments by the same group confirmed the fermentative dissimilation of glycerol by *E. coli* through the use of knockout mutants missing key enzymes involved in respiratory metabolism (Murarka et al., 2008). It was confirmed that glycerol is used in the formation of biomass through carbon labelling and that supplementation of tryptone in the medium was not acting as a source of electron acceptors. The accumulation of H_2 in the environment was found to negatively impact the fermentation.

Since the discovery that *E. coli* can anaerobically ferment glycerol, many studies have been performed involving metabolically engineered strains to enhance the formation of desired value-added products. The production of hydrogen was enhanced 20-fold and ethanol 5-fold through the use of adaptive evolution and chemical mutagenesis (Hu and Wood, 2010). Metabolic engineering was also used to increase the production of succinate from glycerol in *E. coli* fermentation (Blankschien et al., 2010).

Anaerobic fermentation of glycerol by the *Propionibacterium* genus of bacteria has previously been demonstrated in several strains. Fermentation experiments using *Propionibacterium acidipropionici* and *Propionibacterium acnes* were performed using 20 g/L glycerol in rich medium to produce propionic acid (Barbirato et al., 1997). The medium contained both yeast extract and tryptic soy broth. Propionic acid was the main product with minor by-products of succinic acid, acetic acid, formic acid, and n-propanol. Glycerol was fully consumed by *P. acidipropionici* in 73 h. while only 44% of glycerol was consumed by *P. acnes* after 480 h. Further studies were performed on the fermentation of glycerol by *P. acidipropionici* and *Propionibacterium freudenreichii* ssp. *shermanii* (Himmi et al., 2000). Rich medium was again used containing 20 g/L glycerol, and complete glycerol consumption was observed in both strains. The major product was propionic acid, the main by-product was acetic acid, and n-propanol and succinic acid were produced as minor by-products. *P. acidipropionici* followed growth-associated fermentation kinetics while the *P. freudenreichii* strain continued to consume glycerol even after product formation stabilized. This indicates glycerol consumption for cell maintenance. *P. acnes* and *P. acidipropionici* are opportunistic pathogens while *P. freudenreichii* is a non-pathogenic species.

More recently a comparison was performed using pure glycerol, crude glycerol, and whey lactose as carbon sources in fermentation by *P. freudenreichii* ssp. *shermanii* for the production of propionic acid (Kośmider et al., 2010). A salt medium was used with the addition of casamino acid and casitone as a source of amino acids, as well as biotin. Fermentation products included propionic acid, acetic acid, and succinic acid. For both pure and crude glycerol carbon sources, complete substrate utilization was reported within 120 hours using 20 g/L substrate. Upon increasing the substrate concentration to 40 g/L, incomplete utilization was observed for both pure and crude glycerol. The consumption of crude glycerol by *P. freudenreichii* spp. *shermanii* implies that the microorganism can tolerate some of the impurities present in crude glycerol from biodiesel production.

2.3 Microbial fuel cells

2.3.1 Overview of microbial fuel cells

A microbial fuel cell (MFC) uses bacteria as a catalyst to convert chemical energy to electrical energy (Fig 2.7). MFCs are similar in operation to conventional fuel cells except that the anode catalyst is biological in nature instead of metallic. The microorganism is present in the anode chamber where it oxidizes a fuel resulting in the production of electrons. The anode accepts these electrons which travel through an external circuit to the cathode creating a current. A reduction reaction occurs in the cathode chamber. The two most common catholytes in MFCs are oxygen and ferricyanide. Currently the use of ferricyanide produces higher power outputs than dissolved oxygen, but the development of oxygen systems is more desirable from both environmental and economic standpoints (Logan, 2008). The anode chamber of an MFC is kept anaerobic while the cathode is either suspended in an aerobic solution or open to the atmosphere in the case of air cathodes.

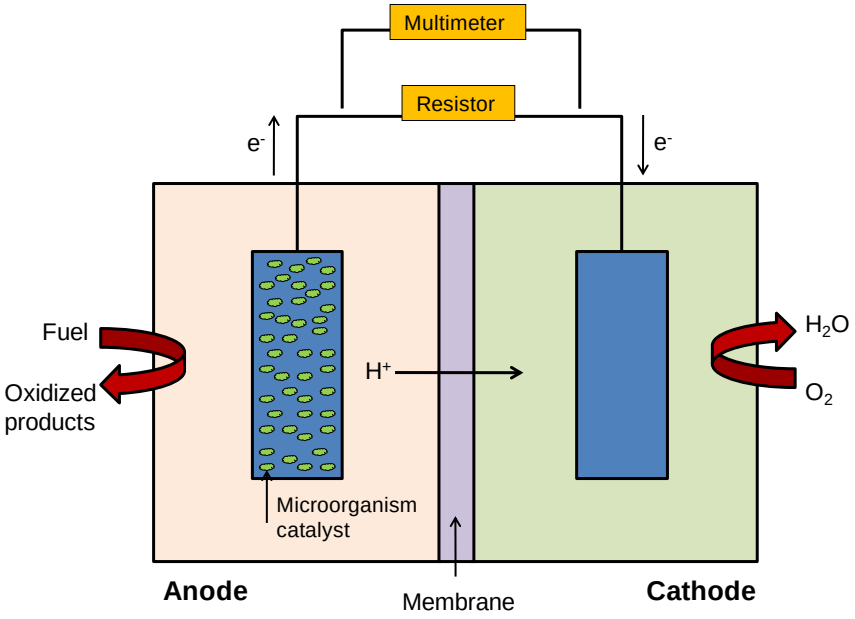

Figure 2.7. Schematic of a microbial fuel cell. Figure adapted from Logan, 2008.

MFCs can be single-chambered or two-chambered systems. In two-chambered MFCs, the cathode and anode chambers are separated by a semi-permeable membrane that restricts oxygen flow but allows proton transfer (Lovely, 2006). One example of a two-chambered system is the H-type fuel cell which is composed of two glass bottles connected by a tube containing a membrane. This type of fuel cell is an inexpensive model and is therefore widely accepted for basic parameter research (Logan et al., 2006). The architecture of H-type MFCs, as well as other two-chambered systems, results in a high internal resistance and therefore limits the power densities achievable by the system.

Practical applications of MFCs will require the use of systems with high Coulombic efficiencies and power outputs but also economical choices of materials and process requirements. MFCs that can be applicable to an industrial scale are still being developed, however, several sophisticated designs have been created that eliminate some of the internal resistance inherent in two-chambered systems. These designs include cube reactors, tubular reactors, and flat plate reactors, and they can achieve higher power densities compared to conventional two-chambered systems. These architectures are single-chambered. A cube reactor, for example, typically consists of a block of acrylic or Lexan with the two electrodes placed at the opposite end of the reactor (Logan, 2008). High power densities are obtainable due to the membraneless configuration and close electrode spacing.

2.3.2 Biocatalysts

The bacteria used as a catalyst in an MFC must be able to transfer their electrons from inside their cell membranes to the anode in order to create a current. Bacteria that can independently transfer their electrons to an electrode are referred to as exoelectrogens. Non-exoelectrogenic bacteria require the use of external mediating compounds that can permeate cell membranes, collect electrons, and transport them to the anode. There are many considerations when choosing a mediating compound in order to not hinder fuel cell performance. The mediator must have a high diffusion coefficient to permeate the bacterial cells and must also be non-toxic to the microorganisms. Furthermore, the potential of the mediator has to be similar to the microorganism but different enough to allow electron transfer to occur. Mediating compounds should also be re-usable and must possess a low

transfer resistance (Bullen et al., 2006). Some examples of mediating compounds include neutral red, methyl blue, and thionine. Chemical mediators are typically expensive, toxic, and can be degraded over time.

The negative environmental and economic implications of chemical mediators on MFC processes have shifted the focus of MFC research to exoelectrogenic bacteria and the development of mediatorless MFCs. There are two known mechanisms of electron transfer in exoelectrogens: the use of bacterial nanowires that attach the cell surface to the electrode and the production of mediating compounds by the cell (Logan, 2008) (Fig. 2.8). Bacterial nanowires are conductive appendages that connect the cells and the electrodes while mediating compounds are excreted from the cells. Mediating compounds carrying electrons are oxidized at the electrode and then return to the cells where they are reduced.

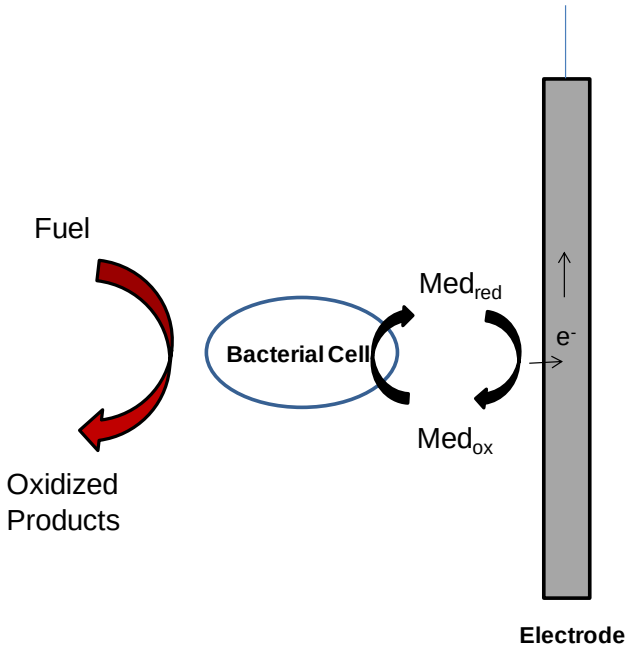

Figure 2.8. Schematic of mediator function in MFCs

Exoelectrogenic activity has been widely documented in the metal-reducing bacterial species *Geobacter* and *Shewanella* (Logan, 2008). *Shewanella putrefaciens,* an iron-reducer, has been shown to be electrochemically active in a fuel cell without the addition of external mediators. Direct electron transfer to the electrode was demonstrated in this species through cyclic voltammetry experiments (Kim et al., 2002). Microorganisms from the *Geobacteraceae* family have been found to be highly enriched on fuel cell anodes placed in sediment MFCs. It was determined that the *Geobacter* microorganisms were able to oxidize organic compounds using solely the anode as an electron acceptor (Bond et al., 2002). A study was then conducted using *Geobacter sulfurreducens* as an MFC biocatalyst with both acetate and hydrogen as fuel. *G. sulfurreducens* was able to completely oxidize both fuels using only the electrode as an electron acceptor and was able to transfer electrons to the electrode without the presence of external mediating compounds (Bond and Lovely, 2003). Further studies demonstrated the use of pilus-like appendages, termed nanowires, as a means of electrons transfer from the cell to the electrode in both *Geobacter* and *Shewanella* species (Logan, 2008).

Pseudomonas aeruginosa has been shown to produce endogenous mediating compounds in MFCs (Rabaey et al., 2004). Enrichment was performed to select for a microbial consortia that can self-mediate electron transfer. *P. aeruginosa* was isolated from the enriched fuel cell and was reported to produce soluble redox mediators. Further studies confirmed the production of pyocyanin and phenazine-1-carboxamide as mediators for electron transfer (Rabaey et al., 2005). It was also determined that the presence of these mediators can enhance the electron transfer rate from other bacterial species present in mixed cultures containing *P. aeruginosa*. *P. aeruginosa* can also function as a pure strain in an MFC (Raghavulu et al., 2011). The use of *P. aeruginosa* as a pure biocatalyst in an MFC with glucose as fuel was found to produce higher current densities than both *E. coli* and mixed cultures derived from distillery wastewater.

Research has been performed using both pure and mixed cultures as MFC anodic catalysts. A summary of selected pure strain biocatalysts used in MFCs is presented in Table 2.4. The use of mixed cultures generally achieves a higher power density (Table 2.5), but

pure culture studies are necessary in order to characterize active microorganisms (Logan et al., 2006). Furthermore, pure cultures have on occasion demonstrated better performance than mixed cultures in the same MFC systems such as the aforementioned example comparing *P. aeruginosa* and mixed cultures derived from distillery wastewater (Raghavulu et al., 2011). A study was performed to compare the pure culture *S. oneidensis* with wastewater as a source of inoculum for several MFC architectures (Watson and Logan, 2010). In all the tested configurations, mixed cultures produced a higher power density. Increases compared to the pure culture ranged from 68-480% depending on the reactor configuration. Before use in MFC experiments, mixed cultures are usually subjected to enrichment for substrate specificity and electrogenic activity. Enrichment for substrate specificity can be performed either outside or inside the fuel cell through successive subculturing into fresh medium containing the desired substrate. Enrichment can also be performed in the fuel cell to obtain an electricity-generating microbial consortium (Kim et al., 2004).

Table 2.4. Selected pure strain bacterial biocatalysts used in MFCs

Genus	Species	Reference
Clostridium	*C. acetobutylicum* (ATCC 824)	Finch et al., 2011
Corynebacterium	*Corynebacterium* sp. MFC03	Liu et al., 2010
Escherichia	*E. coli* K12 (NBRC 3301), K12 (HB101), MTCC 10436, K12 (ATCC 29181), UWE Culture Collection 17	Wang et al., 2007 Zhang et al., 2007 Raghavulu et al., 2011 Qiao et al., 2008 Ieropoulos et al., 2005 Park and Zeikus, 2000 Zhang et al., 2006
Geobacter	*G. metallireducens*	Min et al., 2005
	G. sulfurreducens PCA (ATCC 51573)	Bond and Lovely, 2003 Ieropoulos et al., 2005 Reguera et al., 2006 Ishii et al., 2008 Trinh et al., 2009
Klebsiella	*Klebsiella* sp. ME17	Xia et al., 2010
	K. pneumoniae L17	Zhang et al., 2008
Pseudomonas	*P. aeruginosa* KRP, MTCC 10436	Rabaey et al., 2005 Raghavulu et al., 2011
Rhodoferax	*R. ferrireducens* DSMZ 15236	Liu et al., 2007
Shewanella	*S. japonica* ATCC BAA 316	Biffinger et al., 2011
	S. marisflavi EP1	Huang et al., 2010
	S. oneidensis MR-1, DSP-10,	Lanthier et al., 2008 Biffinger et al., 2008 Watson and Logan, 2010 Luckarift et al., 2010 El-Naggar et al., 2010
	S. putrefaciens MR-1, IR-1, SR-21	Kim et al., 2002

Table 2.5. Comparison of power densities achieved by pure and mixed culture MFCs

Species /Inoculum Sources	Power Density (mW m^{-2})	Substrate	Reference
Escherichia coli	1300	Glucose	Qiao et al., 2008
Geobacter sulfurreducens	418-470	Acetate	Trinh et al., 2009
Pseudomonas aeruginosa	2.7 ± 0.9	Glucose	Rabaey et al., 2005
Shewanella oneidensis MR-1	148 ± 20	Lactate	Watson and Logan, 2010
Shewanella oneidensis DSP10	3000	Lactate	Ringeisen et al., 2006
Foliage/dung soil	5850	Glucose	Rosenbaum et al., 2006
Wastewater	858 ± 9	Lactate	Watson and Logan, 2010
	6860	Acetate	Fan et al., 2008

2.3.3 *E. coli* and *P. freudenreichii* as MFC biocatalysts

A summary of MFC experiments using *E. coli* as the anodic catalyst is presented in Table 2.6. An early study was performed using *E. coli* in an amperometric poised–potential culture system with glycerol as a substrate (Emde et al., 1989). A current of 3.0 to 4.0 mA was achieved; however, it was reported that potassium ferricyanide was required as an electron mediator. It has recently been determined that *E. coli* can be used as a catalyst in mediatorless MFCs through the use of electrochemically-evolved strains (Zhang et al., 2006). Using glucose as the substrate, it was suggested that a natural selection process occurs in electrochemical environments for bacterial cells that can use the anode as a final electron acceptor. A power density of 600 mW m^{-2} was reported using these electrochemically evolved *E. coli* strains. Cyclic voltammetry was employed to confirm the presence of a redox compound excreted from *E. coli* biofilms on carbon-fiber electrodes in a glucose medium (Wang et al., 2007). It was later determined that electrochemically evolved *E. coli* cells produce hydroquinone type compounds as mediators (Qiao et al., 2008). The

highest power density achieved by an MFC containing *E. coli* cells evolved under electrochemical tension is 1300 mW m^{-2}.

Table 2.6. Use of *E. coli* as an anodic MFC catalyst

Strain	Mediator	Substrate	Reference
MTCC 10436	Mediatorless	Glucose	Raghavulu et al., 2011
K12 HB101	Mediatorless	Glucose	Wang et al., 2007 Zhang et al., 2007
K12	Mediatorless	Glucose	Zhang et al., 2006
K12 ATCC 29181	Mediatorless	Glucose	Qiao et al., 2008
UWE Culture Collection 17	Neutral red, methylene blue, thionine, Meldola's blue, 2-hydroxy-1,4-naphthoquinone	Sucrose	Ieropoulos et al., 2005
K12	Neutral red	Glucose	Park and Zeikus, 2000

A study was performed to compare *P. aeruginosa*, *E. coli*, and an anaerobic consortium as catalysts in a fed-batch MFC oxidizing glucose (Raghavulu et al., 2011). It was determined that *P. aeruginosa* achieved the highest current density (264 mA m^{-2}), followed by mixed cultures (166 mA m^{-2}), and then *E. coli* (147 mA m^{-2}). Mixed cultures, however, were able to sustain a biopotential for the longest period of time.

The only report of electrochemical activity in *Propionibacterium* was using a poised-potential amperometric culture system with hexacyanoferrate (III) as a mediator (Emde and Schink, 1990). *P. freudenreichii* was used as a catalyst and glycerol, lactate, and propionate were used as substrates. To date, no research has been published on mediatorless MFCs using *Propionibacteria* as a catalyst.

2.3.4 Performance measures

Microbial fuel cell performance is most commonly assessed by power output and Coulombic efficiency (Logan et al., 2006). Power can be calculated by measuring the voltage (E_{cell}) across a fixed external resistance (R_{ext}). Current (I) can be calculated by Ohm's law as follows:

$$I = E_{cell}/R_{ext} \quad [1]$$

Power (P) can then be determined from the calculated current using the following equation:

$$P = IE_{cell} \quad [2]$$

In order to compare power output between different MFC architectures, power is often normalized to anode surface area (A_{An}), and power density values (P_{An}) are reported.

$$P_{An} = \frac{E_{cell}^2}{R_{ext} A_{An}} \quad [3]$$

Polarization and power density curves are useful tools in the analysis and characterization of a fuel cell (Logan et al., 2006). A polarization curve is a plot of voltage as a function of current. Voltage is measured upon both increasing and decreasing the external resistance, and current is calculated by Ohm's Law. In conventional fuel cells, three zones are generally present on the polarization curve representing activation losses (region A), Ohmic losses (region B), and mass transport losses (region C), respectively (Fig. 2.9). MFCs generally produce a predominantly linear polarization curve allowing the internal resistance (R_{int}) to be determined from the slope. From the polarization curve, the power density curve can be obtained, which allows for the determination of a maximum at a point where the internal resistance is equal to the external resistance (Fig. 2.9). The maximum power density (P_{max}) occurs at the optimal current (i_{opt}). The open circuit voltage (OCV) is

the maximum voltage of the system and is obtained when there is no external load in the fuel cell.

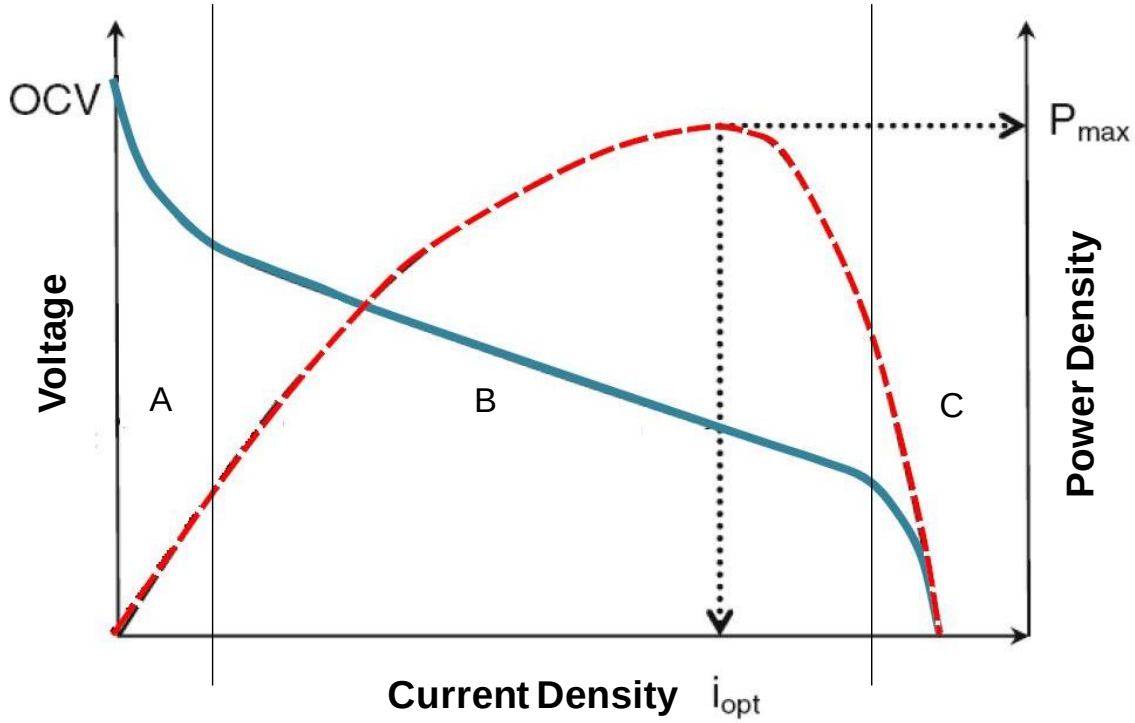

Figure 2.9. Typical polarization and power density curves from a conventional fuel cell. The solid line is voltage as a function of current and is referred to as the polarization curve. The dashed line is power density as a function of current density. Figure adapted from Clauwaert et al., 2008.

As demonstrated by fuel cell polarization curves, performance can be governed by activation losses (A), Ohmic losses (B), and mass transport losses (C). MFCs have additional bacterial metabolic losses compared to conventional fuel cells (Logan et al., 2006). Activation losses in an MFC occur throughout the transport of electrons to the anode from the bacterial cell or from the cathode to the oxidant (Lee et al., 2007). They also occur when heat energy is lost upon initiation of oxidation and reduction reactions (Logan, 2008). Lowering activation losses can be achieved through increasing the electrode surface area, increasing the operating temperature, and establishing a biofilm on the anode surface (Logan et al., 2006).

Bacterial metabolic losses occur when the bacteria acquire energy from substrate oxidation (Logan, 2008). As part of bacterial metabolism, electrons travel through the electron transport chain to a final electron acceptor at a higher potential than the substrate (Logan et al., 2006). The larger the difference between the anode and substrate potential, the greater metabolic energy gain for the bacteria and therefore a lower MFC voltage will be obtained. The anode potential cannot be too low however or electron transport will be repressed.

Ohmic losses are of the greatest concern in an MFC. This can be seen by the predominantly linear nature of polarization curves from MFC systems. Ohmic losses include the resistance towards the flow of electrons through the circuit and the resistance of ions through the membrane (Logan et al., 2006). To decrease the resistance of electron flow, good electrical contacts need to be established between the electrodes and wires, and the space between the electrodes should be minimized. Generally the same membrane materials are used in conventional fuel cells and MFCs; however, in MFCs proton transfer is often inhibited by the cations present in the medium of the anode chamber (Kim et al., 2007a). The salts used in the medium can dissociate into cations such as K^+, Na^+, NH_4^+, Mg^{2+}, and Ca^{2+}. These cations can be transported across the membrane instead of protons resulting in acidification of the anode chamber and an alkalized cathode chamber. The oxidation potential of the microorganisms can be negatively affected by too acidic of an environment, and a basic cathode reduces its potential. Using a membraneless MFC or a low concentration of cations can improve the efficiency of proton transfer to the cathode.

Concentration losses generally occur at high current densities and are a result of poor reactant flux towards the electrode or product flux away from the electrode. Substrate transport towards the anode is generally not an issue in MFCs; however product accumulation, specifically protons, near the anode does occur resulting in a low pH. On the cathode side, oxygen reduction has been shown to be a limiting factor in MFC performance (Kim et al., 2007a). Carbon-based materials are commonly used as cathodes. Graphite, for example, requires a dissolved oxygen concentration of 6.6 mg/L, which is approximately 80% of the concentration in air saturated water. Consequently, oxygen flux to the cathode is

rarely fast enough to avoid being a limiting factor in air cathode fuel cell operations. Solutions to this problem include using a cathode catalyst such as platinum to decrease the necessary dissolved oxygen concentration for a reaction to occur. Hexacyanoferrate is also commonly used as a cathode mediator; however, it is a toxic, non-biodegradable chemical, and therefore its use decreases the sustainability of the technology.

Coulombic efficiency (ε_c) is another important indicator of MFC performance. It is defined as the Coulombs transferred to the anode (C_p) over the theoretical maximum Coulombs (C_t) if all substrate oxidation produced current (Liu and Logan, 2004a).

$$\varepsilon_C = \frac{C_p}{C_t} * 100\% \qquad [4]$$

The total Coulombs produced (C_p) is determined by integrating the current with respect to time, and C_t is determined as follows:

$$C_t = \frac{FnSv}{M} \qquad [5]$$

where F is Faraday's constant (98485 C/mol electrons), n is the moles of electrons produced per mole of substrate consumed (14 in the case of glycerol), S is the substrate concentration, v is the liquid volume, and M is the molecular weight of the substrate. Coulombic efficiency can be greatly affected by cell growth as electrons are used in the production of biomass instead of being transferred to the anode (Logan et al., 2006).

The performance of a fuel cell can also be assessed through electrochemical efficiency (η). The theoretical maximum open circuit voltage (E^o) can be calculated using the following equation:

$$E^\circ = \frac{-G}{nF} \qquad [6]$$

where G is the Gibbs energy of the overall reaction occurring in the fuel cell. The electrochemical efficiency is then calculated by Equation 7 where the OCV is measured from the fuel cell.

$$\eta = \frac{OCV}{E^{\circ}} \qquad [7]$$

One limitation to using electrochemical efficiencies for MFC systems is the assumption that complete oxidation of the fuel is occurring. MFC efficiencies are difficult to predict due to the variety of factors that can influence bacterial metabolism, but the electrochemical efficiency can provide a basis value to assess system performance.

2.3.5 Applications

The development of MFC technologies is still in its infancy, so certain applications are being focused on to break through the market. Currently, the most promising application for MFCs is the recovery of energy from waste. A significant amount of research has focused on the development of MFC technologies for wastewater treatment. It has been shown that it is possible to produce electricity from an MFC using domestic wastewater as a fuel and at the same time lower the chemical oxygen demand (COD) of the wastewater (Liu et al., 2004b). A single-chamber MFC bioreactor was developed that operated under continuous flow. The maximum power generation was 26 mW m^{-2}, and a COD reduction of 80% was achieved using the effluent from the primary clarifier in a wastewater treatment plant for the fuel.

Research on MFC treatment of wastewaters has since been conducted using a variety of different feedstocks. Full strength beer brewery wastewater was able to achieve a power density of 483 mW m^{-2} with a COD removal range of 85-87% when varying the temperature from 20 to 30°C (Wang et al., 2008). A study was performed comparing four different industrial wastewaters (bakery, brewery, dairy, paper) in an MFC inoculated with anaerobic sludge (Velasquez-Orta et al., 2011). It was found that 80% organic matter removal could be achieved with all wastewaters; however, there was only significant current production using the paper wastewater feedstock. Current was found to be limited by the anodic biofilm composition which varied with wastewater feed. The treatment of corn stover has also been performed using an MFC (Wang et al., 2009). A power density of 331 mW m^{-2} was achieved. This was the first time direct production of power was demonstrated from such a complex biomass source.

Using MFCs for both wastewater treatment and the production of secondary fuels has also been proposed. A link was shown for the production of electricity and hydrogen by the treatment of wastewater in an MFC (Oh and Logan, 2005). Using cereal wastewater with a high COD and sugar content, MFC treatment resulted in hydrogen production of 210 mL/L, power generation of 81 mW m^{-2}, and 95% COD removal.

Although a significant amount of research has been conducted on concurrent wastewater treatment and electricity generation using MFCs, this type of technology is not yet being used on an industrial scale. Research has come a long way, but design improvements are still required for economically and technically viable systems on a large scale. The Advanced Water Management Centre has built a pilot scale MFC in Australia using brewery wastewater as a feedstock, and experiments are currently being performed to assess the feasibility of the system.

In addition to the extraction of energy from waste, there are several other promising applications in development for MFCs. These include sediment MFCs (SMFCs) for remote power generation, biosensors, and bioremediation.

SMFCs function by placing an anode into anaerobic marine sediment and placing the cathode in the water above which contains dissolved oxygen (Logan, 2008). They can be used in remote locations such as the bottom of the ocean. An SMFC was the first type of MFC technology to function as a practical device (Franks and Nevin, 2010). Meteorological buoys used to measure real time data such as temperature, pressure and humidity, were powered solely using an SMFC (Tender et al., 2008). These technologies are still being developed for economic improvements; however, this field work shows that SMFCs can be used instead of batteries for low-power marine instruments with longer durability.

The use of MFCs as biosensors for biological oxygen demand (BOD) in wastewater analysis is another promising application for this type of technology. Current output is proportional to the amount of organic matter in the water. This type of technology will allow for real-time monitoring of wastewater effluents instead of the standard BOD_5 test which takes 5 days to obtain results. A two-chambered mediatorless MFC was developed for use as a BOD biosensor and was operated for over 5 years without servicing (Kim et al., 2003). A

single-chamber MFC (SCMFC) was then developed, and the biosensor output exhibited a linear relationship with BOD up to 350 mg BOD cm^{-3}, which was an improvement compared to the two chambered system (Di Lorenzo et al., 2009). The SCMFC was also more compact with lower operational costs due to the use of a direct air cathode.

MFCs are also being developed for concurrent electricity generation and bioremediation. An MFC is being developed as a sustainable power supply that can remediate hexavalent chromium from contaminated sites (Huang et al., 2011). While this type of technology is still in the early development phases, proof of concept has been achieved through the use of a MFC producing power up to 15 W m^{-3} while reducing hexavalent chromium at a rate ranging from 12.4 to 20.6 mg g^{-1} VSS h^{-1}. Another potential bioremediation application of MFCs is for the anaerobic biodegradation of diesel. In a SCMFC study, 82% removal of diesel range organics, along with a power generation of 31 mW m^{-2} cathode area, was achieved (Morris et al., 2009).

2.3.6 Glycerol feedstocks in MFCs

MFC technologies are being developed with a wide range of feedstocks. The most prevalent feedstocks seen in literature are wastewater, glucose, and acetate; however, numerous feedstocks are possible depending on the bacterial strains used as catalysts and their oxidative capacity towards the fuel.

The potential of glycerol as an MFC feedstock is just starting to be realized. Glycerol is a non-toxic, non-volatile, non-flammable chemical. It has a high energy density, and it is highly abundant, making it a promising choice as a fuel (Arechederra et al., 2007). A biofuel cell, or enzymatic fuel cell, is similar to an MFC except that it uses solely an enzyme as an anode catalyst instead of whole microorganisms. Biofuel cells can have high specificity for certain fuels but less versatility to complex feeds compared to MFCs (Davis and Higson, 2007). A biofuel cell was developed using an alcohol dehydrogenase and aldehyde dehydrogenase based anode in order to oxidize glycerol as a fuel (Arechederra et al., 2007). This system was able to produce a power density of 12100 mW m^{-2} which is high compared to ethanol biofuel cells.

There have been two recent reports using glycerol as an MFC feedstock in a single-chambered system. A pure culture system was developed using *Bacillus subtilis* as an anode biocatalyst (Nimje et al., 2011). Pure glycerol was used as a substrate and a maximum power density of 0.06 mW cm^{-2} was achieved at an optimal external resistance of 390 Ω. The other study was performed using biodiesel waste with a COD of 1400 mg/L as a fuel and domestic wastewater as an inoculum source (Feng et al., 2011). A power density of 2100 mW m^{-2} cathode area was achieved.

Chapter 3. Materials and methods

3.1 Chemicals

A list of chemicals used and their suppliers is presented in Table 3.1.

Table 3.1. Suppliers and purities of chemicals

Chemical (purity)	Supplier
1,3-Propanediol (98%)	Sigma Aldrich (St. Louis, MO)
Acetonitrile (HPLC grade)	Fisher Scientific (Fairlawn, NJ)
Glycerol (99.8%)	Fisher Scientific (Fairlawn, NJ)
Hydrogen peroxide (3%)	Fisher Scientific (Fairlawn, NJ)
Sodium hydroxide (1 N)	Acros (Geel, Belgium)
Sulfuric acid (1 N)	Fisher Scientific (Fairlawn, NJ)

3.2 Growth media and stock solutions

Suppliers of growth media and media supplements are described in Table 3.2. Luria-Bertani (LB) broth and Reinforced Clostridial Mediaum (RCM) were prepared as described by the manufacturers. A concentration of 15 g/L Bacto agar was added to LB and RCM media when preparing agar plates. Distilled water (DH_2O) was used in the preparation of all media.

Table 3.2. Suppliers of rich media components and supplements

Medium/Medium Components	Supplier
Bacto agar	BD (Sparks, MD)
Bacto tryptone	BD (Sparks, MD)
Bacto yeast extract	BD (Sparks, MD)
Luria-Bertani broth, Miller	Fisher Scientific (Fairlawn, NJ)
Reinforced Clostridial Medium	Oxoid (Hampshire, UK)

Defined medium and stock solutions wer€e prepared according to the following formulations:

Standard M9 Medium (20 g/L Glycerol)

$Na_2HPO_4 \cdot 7H_2O$	6.4 g
KH_2PO_4	1.5 g
NaCl	0.25 g
NH_4Cl	0.5 g
500 g/L glycerol	20 mL
1M $MgSO_4$	1 mL
1M $CaCl_2$	50 µL
DH_2O	to 500 mL

The basic M9 medium formulation was taken from Sambrook et al. (1989).

Enriched M9 Medium (5 g/L Glycerol)

$Na_2HPO_4 \cdot 7H_2O$	6.4 g
KH_2PO_4	1.5 g
NaCl	0.25 g
NH_4Cl	0.5 g
Tryptone	5 g
500 g/L glycerol	5 mL
1M $MgSO_4$	1 mL
1M $CaCl_2$	50 µL
DH_2O	to 500 mL

1M $CaCl_2$

$CaCl_2$	5.55 g
DH_2O	50 mL

1M $MgSO_4$

$MgSO_4$	6.02 g
DH_2O	50 mL

500 g/L glycerol

Glycerol	39.65 mL
DH_20	60.35 mL

Phosphate Buffer

In DH_2O:

KH_2PO_4	20.3 g in 150 mL
K_2HPO_4	60.96 g in 350 mL

For the preparation of phosphate buffer, the KH_2PO_4 solution was added to the K_2HPO_4 solution until a pH of 7.2 was reached. The buffer was then diluted 100X in DH_2O making a 10 mM solution.

Pfennig's Vitamins*

p-aminobenzoic acid	50 mg
Vitamin B-12	50 mg
Biotin	10 mg
Thiamine	100 mg
DH_2O	1.0 L

*filter sterilized before addition to medium

Standard M9 medium was used for maintenance of enrichment cultures while enriched M9 medium was used for pure cultures. The concentration of tryptone in enriched M9 medium was varied or replaced with yeast extract during media optimization experiments. For the preparation of anaerobic M9 and enriched M9 media, the water was first boiled, and the completed medium was sparged with nitrogen while cooling. Once the

medium reached room temperature, it was dispensed into serum bottles while continuously sparging with nitrogen. Resazurin was added to a final concentration of 1 mg/L to M9 medium when testing anaerobic conditions. Sodium thioglycolate was added to each bottle to a final concentration of 0.5 g/L. Media were sterilized through autoclaving at 121°C for 20 minutes. For the preparation of M9 plates, Bacto agar was added to a final concentration of 15 g/L to enriched M9 medium.

3.3 Pure bacterial strains

Bacterial strains *E. coli* W3110 (ATCC 27325), *P. freudenreichii* ssp. *freudenreichii* (ATCC 6207), and *P. freudenreichii* ssp. *shermanii* (ATCC 9614) were obtained from the American Type Culture Collection. *E. coli* strains TG1, DH5α, and BL21 were obtained from Dr. Christopher Lan (Department of Chemical and Biological Engineering, University of Ottawa). Strains were stored in 15% glycerol solutions at -80°C. *E. coli* strains were stored in LB, and *P. freudenreichii* strains were stored in RCM. For strain maintenance, *E. coli* was grown aerobically on LB agar plates at 37°C, and *P. freudenreichii* was grown on RCM agar plates anaerobically at 30°C in an anaerobic jar containing a CO_2 gas generating kit and an anaerobic indicator all obtained from Oxoid (Hampshire, UK). Fresh plates were streaked monthly and stored at 4°C.

3.4 Enrichment of mixed cultures

Samples of compost were collected from the University of Ottawa's mechanical composting system. Three sources were used: fresh compost, one month old compost, and three month old compost. For each sample, a mass of 2 g of solid compost was diluted into 50 mL of enriched M9 medium in 125 mL serum bottles. The headspaces were filled with nitrogen gas. Cultures were prepared in triplicate for each compost source and were grown at room temperature with gentle shaking. After one week of growth, 0.5 mL subsamples of each culture were diluted into 50 mL of M9 anaerobic medium containing 10 g/L glycerol. Glycerol concentrations were then monitored by HPLC, and once glycerol was depleted, 100X dilutions into fresh M9 anaerobic medium with 20 g/L glycerol were performed (Fig. 3.1). After 5 months of transfers, the triplicate cultures from each source were combined by adding 0.5 mL subsamples to 50 mL anaerobic M9 medium containing 20 g/L glycerol.

Cultures were then maintained by monthly transfer into anaerobic M9 medium with 20 g/L glycerol. The stabilized mixed cultures were named AR1 (fresh compost source), AR2 (one month old compost source), and AR3 (three month old compost source).

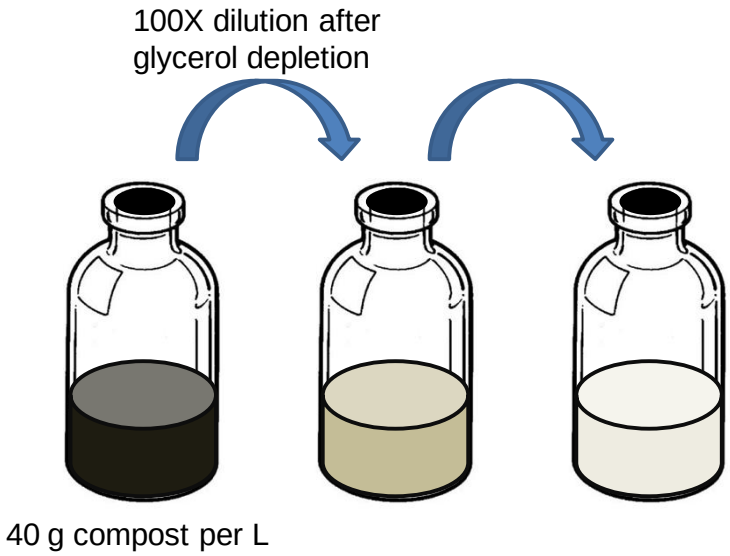

Figure 3.1. Schematic of compost enrichment procedure

3.5 Anaerobic glycerol fermentation

From LB agar plates, *E. coli* colonies were picked and grown overnight at 37°C and 150 rpm in LB liquid medium. From RCM agar plates, *P. freudenreichii* strains were grown in RCM liquid medium in hungate tubes (Bellco, NJ) at 30°C until turbid. These pre-cultures were used to inoculate 50 mL enriched M9 medium in 125 mL serum bottles in order to obtain active, anaerobic, liquid cultures for each strain. Once exponential growth occurred, pre-cultures were used to inoculate fresh 125 mL serum bottles containing 50 mL enriched M9 medium under anaerobic conditions to a final protein concentration of 0.1 mg/L. Fermentations were performed in triplicate for each strain. All experiments were

conducted at 37°C for *E. coli* strains and 30°C for *P. freudenreichii* strains. Samples were taken throughout the fermentation to monitor growth and glycerol conversion. Fermentations were run until a stationary phase was reached. Plates were then streaked and grown anaerobically to check for purity. All samples and inoculations were performed using sterile 23 ½ gauge needles and sterile 1 mL syringes. Samples for HPLC were centrifuged at 13,400 rcf for 2 minutes, and supernatants were stored at -20°C until analysis.

To determine whether 1,3-propanediol (1,3-PDO) was an end product in mixed cultures, fermentations were performed using anaerobic M9 medium with 5 g/L 1,3-PDO as the sole carbon source. Medium was inoculated with active cultures of either AR1, AR2, or AR3, and the cultures were grown anaerobically in serum bottles at room temperature. The concentration of 1,3-PDO in the medium was monitored using high performance liquid chromatography (HPLC).

3.6 Medium optimization experiments

Pure culture anaerobic glycerol fermentation experiments were repeated as previously described with different concentrations of tryptone in enriched M9 medium and also by replacing tryptone with yeast extract. Three different variations of the medium were used: 2 g/L tryptone, 5 g/L tryptone, and 5 g/L yeast extract. Each pure strain was anaerobically grown in triplicate in each type of medium. For *P. freudenreichii* strains, a glycerol concentration of 10 g/L was used, and for *E. coli* strains, a glycerol concentration of 5 g/L was used. Additionally the *P. freudenreichii* strains were grown in M9 medium with 5 g/L glycerol and 1.0 mL/L Pfennig's vitamin solution.

3.7 Analytical methods

To detect and quantify the presence of glycerol and 1,3-PDO, samples were analyzed with HPLC using the RID detector of an Agilent 1200 series system equipped with a Zorbax carbohydrate column. Column and detector temperatures were both 40°C. The mobile phase was a 75% acetonitrile water solution with a flow rate of 1.5 mL/min. Under these conditions, glycerol and 1,3-PDO standards were found to elute at 2.3 minutes and 1.8 minutes, respectively. Samples were run for 5.5 minutes each. Solvents were filtered

through vacuum filtration before use. Water was filtered using a 0.45 µm pore size Millipore MF-Millipore mixed cellulose ester membrane, and acetonitrile was filtered using a Millipore Fluoropore PTFE membrane.

Growth was monitored throughout fermentation experiments by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Thermo Electron Corporation). Sterile enriched M9 medium was used as a blank. The OD_{600} values were correlated to protein concentration as determined by the RC DC™ Protein Assay (BioRad). To obtain samples for the protein assay, strains were grown in enriched M9 medium, and samples were taken at various optical densities. The samples were centrifuged at 13,400 rcf for 2 minutes, and the pellets were resuspended in 1N NaOH and were heated at 90°C for 10 minutes in a water bath. These samples were then used to perform the RC DC™ Protein Assay as described by the manufacturer. Standard curves were prepared for each pure strain correlating protein concentration to OD_{600} using bovine serum albumin (BSA) standards (Appendix A1).

Gas chromatography experiments were solely qualitative. Carbon dioxide was detected in fermentation headspaces using a GOW-MAC series 580 TCD GC system and a Porapak Q column. Column, detector, and injector temperatures were set at 135°C, 165°C, and 160°C, respectively. Current was set at 125 amps. Carbon dioxide peaks were detected at 21 seconds.

3.8 Microbial fuel cell design

Two-chambered MFCs were fabricated using two 250 mL Pyrex solution bottles (Fisher Scientific) fused with an O-ring joint to straight tube adapter (LaSalle Scientifique) (Figs. 3.2 & 3.3). A Nafion membrane (Fuel Cell Store) was clamped between the adapters. Nafion membranes were treated before use by low boiling for 1 hour sequentially in each of the following solutions (4 hours total): 3% hydrogen peroxide, deionized water, 0.5 M sulfuric acid, and deionized water again. Membranes were then stored in deionized water until use. The membrane surface area was 9.6 cm^2. Electrodes were made from untreated carbon cloth (Fuel Cell Store). The cathode and anode areas were 25 cm^2 and 6.5 cm^2, respectively. Titanium wire with a diameter of 0.5 mm (VWR International) was used to

connect the circuit. Connections were secured with alligator clips. The anode chamber was sealed with plug seal caps (Fisher Scientific) and was sparged with nitrogen gas before use. The cathode chamber was open to the atmosphere and continuously sparged with air throughout fuel cell operation. MFCs were autoclaved at 121°C for 20 minutes before use.

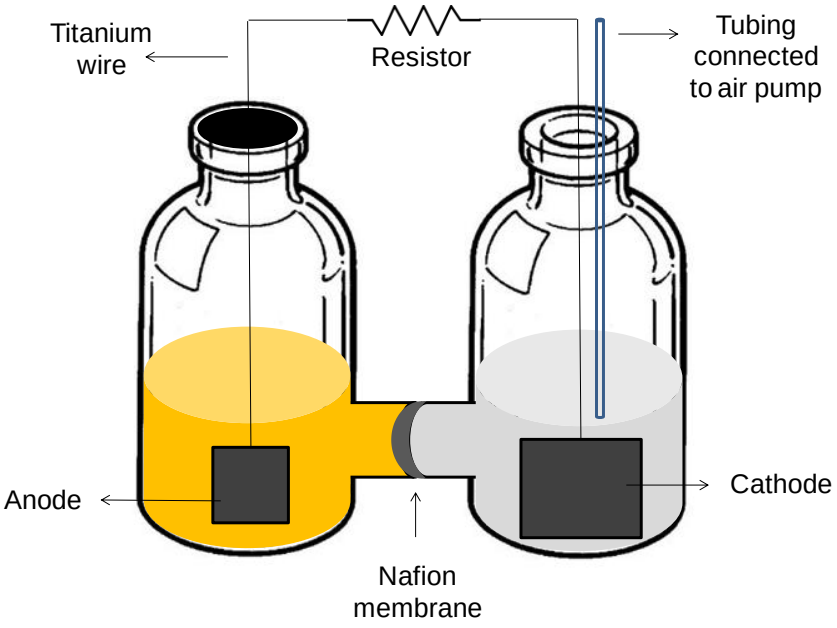

Figure 3.2. Schematic of H-type MFC system. Pyrex solutions bottles were used as the anode and cathode chambers. A Nafion membrane was clamped between the two bottles. Titanium wire was used to connect the circuit, and carbon cloth was used as the electrodes.

Figure 3.3. H-type MFC system. Pyrex solution bottles were used as the anode and cathode chambers.

3.9 Microbial fuel cell operation

The medium in the anode chamber varied depending on the bacterial strain used. For *E. coli*, standard enriched M9 medium was used, for *P. freudenreichii* M9 medium with 10 g/L glycerol enriched with 5 g/L yeast extract was used, and for mixed cultures, enriched M9 medium with 20 g/L glycerol was used. The anode chamber was filled with 150 mL of medium, and the cathode chamber was filled with 150 mL of phosphate buffer. The anode chamber was inoculated with 0.5 mL of either *E. coli*, *P. freudenreichii* or mixed cultures that were actively growing anaerobically in the same medium used in the anode chamber. The temperature of MFC operation varied with biocatalyst. The MFC was operated at 37°C for *E. coli* and 30°C for mixed cultures and *P. freudenreichii*.

3. 10 Electrochemical analysis

For experiments using resazurin as an anaerobic indicator, the MFC electrodes were hooked up to a 1000 Ω resistor, and voltage was measured using a voltmeter (VWR). Samples were taken for HPLC daily throughout the experiment.

For electrochemical analysis without resazurin, the MFCs were connected to a VersaSTAT 3 potentiostat (Princeton Applied Research). The voltage was set to a constant value of 200 mV, and open circuit voltage (OCV) was measured every two hours. The OCV was allowed to stabilize for 30 minutes before returning to a constant voltage of 200 mV. Once the OCV stabilized at the maximum value, polarization curves were run at a scan rate of 0.001 V/s. Throughout fuel cell operation, samples were taken daily for HPLC analysis.

Chapter 4. Strain selection

4.1 Introduction

Bacterial strains were selected for screening as anodic catalysts in an MFC using glycerol as a feedstock. Both pure strains and mixed cultures were chosen for catalyst screening experiments. The following criteria were used as a basis for bacterial strain selection:

- Ability to anaerobically metabolize glycerol
- Non-stringent anaerobe
- Non-pathogenic

The anode chamber of an MFC is kept anaerobic since oxygen acts as an electron acceptor. For this reason, bacteria used as an anodic catalyst in an MFC must be able to anaerobically metabolize the fuel.

Anaerobic bacteria can be divided into three categories: obligate, aerotolerant, and facultative. Obligate anaerobes can't use oxygen for growth and are intolerant to the presence of oxygen in their surroundings. Aerotolerant anaerobes do not use oxygen for growth but are tolerant to oxygen in their surroundings, and facultative anaerobes can utilize oxygen for growth but also can grow under anaerobic conditions. Strictly anaerobic conditions are difficult to achieve, especially on an industrial scale. For this reason, only aerotolerant and facultative anaerobes were selected for screening.

The pathogenicity of bacterial strains is important to consider especially when there is a possibility of future scale up. Pathogenic strains should be avoided in industrial microbiology due to potential health and environmental hazards and also increased handling costs and precautions.

This chapter describes how the aforementioned criteria were applied to the selection of pure strains and the source of mixed cultures. The design of the enrichment culture protocols is justified, and background information is provided on the selected pure strains.

4.2 Strain selection

4.2.1 Pure cultures

In order to select pure cultures for MFC screening, the first criterion applied was to find strains that have been shown in literature to anaerobically metabolize glycerol. Bacterial genera previously shown to grow anaerobically with glycerol as the sole carbon source include *Klebsiella, Citrobacter, Enterobacter, Clostridium, Propionibacterium, Anaerobiospirillum,* and *Escherichia*. The specific strains which have demonstrated an oxidative capacity towards glycerol in literature were considered as potential candidates for anodic biocatalysts. The pathogenicity of the strains and their anaerobic class were then assessed and are summarized in Table 4.1.

Table 4.1. Characteristics of select bacterial strains that can anaerobically metabolize glycerol

Genus	Class of Anaerobe	Species	Pathogenicity
Klebsiella	Facultative[1]	*K. pneumoniae*	Yes[1]
Citrobacter	Facultative[1]	*C. freundii*	Opportunistic[2]
Enterobacter	Facultative[1]	*E. aerogenes*	Opportunistic[1]
		E. agglomerans	Opportunistic[1]
Clostridium	Obligate[3]	*C. butyricum*	No[4]
		C. pasteurianum	No[5]
Propionibacterium	Aerotolerant[6]	*P. freudenreichii*	No[7]
		P. acidipropionici	Opportunistic[7]
		P. acnes	Yes[7]
Anaerobiospirillum	Obligate[7]	*A. succinicipropionici*	Yes[8]
Escherichia	Facultative[1]	*E. coli* K12	No[9]
		E. coli B	No[9]

[1] Bergey and Holt, 1994; [2] Cong et al., 2011; [3]Brook, 2008; [4] Heppner and Möse, 1978; [5] Brown, 2000; [6] Isenberg, 1998; [7] Salminen et al., 2005; [8] Shlaes, et al., 1982; [9] Schneider et al., 2002;

From the bacterial strains that have demonstrated the ability to anaerobically metabolize glycerol, only two species, *E. coli* and *P. freudenreichii*, are non-pathogenic and non-stringent anaerobes. Consequently, these bacteria were selected for use in catalyst screening experiments.

E. coli is a gram negative, rod-shaped bacterium. Although some strains of *E. coli* are pathogenic, there are two commonly used laboratory strains, B and K12, which do not produce the toxic O antigen that confers pathogenicity to the other strains. Both of these strains are commonly used in industrial microbiology since they are well studied and easy to both culture and manipulate. Four different strains of *E. coli* were selected as potential anode biocatalysts: W3110, TG1, DH5α, and BL21. They are all derivatives of the K12 strain except for the latter, BL21, which is a derivative of *E. coli* B. There is less than a 2%

difference between the genomes of *E .coli* strains K12 and B mostly due to transposable insertion sequences (Schneider et al., 2002).

The W3110 derivative was chosen because it was specifically shown to anaerobically metabolize glycerol (Dharmadi et al., 2006). Since the TG1 and DH5α strains are also of the K12 family, they were promising catalyst choices as well. Both strains have also been shown to aerobically metabolize glycerol as a carbon source (Rinas et al., 1995), and since *E. coli* is a facultative anaerobe, switching to an anaerobic environment may still allow the strains to metabolize glycerol. Anaerobic glycerol metabolism has also been demonstrated in *E. coli* B strains (Dharmadi et al., 2006), and the BL21 strain has specifically shown the ability to grow aerobically using glycerol as a substrate in M9 minimal medium (Paliy and Gunasekera, 2007).

P. freudenreichii is a gram-positive bacterium and has been used previously in industrial processes for the manufacturing of swiss cheese. Two subspecies of *P. freudenreichii* were selected for screening as anode catalysts in a glycerol-oxidizing MFC: subspecies *shermanii* and subspecies *freudenreichii*. Both strains have been previously shown to anaerobically metabolize glycerol in literature (Himmi et al., 2000; Emde and Schink, 1990).

4.2.2 Mixed cultures

Mixed cultures were derived from compost samples and were enriched for bacterial communities with a high oxidative capacity towards glycerol. The criteria used to select pure cultures were also employed when choosing the mixed culture source and establishing enrichment protocols. Compost was chosen as a source of inoculum since glycerol is commonly found in a variety of food products and is therefore likely present in food compost. Samples were taken from below the surface of the compost in order to increase the probability of obtaining microorganisms in the mixed cultures that do not require oxygen for growth. It was also unlikely that the samples contained obligate anaerobes since the compost source was exposed to air. Three different sources of compost inoculum were chosen in order to increase the diversity of bacteria in the enrichment cultures. The compost sources were monitored on a regular basis for the presence of pathogenic bacteria at the facility.

The inoculum source was chosen to increase the likelihood of containing non-pathogenic bacteria that can anaerobically metabolize glycerol. An enrichment protocol was established in order to concentrate the specific bacteria in the original mixed cultures with high anaerobic oxidative capacities towards glycerol and to dilute out bacteria that are not tolerant to the desired conditions. The culturing conditions of the enrichment procedure were chosen keeping in mind future scale-up. For this reason, minimal medium and room temperature conditions were used. Selecting for cultures that are tolerant to minimal conditions will reduce operating costs in any future industrial applications. A glycerol concentration of 20 g/L was selected for the enrichment medium in order to ensure that the surviving bacteria possess a high tolerance towards the substrate.

4.3 Conclusions

Six pure cultures and three mixed cultures were selected as potential anode catalysts for a glycerol-oxidizing MFC. Four strains of *E. coli* and two strains of *P. freudenreichii* were chosen as pure strains, and three different sources of compost were selected as inocula for mixed enrichment cultures. In the following chapters the anaerobic glycerol conversion capabilities of the strains will be confirmed and compared in order to select the most promising biocatalysts. Based on glycerol conversion studies, strains were tested in H-type MFCs for power generation.

Chapter 5. Anaerobic glycerol conversion in pure and mixed cultures

5.1 Introduction

This chapter focuses on confirming and comparing the anaerobic oxidative capacity of both pure bacterial strains and mixed cultures towards glycerol through fermentation experiments. Potential industrial applications of bacteria that can metabolize glycerol anaerobically include both the fermentation of glycerol to value-added products and use as anodic catalysts in a glycerol-oxidizing MFC. This chapter will focus on applications in fermentation and also using fermentation data as an indicator of future MFC performance.

For analysis of pure strains, anaerobic fermentation experiments were performed using glycerol as the sole carbon source. Four strains of *E. coli* (W3110, TG1, DH5α, and BL21) and two strains of *P. freudenreichii* (subspecies *freudenreichii* and subspecies *shermanii*) were studied. The strain selection process was described in detail in Chapter 4. Growth and glycerol consumption were monitored throughout the fermentation experiments and were used to assess the conversion capacity and tolerance of each strain towards glycerol. Glycerol conversion was also monitored in mixed cultures derived from compost and compared to pure culture values. The mixed culture selection and enrichment process was explained in Chapter 4.

This chapter also explores the effect of several medium additives on the anaerobic glycerol conversion capacities of the pure strains. In addition, an analysis of the production of carbon dioxide and 1,3-PDO as glycerol metabolites was performed. The investigation of fermentation by-products allowed for the elucidation of conversion pathways in the strains and gave an indication of the feasibility of using glycerol fermentation for the production of value-added metabolites.

5.2 Batch fermentation

5.2.1 Pure strain anaerobic fermentation

Pure bacterial strains were grown anaerobically in M9 minimal medium with glycerol as the sole carbon source. Growth was monitored by measuring OD_{600}. In order to compare growth between the pure strains of different species, optical density values were correlated to protein concentration (Appendix A) which was then used as a means of monitoring growth. Glycerol consumption was observed throughout the fermentation experiments using HPLC. Growth and glycerol consumption were demonstrated anaerobically in all six pure strains (Figs. 5.1-5.6).

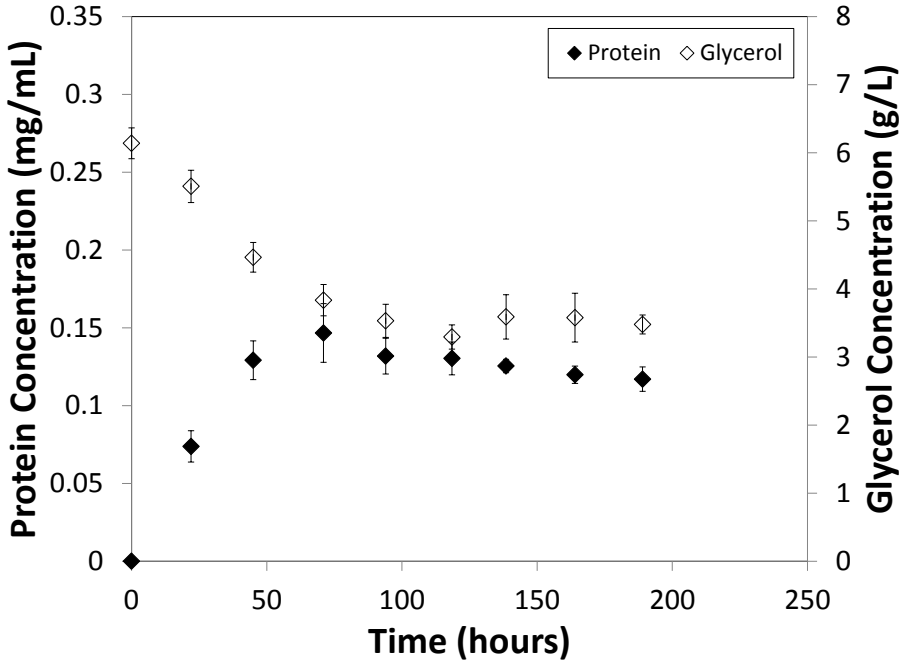

Figure 5.1. Anaerobic growth and glycerol consumption of *E. coli* TG1. *E. coli* was grown anaerobically in M9 medium supplemented with 10 g/L tryptone and glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

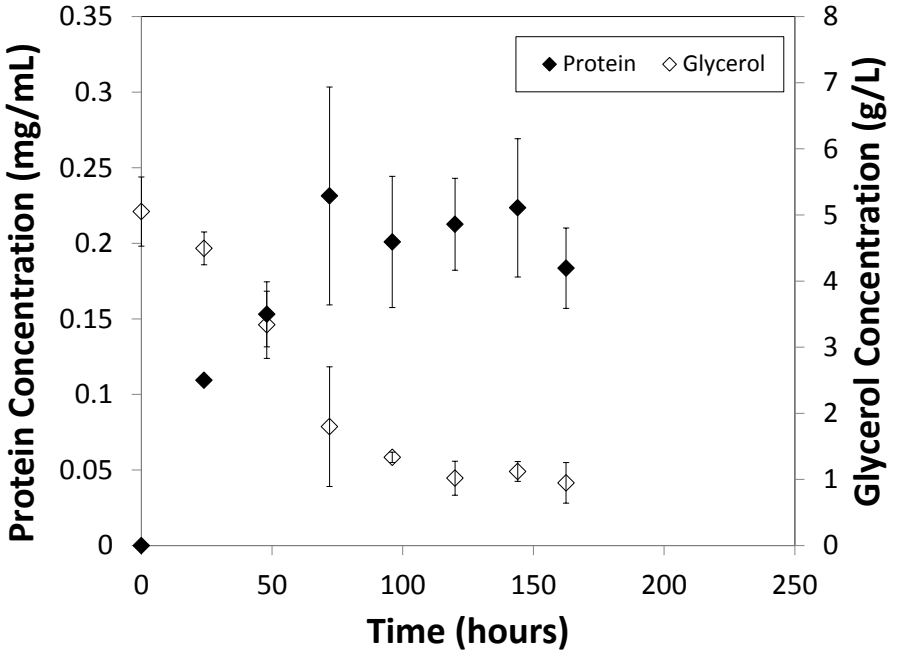

Figure 5.2. Anaerobic growth and glycerol consumption of *E. coli* W3110. *E. coli* was grown anaerobically in M9 medium supplemented with 10 g/L tryptone and glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

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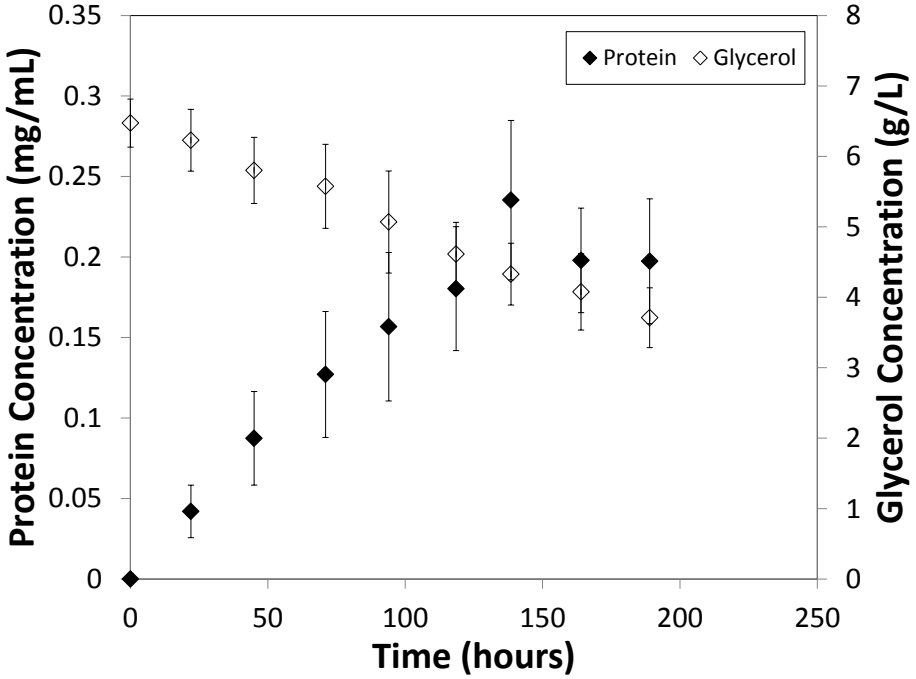

Figure 5.3. Anaerobic growth and glycerol consumption of *E. coli* BL21. *E. coli* was grown anaerobically in M9 medium supplemented with 10 g/L tryptone and glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

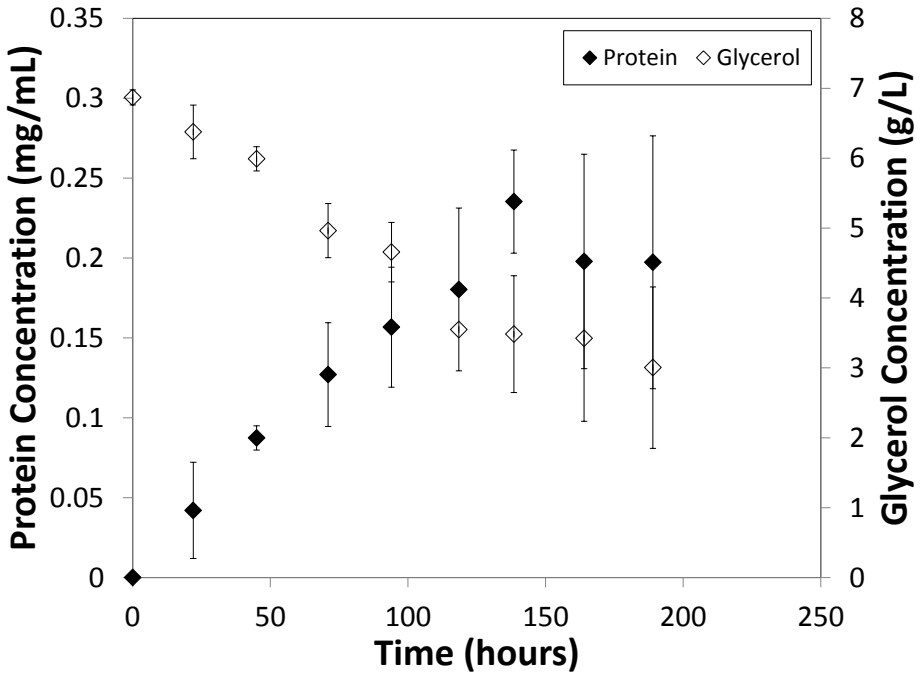

Figure 5.4. Anaerobic growth and glycerol consumption of *E. coli* DH5α. *E. coli* was grown anaerobically in M9 medium supplemented with 10 g/L tryptone and glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

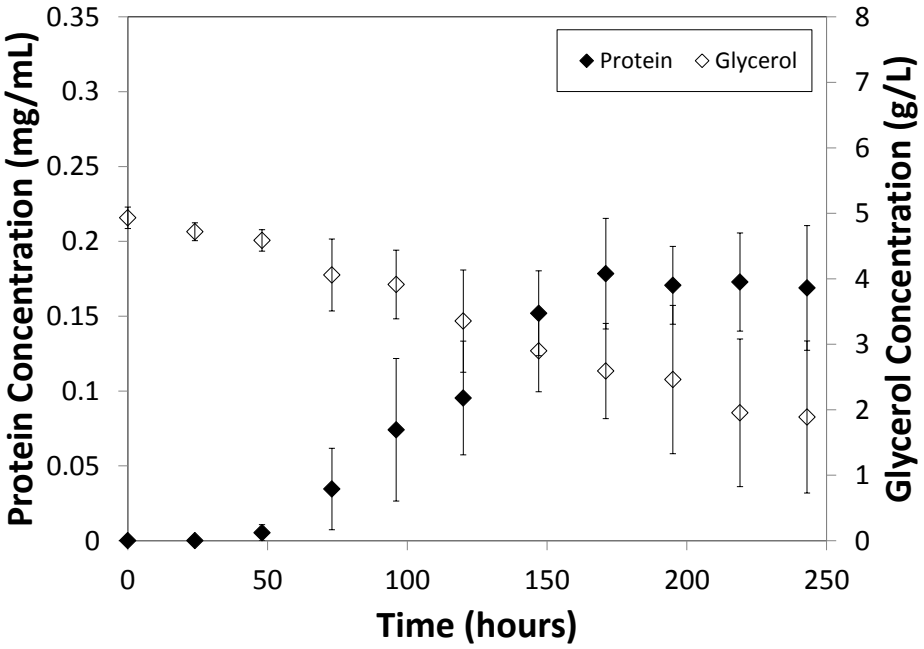

Figure 5.5. Anaerobic growth and glycerol consumption of *P. freudenreichii* ssp. *freudenreichii*. *P. freudenreichii* was grown anaerobically in M9 medium supplemented with 10 g/L tryptone and glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

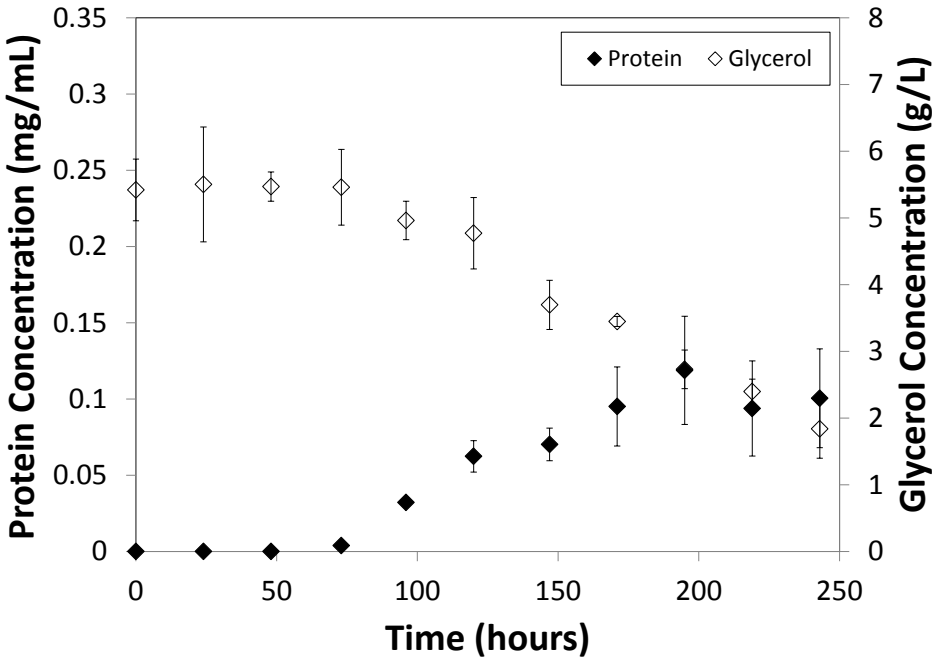

Figure 5.6. Anaerobic growth and glycerol consumption of *P. freudenreichii* ssp. *shermanii*. *P. freudenreichii* was grown anaerobically in M9 medium supplemented with 10 g/L tryptone and glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

Both *E. coli* and *P. freudenreichii* have been shown in literature to anaerobically metabolize glycerol however not using such a minimal growth medium as used in this study (Dharmadi et al., 2006; Himmi et al., 2000). Anaerobic growth with glycerol as a carbon source was demonstrated using *P. freudenreichii* in rich medium containing tryptic soy broth, yeast extract, and potassium salts (Himmi et al., 2000). Similar experiments were performed with *E. coli* using MOPS medium supplemented with yeast extract, tryptone, and selenite (Dharmadi et al., 2006). A direct comparison of anaerobic growth and glycerol conversion has never been performed between the strains.

Batch fermentation experiments were initially performed using M9 minimal medium without any rich nutrient supplementation. Anaerobic growth and glycerol conversion were not significant in any of the pure strains. After the addition of tryptone in the medium, anaerobic growth and glycerol conversion became apparent. Tryptone provides a source of amino acids and peptides, some of which must be necessary for anaerobic glycerol

conversion in these strains. *E. coli* was able to grow aerobically in M9 minimal medium with glycerol as the sole carbon source without the addition of tryptone (results not shown).

A summary of glycerol conversion values, protein yields, and growth rates is presented in Table 5.1. All pure culture fermentation experiments were performed in triplicate and standard deviations are shown. Glycerol conversion values were determined by calculating the glycerol concentration difference throughout the entire fermentation as indicated by HPLC analysis. Fermentation experiments were terminated during the stationary phase of growth. The highest glycerol conversion was achieved using the *E. coli* W3110 and *E. coli* DH5α strains with values of 4.1 ± 0.3 g/L and 3.9 ± 0.4 g/L, respectively. *E. coli* W3110 exhibited a faster conversion compared to *E. coli* DH5α since the glycerol concentration stabilized around 96 hours compared to 118.5 hours with the DH5α strain.

Table 5.1. Summary of batch fermentation parameters of pure strains[1]

Bacterial strain	Fermentation time (h)	Glycerol conversion (g/L)	Protein Yield (g protein/g glycerol)	Growth rate (h^{-1})
E. coli TG1	189	2.7 ± 0.1	0.046 ± 0.006	0.014 ± 0.004
E. coli W3110	162.5	4.1 ± 0.3	0.053 ± 0.008	0.016 ± 0.006
E. coli BL21	189	2.8 ± 0.1	0.042 ± 0.016	0.011 ± 0.007
E. coli DH5α	189	3.9 ± 0.4	0.051 ± 0.026	0.015 ± 0.006
P. freudenreichii ssp. *freudenreichii*	243	3.0 ± 0.6	0.069 ± 0.025	0.034 ± 0.010
P. freudenreichii ssp. *shermanii*	243	3.6 ± 0.3	0.031 ± 0.012	0.039 ± 0.008

[1] Fermentations were performed in triplicate with averages and standard deviations shown

Following *E. coli* W3110 and *E. coli* DH5α, the highest anaerobic glycerol conversion was achieved by the two *P. freudenreichii* strains at 3.6 ± 0.3 g/L and 3.0 ± 0.6 g/L for the *shermanii* and *freudenreichii* subspecies, respectively. When taking into account standard deviations, the difference in conversion levels of the two subspecies is not significant. The lowest glycerol conversion was demonstrated by the *E. coli* TG1 and *E. coli* BL21 strains with values of 2.7 ± 0.1 g/L and 2.8 ± 0.1 g/L, respectively. The differences in

conversion levels between the pure strains can be attributed to a variety of factors, such as different metabolic pathways, glycerol tolerance levels, product inhibition, or other limiting factors in the medium. The differences in conversion between the *E. coli* strains cannot be attributed to the genetic differences between the B and K12 families as TG1, W3110, and DH5α are all from the K12 family and BL21 is a member of the *E. coli* B family. Anaerobic glycerol conversion appears to be both strain and species specific.

All of the pure strains exhibited incomplete glycerol conversion. In most cases, glycerol consumption plateaued once growth reached the stationary phase. However, in Figs. 5.3 and 5.6, glycerol consumption continued once growth became stationary. These figures correspond to the *E. coli* BL21 and *P. freudenreichii* ssp. *shermanii* strains, respectively. These two strains are exhibiting non-substrate associated growth and are likely using glycerol for cell maintenance. *P. freudenreichii* ssp. *shermanii* has previously demonstrated similar behaviour in glycerol fermentation experiments (Himmi et al., 2000).

Incomplete glycerol conversion could be due to other limiting factors in the medium or glycerol intolerance in the strains. Anaerobic glycerol fermentation experiments in literature using *E. coli* and *P. freudenreichii* have exhibited higher conversion values than those achieved in this study (Dharmadi et al., 2006; Himmi et al., 2000). An experiment was performed using *E. coli* to ferment 10 g/L glycerol anaerobically and only 1.2 g/L remained unconverted in the medium. The medium contained two rich nutrient supplements, 10 g/L tryptone and 5 g/L yeast extract, and 1 µM selenite, compared to only 10 g/L tryptone in this study. Selenite is hypothesized to activate an enzyme contributing to anaerobic glycerol fermentation in *E. coli* (Dharmadi et al., 2006). Furthermore, the minimal medium used was MOPS as opposed to M9. MOPS medium contains additional micronutrients and buffer compared to M9. The differences between the two media are outlined in detail by Neidhardt et al. (1974). *P. freudenreichii* has been shown to anaerobically ferment 20 g/L glycerol with both yeast extract (10 g/L) and tryptic soy broth (5 g/L) in the medium. Tryptic soy broth contains both glucose and digest of casein, a source of amino acids. These studies suggest that the use of non-minimal medium will enhance the anaerobic glycerol conversion in *E. coli* and *P. freudenreichii.*

Protein yields were calculated one day after stationary phase was reached and values were used to estimate the amount of glycerol consumed that is being used for cell growth. The protein yield data is presented in Table 5.1. The four *E. coli* strains demonstrated similar protein yields when taking into account variation in the average values. Large variations in growth between triplicate data were observed in the *E. coli* BL21, *E. coli* DH5α and *P. freudenreichii* ssp. *freudenreichii* strains. After assessing the three individual cultures for each strain, it was observed that two cultures followed similar growth trends while one either lagged behind or grew slightly faster. This is common for biological growth experiments since cultures are sensitive to a variety of factors that cause large standard deviations in the growth data. The similar protein yields with different conversion levels in the *E. coli* strains indicate that some form of inhibition towards glycerol conversion is occurring in the *E. coli* TG1 and *E. coli* BL21 strains.

There was a significant difference in protein yields between the two subspecies of *P. freudenreichii*. *P. freudenreichii* ssp. *freudenreichii* had a protein yield of 0.069 ± 0.025 g protein/g glycerol, and *P. freudenreichii* ssp. *shermanii* had a protein yield of 0.031 ± 0.012 g protein/ g glycerol. The higher protein yield in the *freudenreichii* subspecies without an increase in glycerol conversion makes the *shermanii* species a more attractive strain for anaerobic glycerol conversion processes. Less biomass is appealing since more glycerol is available for the production of value-added products.

The *P. freudenreichii* strains demonstrated longer lag phases compared to the *E. coli* strains resulting in longer fermentation times. This was to be expected since *E. coli* is a highly adaptive strain that can grow and thrive under a variety of conditions. Since *E. coli* is facultatively anaerobic, it can consume any trace amounts of oxygen initially present in the medium to initiate growth. Although the lag phases were longer in the *P. freudenreichii* strains, their growth rates ended up being significantly higher than the *E. coli* strains (Table 5.1), so the slightly longer fermentation times shouldn't be viewed as an undesirable trait when comparing the strains.

Anaerobic fermentation data was used to determine the most promising strain of *E. coli* and the most promising strain of *P. freudenreichii* in terms of glycerol conversion. By

solely looking at the glycerol conversion average values, the highest *E. coli* conversion was achieved by the W3110 strain, and the highest *P. freudenreichii* conversion was achieved by the *shermanii* subspecies. When taking into account standard deviations, the glycerol conversion of *E. coli* W3110 is similar to that of *E. coli* DH5α; however, the faster conversion time of W3110 corroborates the conversion data making W3110 the most promising *E. coli* strain. The glycerol conversion values of the *P. freudenreichii* subspecies are also similar when taking into account the associated error. However, the higher protein yield of the *freudenreichii* subspecies without an increase in conversion makes it a less attractive candidate.

5.2.2 Glycerol conversion in mixed cultures

Mixed cultures from three compost sources were enriched for bacterial communities with a high anaerobic oxidative capacity towards glycerol. The three compost sources were fresh (AR1), one month old (AR2), and three months old (AR3). Cultures were grown and enriched in M9 minimal medium with no rich nutrient additives at room temperature in order adapt to anaerobic glycerol consumption under minimal conditions.

In a four week time frame, the highest conversion was achieved by AR2 (7.7 g/L), followed by AR3 (6.9 g/L), and finally AR1 (3.9 g/L). With the addition of 10 g/L of tryptone, conversion was confirmed to reach 20 g/L using AR2. This is a much greater conversion compared to the pure cultures as the highest value was only 4.1± 0.3 g/L by *E. coli* W3110. A higher conversion level in mixed cultures was expected since they were specifically enriched to have a high anaerobic oxidative capacity towards glycerol, and strains with a low tolerance towards glycerol were likely diluted out during the enrichment process.

The AR1 culture likely had a lower conversion level since it was fresh from the mechanical composter. The mechanical composter was aerated and therefore would enrich for aerobic bacteria. Furthermore, the composter was not working optimally and resulted in large amounts of undigested material in the samples. Anaerobic bacterial communities were given time to grow and adapt to the conditions in the other two compost sources (AR2 and

AR3). Glycerol is largely present in food products and therefore was likely present in the compost itself.

Mixed culture fermentations are better suited to industrial scale applications compared to pure cultures. Contamination of pure cultures on a large scale is difficult to avoid, and mixed cultures are generally easier to maintain. For these reasons, in addition to the higher conversion rates, the mixed cultures derived from compost are promising candidates for use in glycerol fermentation. The most promising mixed culture based on glycerol conversion is AR2.

5.3 Medium optimization

Based on the literature review, it was determined that higher glycerol conversion values are obtainable in the pure strains under different growth conditions, particularly using a medium with a richer source of nutrients (Dharmadi et al., 2006; Himmi et al., 2000). For this reason, the effects of rich nutrient additives to the growth medium on anaerobic glycerol conversion in the pure strains were investigated. The standard growth medium contained 10 g/L tryptone as a rich nutrient additive. Three different variations of the standard medium were used for all the pure strains: 2 g/L tryptone, 5 g/L tryptone, and 5 g/L yeast extract. If anaerobic growth and glycerol conversion can occur with lower concentrations of tryptone, operating costs will be reduced in industrial applications.

5.3.1 Medium optimization with *P. freudenreichii*

The effects of using different growth media on anaerobic glycerol conversion with *P. freudenreichii* ssp. *freudenreichii* and *P. freudenreichii* ssp. *shermanii* are shown in Figures 5.7 and 5.8, respectively. Batch fermentation experiments were performed with 10 g/L of glycerol as the sole carbon source in the medium in order to see if the anaerobic oxidative capacity of the strains towards glycerol could be improved. The glycerol conversion values cannot be directly compared to those obtained with 10 g/L tryptone since for the medium optimization experiments, a higher starting concentration of glycerol was used in the medium (5 g/L glycerol was used previously).

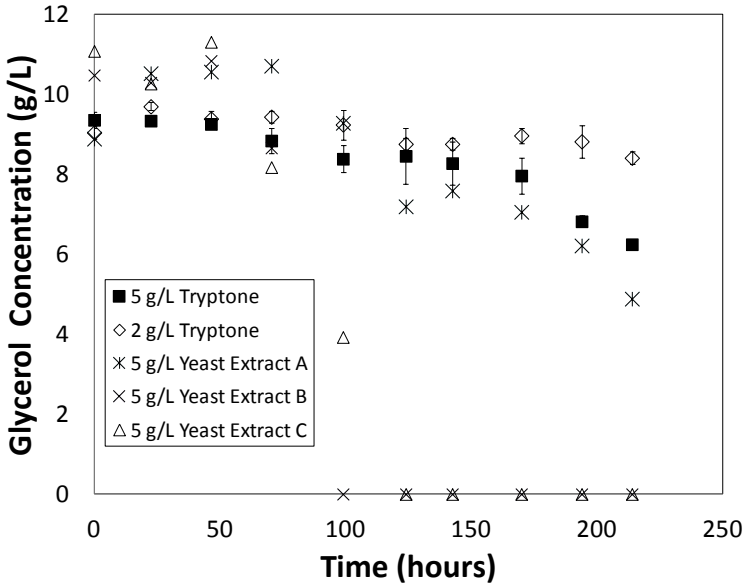

Figure 5.7. Anaerobic glycerol consumption of *P. freudenreichii* ssp. *freudenreichii* using different concentrations of rich nutrient medium additives. *P. freudenreichii* was grown anaerobically in M9 medium with glycerol as the sole carbon source. For the tryptone experiments, average values are presented (n = 3, ± standard deviation).

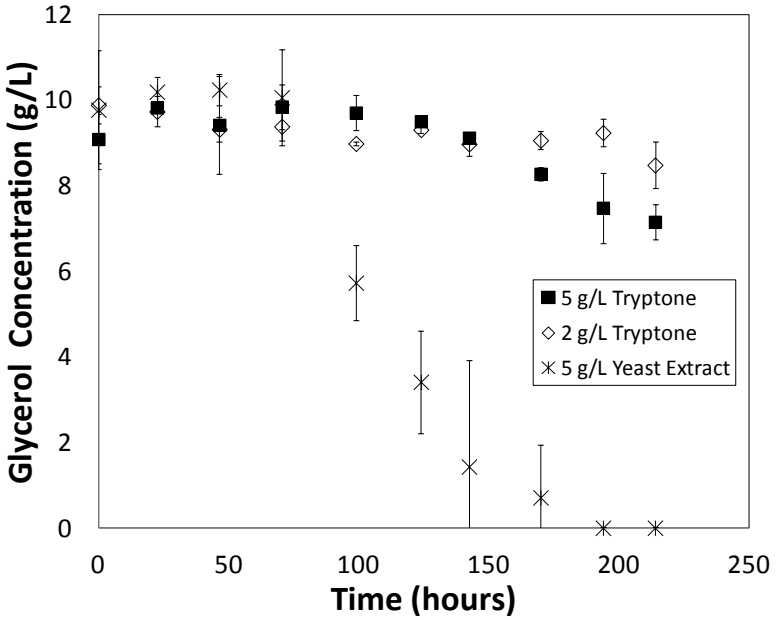

Figure 5.8. Anaerobic glycerol consumption of *P. freudenreichii* ssp. *shermanii* using different concentrations of rich nutrient medium additives. *P. freudenreichii* was grown anaerobically with glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

Comparing the fermentation experiments using 5 g/L tryptone and 2 g/L tryptone, a slightly higher anaerobic glycerol conversion was observed with the higher tryptone concentration for both strains. In the *freudenreichii* subspecies, the glycerol conversion decreased from 3.1 ± 0.1 g/L to 0.6 ± 0.1 g/L when decreasing the tryptone concentration from 5 g/L to 2 g/L. In the case of the *shermanii* subspecies, there was a less significant difference as the anaerobic glycerol conversion only decreased from 1.9 ± 0.1 g/L to 1.4 ± 0.1 g/ with the decrease in tryptone. In both cases however, there was an improvement in anaerobic glycerol conversion with the higher concentration of tryptone. This is to be expected as tryptone is a rich source of amino acids and peptides which can improve growth. It has been shown that *Propionibacterium* can grow in basal medium, however, growth is greatly improved as amino acid sources are added (Stackebrandt et al., 2006).

Although there were differences in the starting glycerol concentration between the medium optimization experiments and those performed previously with 10 g/L tryptone, the conversion values can still be compared indirectly. In the case of the *freudenreichii* subspecies, there wasn't a significant difference between the conversion values obtained at 10 g/L tryptone (3.0 ± 0.6 g/L glycerol) and 5 g/L tryptone (3.1 ± 0.1 g/L). This suggests that another factor could become limiting at this point or that the strain has an intolerance to the higher concentrations of glycerol in the medium. For the *shermanii* species the conversion increased from 1.9 ± 0.1 g/L to 3.6 ± 0.3 g/L with the higher tryptone concentration indicating that the increase in amino acids and peptides is beneficial to anaerobic glycerol conversion.

The replacement of tryptone with 5 g/L yeast extract improved anaerobic glycerol conversion in both strains. In addition to amino acids and peptides, yeast extract also contains vitamins and carbohydrates and is therefore a richer source of nutrients than tryptone. The yeast extract fermentations for the *freudenreichii* subspecies are presented individually instead of as an average of the three trials. Two of the trials followed similar glycerol conversion trends, however, one culture lagged behind the others resulting in large standard deviations. In all three cases, the glycerol conversion was higher with yeast extract than tryptone. Two of the cultures achieved complete conversion of the glycerol in the

medium (~10 g/L) within 125 hours of growth. The other culture reached a conversion value of 5.6 g/L within 214 hours. The three *shermanii* cultures were all able to achieve complete glycerol conversion using yeast extract in the medium within 200 hours of growth.

It is evident that yeast extract improves anaerobic glycerol conversion in both *P. freudenreichii* subspecies. This is likely due to the additional nutrients provided in yeast extract compared to tryptone and suggests that the limitations in glycerol conversion using the tryptone enriched media were not due to glycerol intolerance but the requirement of other nutrients in the medium. This theory is supported by a previous study where complete consumption of 20 g/L glycerol was achieved using a rich medium containing tryptic soy broth and yeast extract (Himmi et al., 2000).

The maximum protein concentrations increased proportionally to glycerol conversion between the initial fermentations shown in section 5.2 (10 g/L tryptone) and those performed with 5 g/L yeast extract in the medium since the protein yields remained similar (Table 5.2). This corroborates the previous result that the *freudenreichii* subspecies has a higher protein yield compared to the *shermanii* subspecies. Growth rate values remained similar in both media for the two species.

Table 5.2. Comparison of batch fermentation parameters using 10 g/L tryptone or 5 g/L yeast extract as rich nutrient additives

	Protein Yield (g protein/g glycerol)		**Growth rate (h^{-1})**	
	10 g/L Tryp.	5 g/L Y.E	10 g/L Tryp.	5 g/L Y.E
P. freudenreichii ssp. *freudenreichii*	0.069 ± 0.025	0.064[1]	0.034 ± 0.010	0.034[1]
P. freudenreichii ssp. *shermanii*	0.031 ± 0.012	0.041 ± 0.006	0.039 ± 0.008	0.034 ± 0.009

[1]average of trials B & C as seen on Fig. 5.7

Both tryptone and yeast extract provide a source of peptides and amino acids, but yeast extract provides an additional source of vitamins. To see the effect of vitamins on anaerobic glycerol conversion in *P. freudenreichii*, batch fermentations were then performed

using M9 medium supplemented with Pfennig's vitamin solution. No anaerobic glycerol conversion was observed in either strain. This indicates the need for an additional source of peptides or amino acids to the minimal medium. The presence of both vitamins and amino acids appears to enhance anaerobic glycerol conversion in *P. freudenreichii*.

5.3.2 Medium optimization with *E. coli*

Anaerobic glycerol conversion in the *E. coli* strains TG1, W3110, BL21, and DH5α was investigated using M9 minimal medium supplemented with either 5 g/L tryptone, 2 g/L tryptone, or 5 g/L yeast extract. Glycerol concentration was monitored throughout the fermentations and is presented in Figures 5.9 to 5.12 for each strain. In all cases, the lowest conversion was observed using 2 g/L tryptone as a rich nutrient additive. Glycerol conversion improved using 5 g/L tryptone or 5 g/L yeast extract compared to 2 g/L tryptone. There were no significant differences in glycerol conversion between using 5 g/L tryptone or 5 g/L yeast extract. Furthermore, these conversion values are not significantly different from those obtained in the previous experiments using 10 g/L of tryptone. Anaerobic glycerol conversion in *E. coli* appears to be more dependent on having a source of amino acids and peptides as opposed to the other components of yeast extract, such as vitamins.

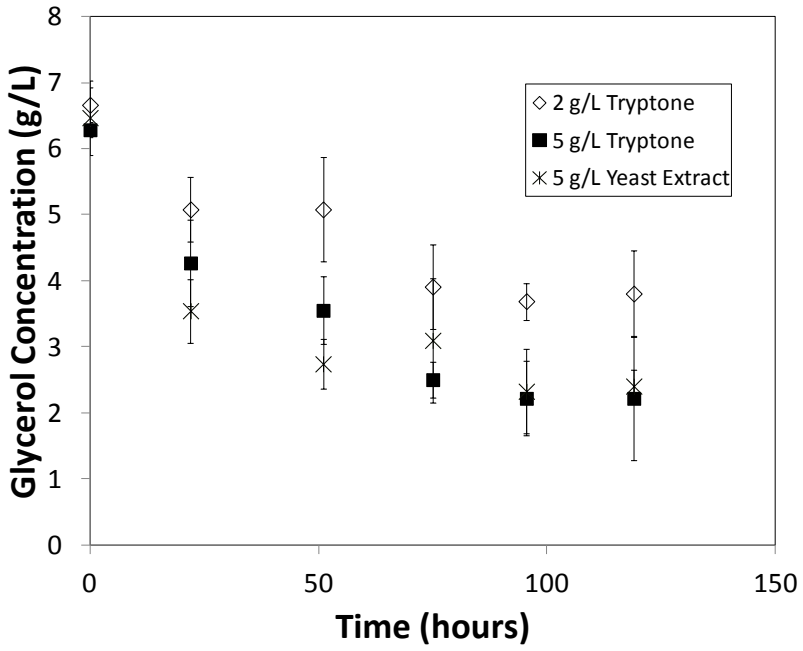

Figure 5.9. Anaerobic glycerol consumption of *E. coli* TG1 using different concentrations of rich nutrient medium additives. *E. coli* was grown anaerobically with glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

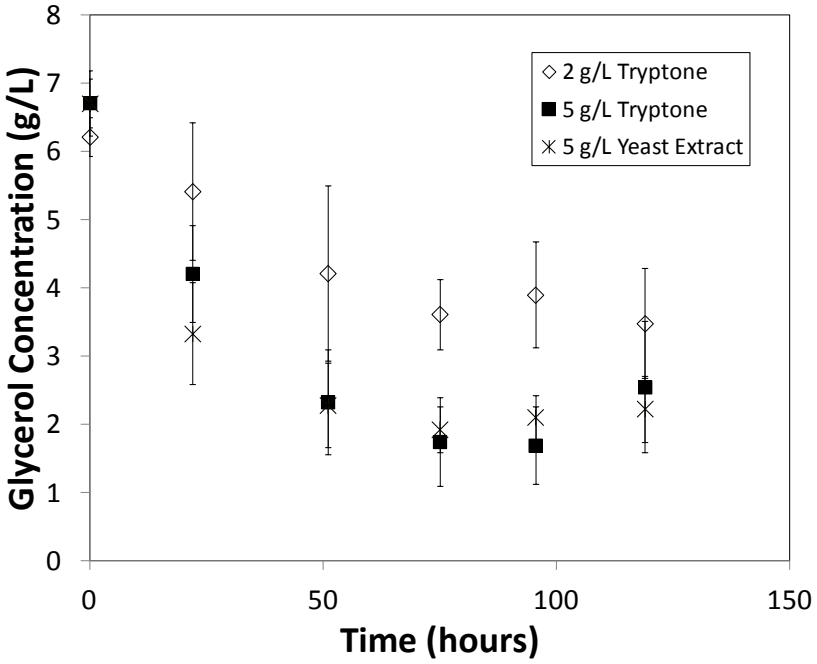

Figure 5.10. Anaerobic glycerol consumption of *E. coli* W3110 using different concentrations of rich nutrient medium additives. *E. coli* was grown anaerobically with glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

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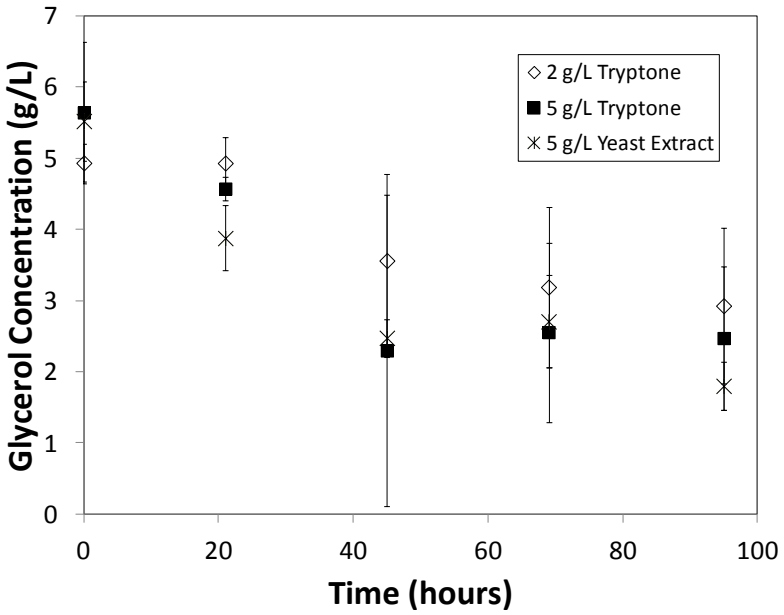

Figure 5.11. Anaerobic glycerol consumption of *E. coli* BL21 using different concentrations of rich nutrient medium additives. *E. coli* was grown anaerobically with glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

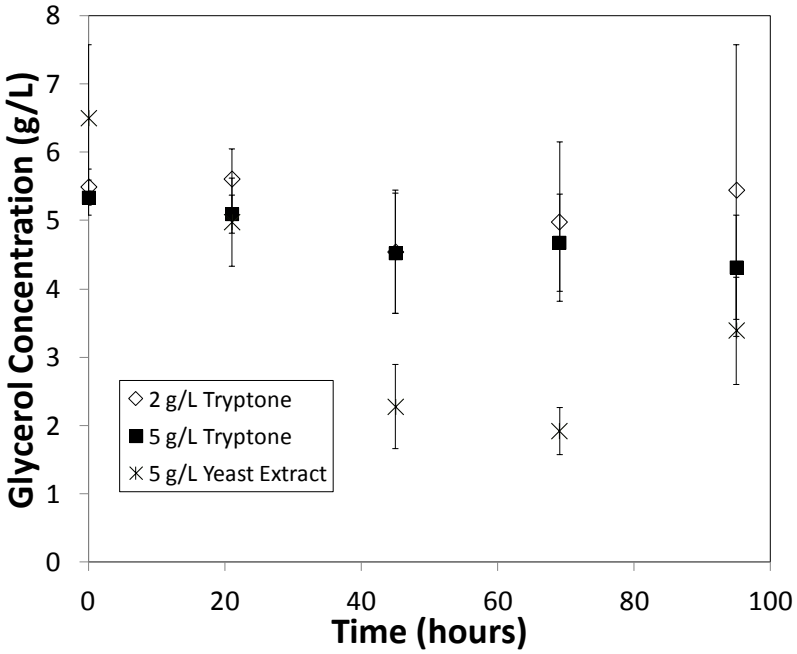

Figure 5.12. Anaerobic glycerol consumption of *E. coli* DH5α using different concentrations of rich nutrient medium additives. *E. coli* was grown anaerobically with glycerol as the sole carbon source. Average values are presented (n = 3, ± standard deviation).

5.4 Conversion products

A qualitative analysis of the production of 1,3-propanediol (1,3-PDO) and carbon dioxide (CO_2) as metabolites of anaerobic glycerol conversion was performed in all the pure strains and mixed cultures. The production of 1,3-PDO was detected through HPLC, and the production of CO_2 was detected through GC. A summary of metabolite production is presented in Table 5.3. It was observed that three different combinations of the metabolites were produced for the three different bacterial groups: *E. coli*, *P. freudenreichii*, and mixed cultures. This indicates that between the three different groups, the metabolic pathways for anaerobic glycerol consumption vary. This could account for the differences in anaerobic glycerol conversion between the bacterial groups.

Table 5.3. Qualitative analysis of the production of 1,3-PDO and CO_2 as metabolites of anaerobic glycerol conversion

Strain or mixed culture	1,3-PDO production	CO_2 production
E. coli TG1	-	✓
E. coli W3110	-	✓
E. coli BL21	-	✓
E. coli DH5α	-	✓
P. freudenreichii ssp. *freudenreichii*	-	-
P. freudenreichii ssp. *shermanii*	-	-
AR1	✓	✓
AR2	✓	✓
AR3	✓	✓

This metabolite analysis is by no means exhaustive but is indicative of the conversion pathways occurring in the strains/mixed cultures and also the feasibility of performing anaerobic glycerol fermentation for the production of value-added products. The production of 1,3-PDO as a fermentation by-product indicates simultaneous oxidative and reductive pathways are occurring from glycerol dissimilation (Fig. 2.5) (Yazdani and Gonzalez, 2007). The chemical 1,3-PDO is produced from the reductive pathway and is of interest since it has a higher market value compared to glycerol. Currently, 1,3-PDO is priced at 0.8 US $/lb compared to 0.45 $/lb for pure glycerol (ICIS, 2011). Some applications for 1,3-PDO include the production of plastics, textile fibres, resins, adhesives, and sealants (Willke and Vorlop,

2008). Additional value can be obtained from the oxidative products of the fermentation (Fig. 2.6). Further metabolite profiling must be performed to determine what products other than CO_2 are being generated. Potential by-products include succinic acid, butanol, ethanol, formic acid, hydrogen, and propionic acid. It is likely that a larger range of products are being produced from the mixed cultures compared to the pure cultures due to the variety of microorganisms present.

It was confirmed that 1,3-PDO is being produced as an end-product of anaerobic glycerol fermentation in the mixed cultures. Fermentations were performed with 1,3-PDO as the sole carbon source in M9 minimal medium. No growth or 1,3-PDO consumption occurred.

In the *E. coli* species, carbon dioxide was produced as a fermentation product. *E. coli* has previously been shown to ferment glycerol in a 1,3-PDO independent manner (Yazdani and Gonzalez, 2007). Anaerobic glycerol fermentation using *E. coli* under similar conditions has been shown to produce succinic acid as a predominant product (Dharmadi et al., 2006) which is primarily used as a sweetener in the food industry. A complete metabolite profile should be created in order to assess feasibility under these fermentation conditions.

P. freudenreichii produced neither fermentation product from glycerol. A previous study has confirmed the production of propionic acid as the major product of anaerobic glycerol fermentation (Himmi et al., 2000). The medium used in this study also contained glucose from tryptic soy broth, so propionic acid could also be the product of glucose. Propionic acid is currently of similar value to glycerol, so its production from the fermentation of glycerol may not be feasible. A complete analysis of metabolites should still be performed under these conditions to confirm the presence of propionic acid and any other potentially valuable products.

5.5 Overall discussion and conclusions

The purpose of the anaerobic fermentation experiments was two-fold: to assess the pure strains and mixed cultures for use in the anaerobic fermentation of glycerol to value-added products and to predict their performance as anodic biocatalysts in glycerol-oxidizing

MFCs. In both cases, high glycerol conversion capabilities and complete glycerol consumption are attractive characteristics.

Anaerobic fermentation experiments demonstrated that *E. coli* strains TG1, W3110, BL21, DH5α, and *P. freudenreichii* subspecies *freudenreichii* and *shermanii*, along with the mixed cultures derived from compost, can all grow and consume glycerol anaerobically. Anaerobic glycerol conversion varied depending on the strain or mixed culture used. Using minimal medium with 10 g/L tryptone, the highest glycerol conversion of the pure strains was achieved by *E. coli* W3110 and DH5α which reached conversion levels of 4.1 ± 0.3 g/L and 3.9 ± 0.4 g/L, respectively. The mixed culture AR2, however, was able to anaerobically consume 20 g/L glycerol. Mixed cultures were enriched in minimal medium for a high oxidative capacity towards glycerol, so it is not surprising that they were able to achieve a higher glycerol conversion compared to the pure strains under minimal conditions. Complete glycerol conversion was only observed using the mixed cultures with 10 g/L tryptone as a rich nutrient additive.

Glycerol conversion was improved in both species of *P. freudenreichii* when using yeast extract as a rich nutrient medium additive instead of tryptone. Complete conversion of 10 g/L glycerol was observed. Yeast extract provides additional nutrients compared to tryptone, such as vitamins, and their absence was limiting anaerobic growth and glycerol conversion when using tryptone. Conversion in *E. coli* species was not greatly affected by changing the rich nutrient additive in the medium. *E. coli* is an extremely versatile bacterium and can therefore grow and thrive under fairly minimal conditions. In literature, anaerobic glycerol conversion in both *E. coli* and *P. freudenreichii* have reached higher values than demonstrated in this study (Dharmadi et al., 2006; Himmi et al., 2000); however, those experiments used richer media. The advantage of using minimal medium in both fermentation and MFC applications is lower operating costs. A feasibility study would have to be performed to determine if additional medium components are worth any increases in glycerol conversion. Furthermore, the use of continuous or fed-batch fermentations could produce higher conversion values.

The production of 1,3-PDO and CO_2 were also studied as anaerobic glycerol conversion products. Three different combinations of products were observed between the three bacterial groups indicating that there are different glycerol dissimilation pathways occurring. This can contribute to the differences in conversion observed between the strains and mixed cultures. The production of 1,3-PDO is desirable since it is a value-added product compared to glycerol. Only the mixed cultures produced 1,3-PDO as a metabolite, but since the analysis of by-products was not exhaustive, it has not been determined whether the pure strains are also producing value-added products. Further metabolite profiling should be performed.

The production of CO_2 is attractive in terms of MFC applications. Complete oxidation of glycerol results in the production of CO_2, so the presence of CO_2 indicates that the maximum number of moles of electrons is being produced per mole of glycerol being consumed by the bacteria. Although the production of CO_2 is promising for MFC applications, it does not mean the voltage will be proportional to the amount of CO_2. Even though electrons are being produced, they may not efficiently be transported through the circuit, so MFC studies must be performed to get a true indication of the most promising anodic biocatalyst. The production of value-added metabolites is also desirable in MFC applications as fermentation products can be recovered concurrently with electricity generation.

In terms of glycerol fermentation applications, the most promising candidate is the mixed cultures as AR2 was able to achieve the highest glycerol conversion. Mixed cultures also offer advantages on an industrial scale such as easier maintenance and handling. In terms of MFC applications, one strain or mixed culture was selected from each group for testing as anodic biocatalysts in a glycerol-oxidizing MFC. It was important to select one catalyst from each group because MFC performance is difficult to predict based on solely fermentation experiments. High glycerol conversion doesn't necessarily correlate to high MFC voltage as the electron transfer mechanisms in the strains have not been elucidated. For the *E. coli* strains the highest anaerobic glycerol conversion was achieved by the W3110 and DH5α strains; however, the conversion was slightly faster with the W3110 strain, so it

was selected for MFC studies. From the *P. freudenreichii* strains, the *shermanii* subspecies more consistently was able to completely convert 10 g/L of glycerol (all three trials compared to two with the *freudenreichii* subspecies). Consistently high conversion, along with lower protein yields, resulted in the *shermanii* subspecies being selected for MFC studies. Out of the mixed cultures, the fastest and highest conversion was achieved by AR2, so it was selected as a potential MFC anodic biocatalyst. The use of the aforementioned strains as anodic catalysts in a glycerol-oxidizing MFC will be discussed in Chapter 6.

Chapter 6. Screening potential biocatalysts for a glycerol-oxidizing microbial fuel cell

6.1 Introduction

The following chapter explores the use of *E. coli*, *P. freudenreichii*, and mixed cultures derived from compost as anodic biocatalysts in a glycerol-oxidizing MFC. Batch fermentation experiments described in Chapter 5 were used to select one catalyst from each group of bacteria (*E. coli*, *P. freudenreichii*, and mixed cultures) for screening in an MFC system. The chosen biocatalysts were *E. coli* W3110, *P. freudenreichii* ssp. *shermanii*, and AR2 (mixed culture).

An H-type MFC was designed in order to test the bacterial strains as anodic catalysts using pure glycerol as the fuel. Proof-of-concept experiments were performed for each biocatalyst using an anaerobic indicator, resazurin, in the anodic medium. Resazurin also acts as an electron mediator. Once proof-of-concept was achieved for both the MFC system design and the catalysts, resazurin was removed from the medium, and the experiments were repeated using a mediatorless system. Power density curves were used to determine the maximum power density obtainable by each catalyst in the system, and these values were used as the major parameter of comparison between the strains.

6.2 H-type fuel cell design

In order to compare performance of the different biocatalysts, an MFC system was designed. Several aspects of the design had to be considered: architecture, membrane material, electrode material, media, and type of wire. A variety of MFC architectures have been described in the literature. The H-type MFC is a widely used and inexpensive design that is appropriate for basic parameter research (Logan et al., 2006). It is a two-chambered system consisting of two bottles connected by a tube. The tube typically contains a cation exchange membrane (CEM) which separates the anode and cathode chambers. This system allows for easy manipulation of several parameters and is also not difficult to sterilize for pure culture work. A disadvantage of this type of design is that it produces low power densities due to the high internal resistance inherent in the system. More sophisticated and

expensive designs have been developed with lower internal resistances, such as single-chamber cube and tubular reactors (Logan, 2008). These systems are suitable for optimizing the power output of previously studied parameters but are not necessary for proof-of-concept experiments. An H-type system was selected for this study (Fig. 3.2) since it is in the initial research stages. After promising catalysts are selected, more sophisticated systems can be used to maximize power.

Membrane selection was based on previous MFC studies in literature. A membrane material was chosen that has already been shown to work in MFC systems in order to not affect the proof-of-concept experiments for the anodic biocatalysts. The most commonly used membranes in MFC research are Nafion and CMI-7000 (Logan, 2008). A comparison of different membranes in MFC systems demonstrated that Nafion produced a lower internal resistance and a higher maximum power density compared to CMI-7000 using a bottle type system (Kim et al., 2007b). For this reason, Nafion was chosen for this design. It is also a highly characterized material since it is commonly used in conventional fuel cell systems.

Electrode material selection was also based on MFC studies found in literature. Carbon-based materials are the most prevalent since they are highly conductive, inexpensive and also conducive to bacterial growth (Logan, 2008). Carbon cloth was chosen as the material for both the cathode and the anode since it porous, flexible, and fairly inexpensive. When trying to optimize power production of an MFC, the cathode is typically loaded with a Pt catalyst, especially when using oxygen as a catholyte. The presence of platinum decreases the amount of dissolved oxygen necessary for a reduction reaction to occur on the cathode (Kim et al., 2007a). For this study, it was decided not to use a cathode catalyst since they are expensive and the goal of this investigation was solely comparative in nature.

Medium selection for both the anode and cathode chambers is an important aspect of MFC operational design. In the case of the anodic medium, optimization experiments were performed and are outlined in Chapter 5. In order to maintain a similar ionic strength between the anode and cathode, M9 medium was also used in the cathode chamber without the addition of a carbon source or any rich nutrient additives. Cathode contamination was

occurring, so the cathode medium was switched to phosphate buffer, and the ions were allowed to equilibrate across the membrane for one day before experiments were conducted.

Titanium wire was chosen to connect the MFC circuit. Copper wire should not be used as it corrodes over time and can be inhibitory towards bacterial growth (Logan, 2008). Other materials used in literature include platinum and titanium. Titanium is less expensive, so it was chosen for this design.

In general, the goal was to design a simple and inexpensive MFC that is easy to manipulate and sterilize. Standard MFC materials were chosen to avoid introducing additional variables to the experiments. Once anodic biocatalyst selection is complete, other aspects of the MFC design can be optimized in order to maximize performance.

6.3 Proof-of-concept experiments

Once the MFC design was complete, experiments were performed to both test the MFC design and to screen each catalyst. In order to confirm that the anode chamber was anaerobic, the redox indicator, resazurin, was added to the medium. The oxidized form of resazurin is pink or blue while the reduced form is colourless. Oxygen is an electron acceptor, so its presence in the anode chamber would inhibit MFC performance. Resazurin can also act as an electron mediator between the bacterial cells and the electrodes since it can easily be oxidized and reduced. Initial screening experiments were therefore performed in the presence of an electron mediator and did not screen for exoelectrogenic activity.

The H-type MFCs were inoculated with actively growing anaerobic cultures of either AR2, *E. coli* W3110, or *P. freudenreichii* ssp. *shermanii*. The anode and cathode were connected to an external load of 1000 Ω, and voltage was measured with a voltmeter across the resistor as a function of time. Once the voltage stabilized, the resistor was varied from 510 Ω to 10,000 Ω to find the maximum power density attainable with each catalyst. Samples were also taken from the anode chamber at various points throughout fuel cell operation to monitor glycerol consumption.

A voltage was produced for each of the three catalysts confirming proof-of-concept for each strain in an electron-mediated MFC system using pure glycerol as the fuel. The

amount of glycerol in the medium varied based on the medium optimization experiments described in Chapter 5. The starting glycerol concentrations in the medium were 17 g/L, 5 g/L, and 10 g/L for AR2, *E. coli* W3110, and *P. freudenreichii* ssp. *shermanii*, respectively. Complete glycerol consumption was observed with in all three biocatalysts. Once the glycerol in the medium was depleted, additional glycerol was injected into the anode chamber. No further significant glycerol consumption occurred using the *E. coli* W3110 and *P. freudenreichii* ssp. *shermanii* strains. This could be due to other limiting factors in the medium, and therefore glycerol consumption could be enhanced through the use of fed-batch or continuous operation. The mixed culture, however, was able to continue to consume glycerol after injection reaching a total consumption of 27 g/L (Fig. 6.1).

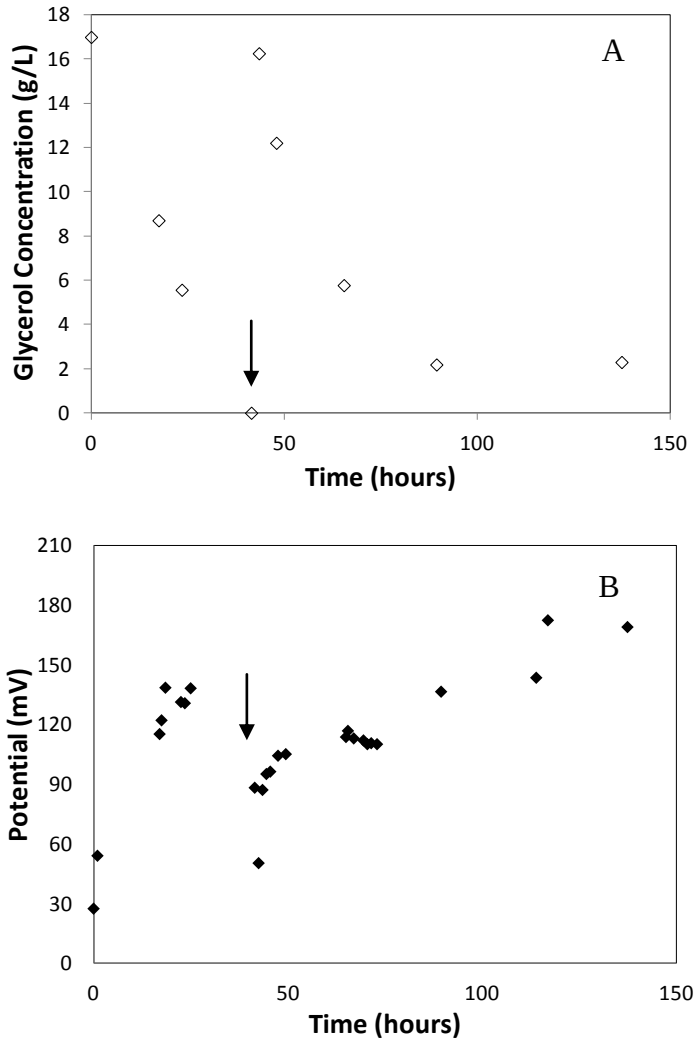

Figure 6.1. Glycerol concentration (A) and potential (B) as a function of time using an AR2 anodic catalyst in an electron-mediated system. MFC was inoculated with AR2 using M9 medium with 17 g/L glycerol and 10 g/L tryptone in the anode chamber. The external load was a 1000 Ω resistor. Arrow indicates when additional glycerol was injected in the medium.

Throughout the depletion of the initial glycerol present in the medium, the maximum voltage achieved using a 1000 Ω load was 138.5 mV after only 18.5 hours of growth. The voltage then began to deplete due to lack of fuel, but after glycerol injection, it rose again to a maximum value of 172.4 mV. The injected glycerol never fully depleted. This again is likely due to other limiting nutrients in the medium resulting in the system no longer being able to sustain growth. Since AR2 is a mixed culture, it can adapt in a fuel cell environment and enrich for bacteria that can use the electrode as a final electron acceptor. This explains

why a higher working voltage was achieved throughout the second round of glycerol consumption compared to the depletion of the initial glycerol in the medium.

The maximum voltages obtained using a 1000 Ω resistor for each catalyst are summarized in Table 6.1. The highest voltage was achieved by the mixed culture (AR2) with a value of 172.4 mV, followed closely by *P. freudenreichii* ssp. *shermanii* with a voltage of 165.5 mV. The lowest voltage (78.1 mV) was produced using *E. coli* W3110 as a catalyst.

Table 6.1. Maximum working voltage of catalysts using a 1000 Ω load

Catalyst	**Maximum Voltage (mV)**
AR2	172
E. coli W3110	78
P. freudenreichii ssp. *shermanii*	166

The order of catalyst performance based on working voltage corresponds to the amount of glycerol consumed by each catalyst in the fuel cell. The highest glycerol conversion was achieved by AR2, followed by *P. freudenreichii* ssp. *shermanii*, and finally *E. coli* W3110. Using a system with a mediator, it is expected that glycerol conversion would be somewhat proportional to voltage, but it is also dependent on glycerol conversion pathways (Fig. 2.6). For example, complete oxidation of glycerol to CO_2 will produce the highest number of moles of electrons per mole of glycerol consumed. Exoelectrogenic behaviour of the catalysts could also enhance voltage compared to non-exoelectrogenic strains. Non-exoelectrogenic bacteria are solely dependent on the chemical mediator to transfer electrons from the bacterial cells to the electrodes while exoelectrogenic bacteria can use both the chemical mediators and excreted cellular mediators or bacterial nanowires.

When the fuel cells were operating at their maximum voltage, polarization and power density data were obtained by varying the external load. This data is presented in Figs. 6.2 to 6.4 for each catalyst. The polarization and power density data is by no means exhaustive as

only a range of resistances were used as an external load; however, these experiments allow for a comparison of performance between the strains.

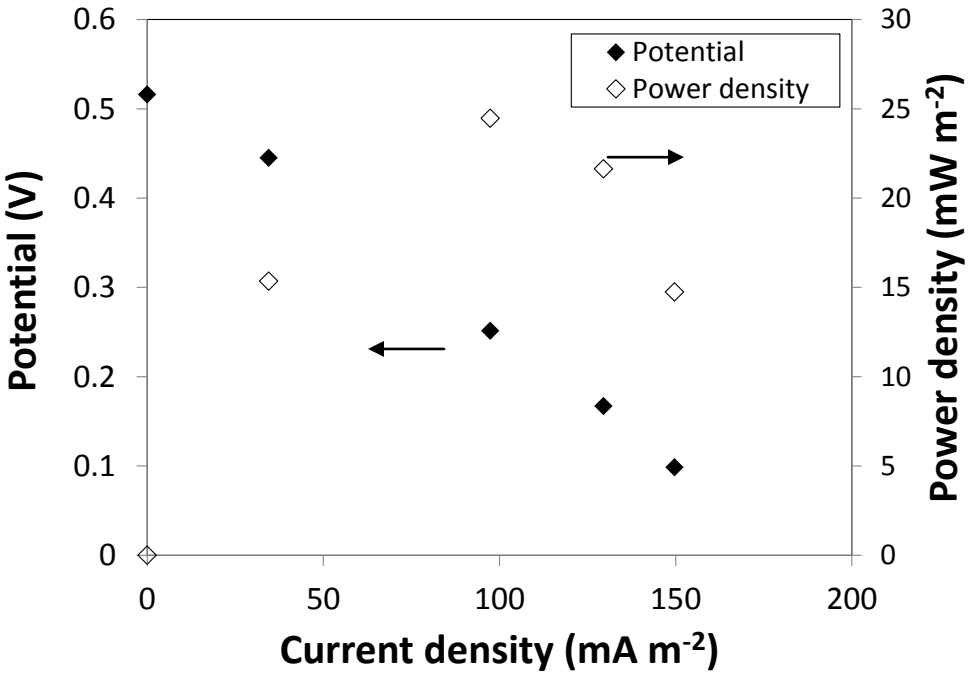

Figure 6.2. Polarization and power density data using AR2 as an anodic biocatalyst in an electron-mediated MFC system. The anodic medium used was M9 with 17 g/L glycerol and 10 g/L tryptone. The fuel cell was operated at 30°C.

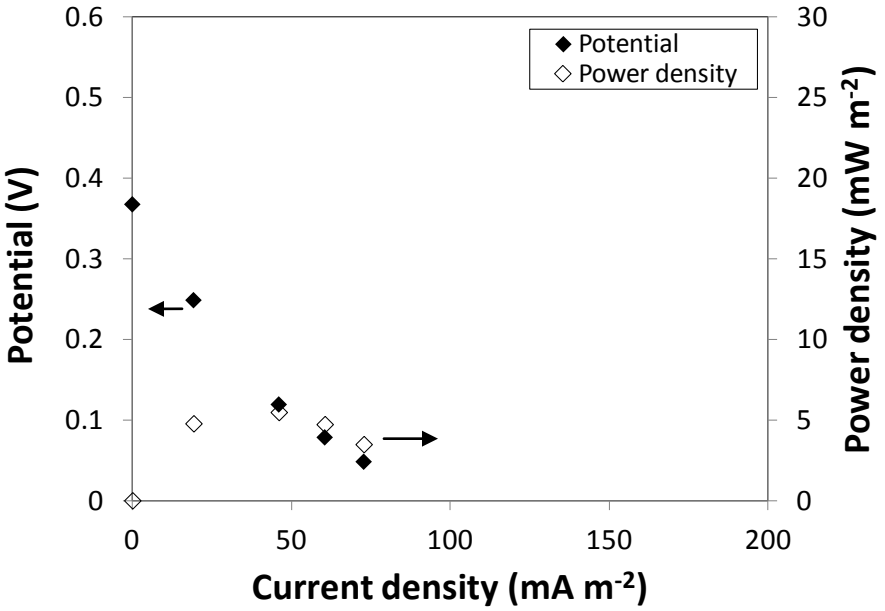

Figure 6.3. Polarization and power density data using *E. coli* W3110 as an anodic biocatalyst in an electron-mediated MFC system. The anodic medium used was M9 with 5 g/L glycerol and 10 g/L tryptone. The fuel cell was operated at 37°C.

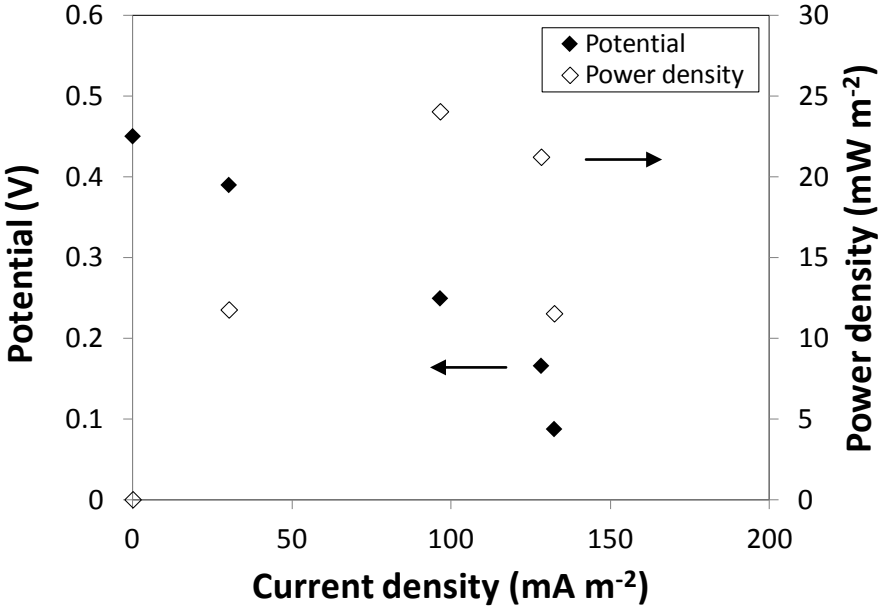

Figure 6.4. Polarization and power density data using *P. freudenreichii* ssp. *shermanii* as an anodic biocatalyst in an electron-mediated MFC system. The anodic medium used was M9 with 10 g/L glycerol and 5 g/L yeast extract. The fuel cell was operated at 30°C.

The highest power density was obtained using an external load of 2000 Ω for all of the catalysts; however, since continuous polarization data wasn`t taken, it can only confidently be concluded that the maximum power density occurs in the range of 1000 to 10,000 Ω. The maximum power density occurs when the internal resistance is equal to the external resistance (Logan et al., 2006). In MFC systems, the internal resistance is typically dominated by Ohmic losses inherent in the system, so it is expected that the maximum power density for each catalyst should occur using similar external loads since the architectural design was kept the same.

The maximum power density generated for each catalyst was 24.5 mW m^{-2} for AR2, 5.5 mW m^{-2} for *E. coli* W3110, and 24.0 mW m^{-2} for *P. freudenreichii* ssp. *shermanii*. The OCV values for the three catalysts were 516 mV, 367 mV, and 450 mV for AR2, *E. coli* W3110, and *P. freudenreichii* ssp. *shermanii*. It is difficult to analyze the shape of the polarization curve based on so few data points, but the data sets for the three catalysts are exhibiting three different shapes. For the mixed culture (Fig. 6.2), the polarization data is fairly linear indicating that the system is being dominated by Ohmic resistances. This is typical for MFC systems (Logan, 2008). The *E. coli* catalyst (Fig. 6.3) is demonstrating distinct regions of activation and then Ohmic losses, and the *P. freudenreichii* catalyst (Fig. 6.4) is exhibiting a region of concentration losses at higher current densities.

Continuous polarization curves and replicate experiments should be performed to get a better indication of system performance, but this data allowed for proof-of-concept and a means of comparison between the strains. All three bacterial cultures worked as anodic biocatalysts in a glycerol-oxidizing MFC system containing an electron mediator. The best performance was exhibited by AR2, followed by *P. freudenreichii* ssp. *shermanii*, and finally *E. coli* W3110. This corresponded to the anaerobic glycerol conversion capacities described in Chapter 5. However, it is important to note that even in MFC systems using a mediator, voltage doesn't directly correlate to glycerol conversion as there are a number of different factors that can contribute to system performance. The power densities achieved using AR2 and *P. freudenreichii* ssp. *shermanii* were similar even though the mixed culture was able to

consume significantly more glycerol suggesting that *P. freudenreichii* is capable of a higher Coulombic efficiency.

6.4 Mediatorless MFCs

Once proof-of-concept experiments were completed for each strain, MFCs were inoculated with the three catalysts using the same medium as the previous experiments but without the presence of a mediator. Voltage was again produced using all three catalysts. This demonstrates that AR2, *E. coli* W3110, and *P. freudenreichii* ssp. *shermanii* are all exoelectrogenic cultures. Exoelectrogenic activity has previously been reported in *E. coli* using glucose systems (Zhang et al., 2006; Qiao et al., 2008), but this is the first time that *P. freudenreichii* and AR2 have been used as an MFC catalyst in a mediatorless system.

The MFCs were hooked up to a potentiostat to monitor OCV as a function of time and to obtain continuous polarization data for each catalyst. Glycerol concentration was also monitored periodically throughout MFC operation. Plots of OCV and glycerol concentration in the anode chamber as a function of time are presented in Figures 6.5-6.7.

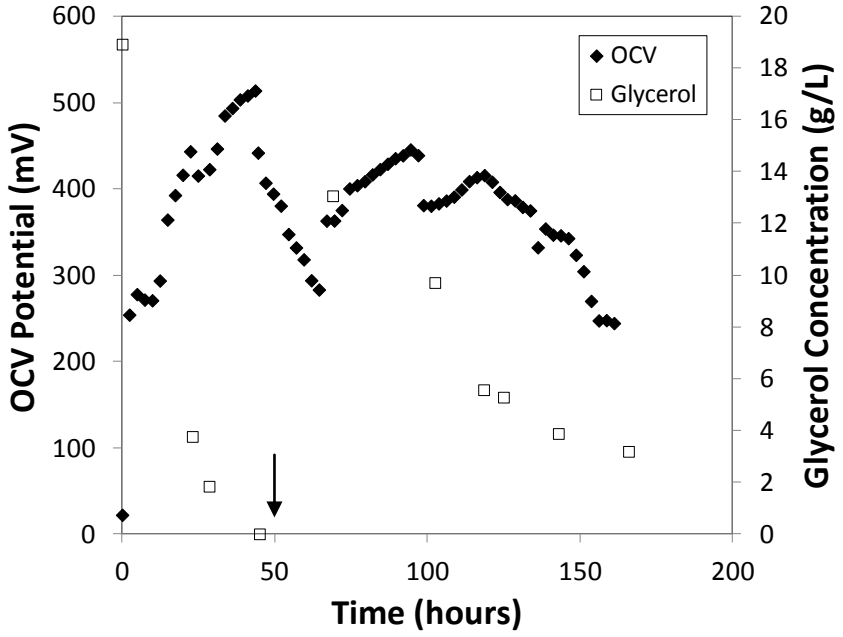

Figure 6.5. OCV and glycerol concentration as a function of time in a mediatorless MFC using AR2 as the anodic biocatalyst. M9 medium with 19 g/L glycerol and 10 g/L tryptone was used as the anodic medium. The fuel cell was operated at 30°C. The arrow indicates the addition of more glycerol to the system.

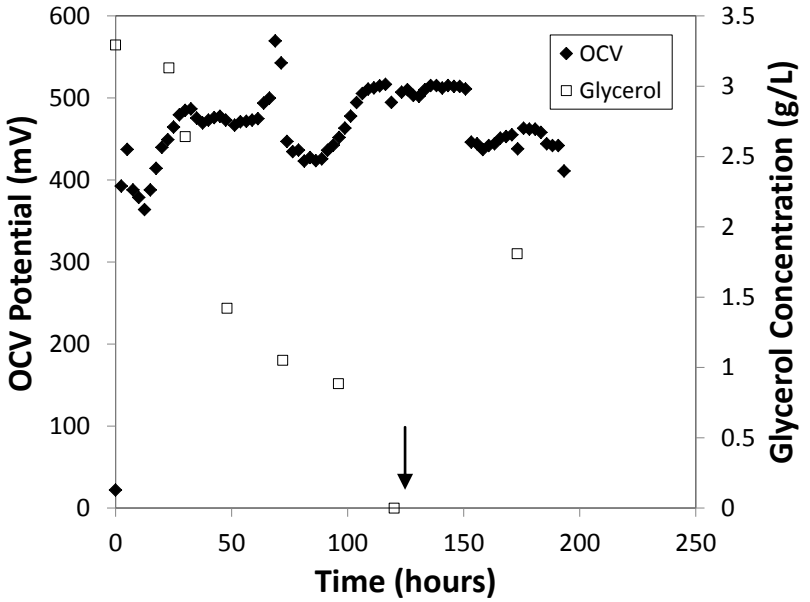

Figure 6.6. OCV and glycerol concentration as a function of time in a mediatorless MFC using *E. coli* W3110 as the anodic biocatalyst. M9 medium with 3.3 g/L glycerol and 10 g/L tryptone was used as the anodic medium. The fuel cell was operated at 37°C. The arrow indicates the addition of more glycerol to the system.

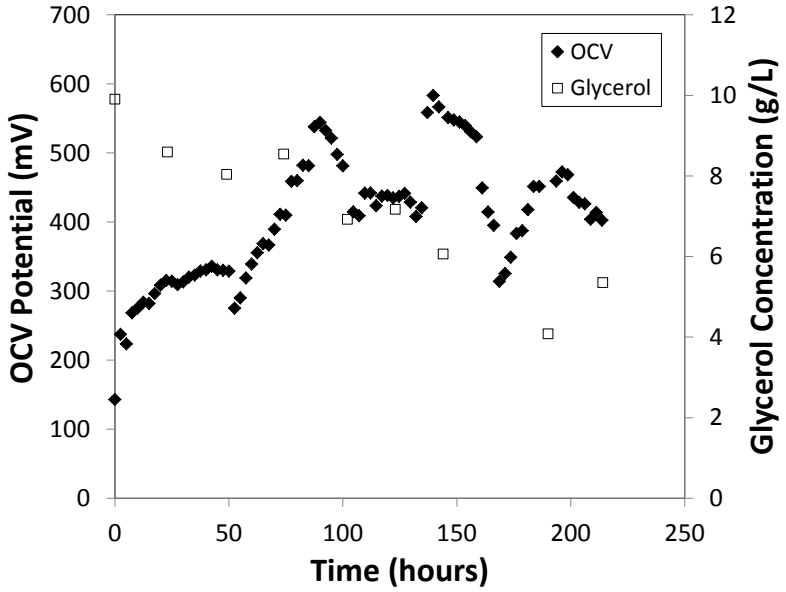

Figure 6.7. OCV and glycerol concentration as a function of time in a mediatorless MFC using *P. freudenreichii* ssp. *shermanii* as the anodic biocatalyst. M9 medium with 10 g/L glycerol and 5 g/L yeast extract used as the anodic medium. The fuel cell was operated at 30°C.

Complete glycerol conversion was observed using AR2 as the anodic catalyst (Fig. 6.5). Once glycerol depleted in the medium, the OCV began to decrease. This is to be expected as there was no longer fuel in the medium. After additional glycerol was injected to the medium, the OCV started to increase again but never reached the previous level. Glycerol consumption then began to plateau, and the OCV dropped again. The production of 1,3-PDO was confirmed in the fuel cell as glycerol depleted by was not quantitatively analyzed. The use of a fed-batch or continuous system could improve performance compared to batch operation since there would be a fresh supply of all nutrients required for growth.

Complete glycerol consumption was also demonstrated using *E. coli* W3110 as the catalyst (Fig. 6.6). Once the initial glycerol in the medium was consumed, additional glycerol was added to a concentration of 2 g/L; however, this glycerol was not consumed, and the voltage began to drop. Again, the use of a fed-batch or continuous system should be explored.

Using *P. freudenreichii* ssp. *shermanii* as the anodic catalyst, incomplete glycerol conversion was observed. Approximately half of the initial 10 g/L of glycerol was left unconsumed in the medium. In Chapter 5, it was demonstrated that this strain can consume at least 10 g/L glycerol under the same growth conditions. The state of the pre-culture could have contributed to the lower amount of glycerol consumption, and the experiment should be repeated in the future.

When the MFCs were operating at their maximum OCV values, polarization data was taken. Polarization and power density curves are shown in Figs. 6.8 - 6.10. The best performance was observed by *P. freudenreichii* ssp. *shermanii*, followed by AR2, and finally *E. coli* W3110. The external resistances where optimal performance occurred were 3445 Ω, 2435 Ω, and 4069 Ω for AR2, *E. coli* W3110, and *P. freudenreichii* ssp. *shermanii*, respectively. These results corroborate the range of optimal external resistances found using the electron-mediated system, and the high values indicate that there is a lot of room for system design improvements.

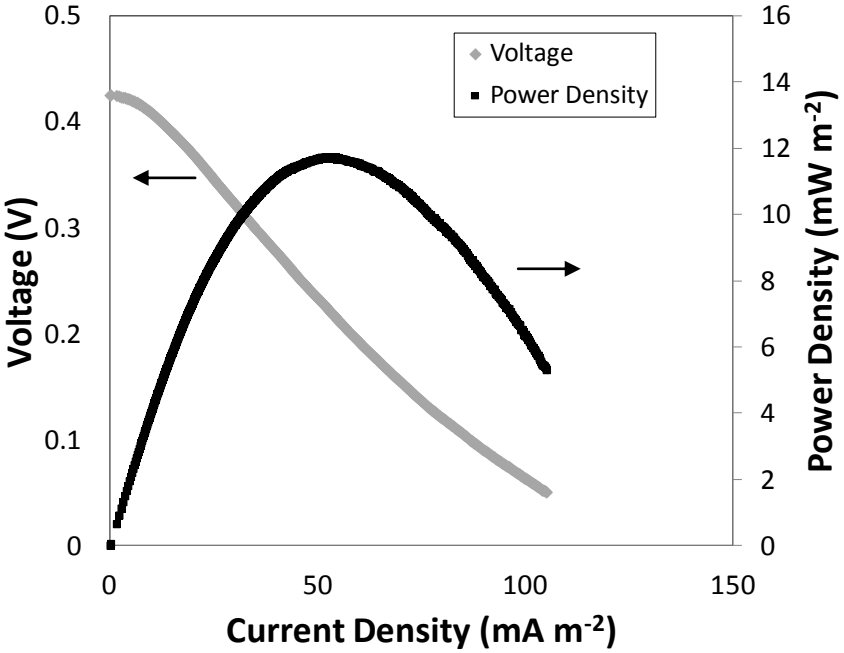

Figure 6.8. Polarization and power density curves using AR2 as the anodic biocatalyst. The fuel cell was operated at 30°C using 19 g/L glycerol and 10 g/L tryptone in the anodic medium.

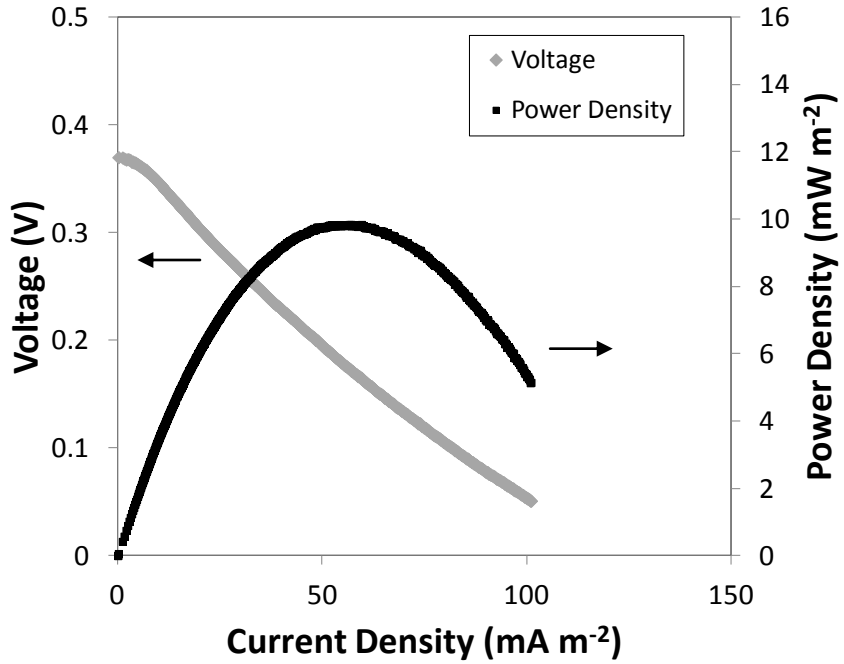

Figure 6.9. Polarization and power density curves using *E. coli* W3110 as the anodic biocatalyst. The fuel cell was operated at 37°C using 3.3 g/L glycerol and 10 g/L tryptone in the anodic medium.

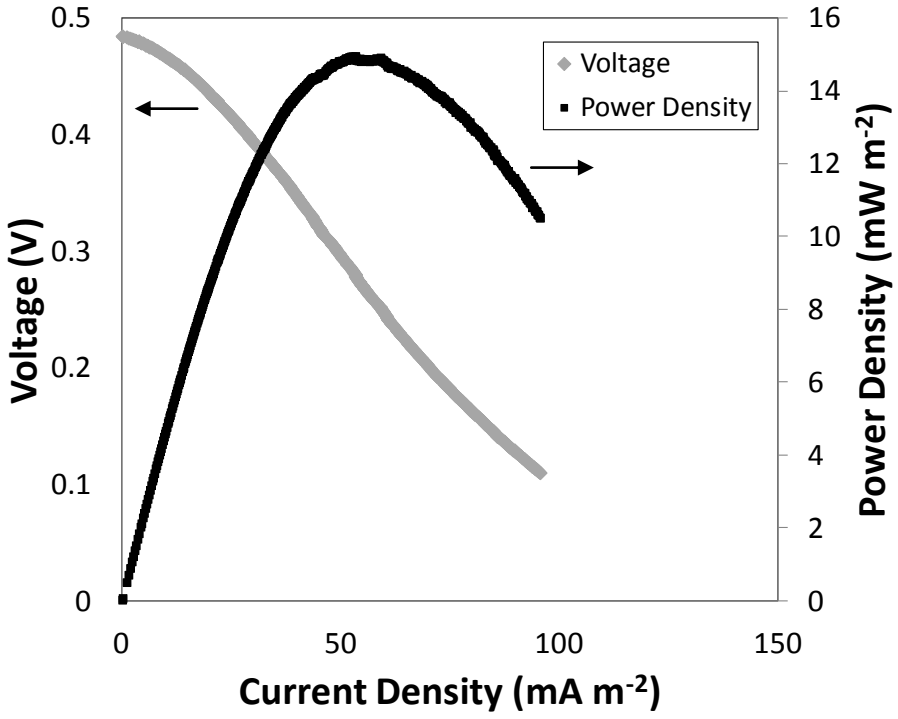

Figure 6.10. Polarization and power density curves using *P. freudenreichii* ssp. *shermanii* as the anodic biocatalyst. The fuel cell was operated at 30°C using 10 g/L glycerol and 5 g/L yeast extract in the anodic medium.

The MFC inoculated with AR2 produced a maximum power density of 11.7 mW m^{-2} and an OCV of 425 mV (Fig. 6.8). These values are lower compared to the values obtained previously using a mediator (Table 6.2). These results show that the mixed cultures possess exoelectrogenic activity, but electron transfer is enhanced by the presence of resazurin as a mediator.

Table 6.2. Comparison of OCV and maximum power density with and without the presence of an electron mediator

Anodic Catalyst	OCV mediator (mV)	OCV mediatorless (mV)	Maximum power density mediator (mW m^{-2})	Maximum power density mediatorless (mW m^{-2})
AR2	516	425	24.5	11.7
E. coli W3110	367	370	5.5	9.8
P. freudenreichii ssp. *shermanii*	450	485	24.0	14.9

The maximum power density and OCV values of the MFC inoculated with *E. coli* W3110 were 9.8 mW m^{-2} and 370 mV in the mediatorless system (Fig. 6.9) and 5.5 mW m^{-2} and 367 mV with the presence of resazurin. The OCV didn't change significantly when the mediator was removed from the medium, but the maximum power density was higher using a mediatorless system. This indicates that the electron transfer efficiency is better without the presence of resazurin in the medium.

The exoelectrogenic activity of *E. coli* has previously been documented in the literature using glucose MFC systems (Zhang et al., 2006; Qiao et al., 2008). Further analysis on the electron transfer mechanism of *E. coli* demonstrated the production of soluble mediator compounds (Qiao et al., 2008). The performance of the glycerol-oxidizing MFC was not significantly enhanced with the presence of resazurin. Chemical mediators can have different effects on different microorganisms. The potential of the chemical mediator has to be similar to the microorganism but different enough to allow electron transfer to occur (Bullen et al., 2006). For this reason, chemical mediators can exhibit different levels of performance using different microorganisms. Mediators can also have toxic effects towards the microorganisms. Different strains can be more tolerant to the presence of resazurin in the medium.

The MFC inoculated with *P. freudenreichii* ssp. *shermanii* produced a maximum power density of 14.9 mW m^{-2} and an OCV of 485 mV (Fig. 6.10). This catalyst exhibited better performance with the mediator as the maximum power density and OCV values were 24 mW m^{-2} and 450 mV respectively when resazurin was present.

Although the performance of the catalysts was mostly enhanced with the presence of a chemical mediator, the use of mediatorless MFCs is still preferred especially in an industrial setting. Chemical mediators are expensive and toxic. Furthermore, their performance degrades over time necessitating their replacement. The performance of mediatorless MFCs can be enhanced through other means, such as optimization of architecture and medium.

In terms of power density, the best performance was exhibited using *P. freudenreichii* ssp. *shermanii* as the anodic catalyst. These results suggest that *P. freudenreichii* has strong exoelectrogenic behaviour compared to the other catalysts. Even though only 5 g/L of glycerol was consumed by *P. freudenreichii*, it was still able to produce higher power densities compared to AR2 which was able to consume 29 g/L of glycerol throughout the duration of the experiment (including the injection of additional glycerol). Since it was shown in Chapter 5 that *P. freudenreichii* ssp. *shermanii* has an oxidative capacity of at least 10 g/L, it would be interesting to repeat these experiments to see the true potential of the catalyst.

Electrochemical efficiencies were calculated for each catalyst as described by equations 6 and 7 in Chapter 2. The complete calculation procedure is outlined in Appendix B. Efficiency values were determined assuming that all glycerol was converted to carbon dioxide as shown in the following reaction (n=14):

$$C_3H_8O_3 + 7/2\ O_2 \rightarrow 3CO_2 + 4H_2O$$

The calculated efficiency values ranged from 31 - 43% (Table 6.3) indicating that improvements to system design should greatly improve the performance of all biocatalysts. Some possible design improvements include the use of a cathode catalyst, closer electrode spacing, and the use of a membraneless system. Although the efficiency values serve as a performance indicator, they are not truly indicative of the system. The theoretical maximum value was calculated assuming that all glycerol was converted to carbon dioxide and therefore that no glycerol was used for biomass formation. The maximum is therefore never attainable with this type of system.

Table 6.3. Electrochemical efficiencies with and without the presence of a mediator

Anodic biocatalyst	Electrochemical efficiency (mediator)	Electrochemical efficiency (mediatorless)
AR2	0.43	0.35
E. coli W3110	0.31	0.31
P. freudenreichii ssp. *shermanii*	0.37	0.40

Although the best performance was exhibited by the *P. freudenreichii* catalyst, AR2 is still a promising choice and should be further investigated. The use of mixed cultures as catalysts in MFCs offers advantages since pure cultures can be difficult to maintain on a large scale. The mixed cultures derived from compost have also been shown to produce 1,3-PDO from glycerol, and 1,3-PDO is a value-added by-product. The production of 1,3-PDO was confirmed in the fuel cell system. The metabolite analysis was described in Chapter 5. The complete conversion of high concentrations of glycerol is also a desirable characteristic, and the mixed cultures are more likely to be able to adapt to the impurities present in crude glycerol.

The use of *E. coli* as the anodic catalyst in an MFC using glycerol as a fuel is novel. Although the performance of *E. coli* W3110 as a catalyst was lower than AR2 and *P. freudenreichii* ssp. *shermanii*, it still has the potential to be optimized. The benefit of using *E. coli* on an industrial scale is that it is easy to grow and manipulate.

6.5 Discussion and conclusions

This chapter has introduced two novel exoelectrogenic catalysts for glycerol-oxidizing MFCs: *P. freudenreichii* ssp. *shermanii* and AR2 (mixed culture). It has also demonstrated the use of a previously known exoelectrogen, *E. coli*, as an MFC catalyst using glycerol as a fuel. The use of *E. coli* as an MFC catalyst has only been previously demonstrated using glucose feedstocks (Zhang et al., 2006; Qiao et al., 2008).

The three catalysts were compared using both electron-mediated systems and mediatorless systems. Using resazurin as an electron mediator, the best performance was

exhibited by AR2, followed by *P. freudenreichii* ssp. *shermanii*, and finally *E. coli* W3110. The power densities obtained by AR2 and *P. freudenreichii* ssp. *shermanii* were similar as the values were 24.5 mW m^{-2} and 24.0 mW m^{-2}, respectively. Since continuous polarization data was not taken, it is hard to distinguish a difference in performance based on these values, however, they both exhibited improved performance compared to *E. coli* W3110 which only produced a maximum power density of 5.5 mW m^{-2}.

In the mediatorless system, the best performance was observed by *P. freudenreichii* ssp. *shermanii* with a power density of 14.9 mW m^{-2}, followed by AR2 with a power density of 11.7 mW m^{-2}, and finally *E. coli* W3110 with a power density of 9.8 mW m^{-2}. *P. freudenreichii* ssp. *shermanii* exhibited a higher electron transfer efficiency compared to AR2 since less glycerol was consumed but a higher power density was obtained.

The use of mixed cultures as MFC catalysts holds several advantages on an industrial scale, such as ease of handling and avoidance of contamination issues. Pure cultures studies are still necessary to identify active strains. The addition of active pure cultures to mixed cultures could be explored as well as the identification of the genes controlling exoelectrogenicity. Genetic modifications of catalysts for improved performance can then be considered. For these reasons and the aforementioned performance comparisons, the use of both AR2 and *P. freudenreichii* ssp. *shermanii* should be actively further researched for use in glycerol-oxidizing MFCs.

All experiments were performed using pure glycerol, so the tolerance of the catalysts towards crude glycerol from biodiesel production must still be determined in an MFC system. *P. freudenreichii* ssp. *shermanii* has previously been shown to ferment 20 g/L of crude glycerol from biodiesel production containing 86% glycerol content (Kośmider et al., 2010). This strain must therefore be able to at least tolerate some of the contaminants in crude glycerol from biodiesel production. The benefit of using mixed cultures is that they can be further enriched using crude glycerol substrates to enhance the cultures for tolerance towards the impurities.

The shape of the polarization data differed between the resazurin-mediated and the mediatorless systems. When operating the mediatorless system, continuous polarization data

was taken instead of just a few points, so this data is more indicative of the true nature of the system. All three catalysts had a short period of activation losses, but the polarization curves were mostly dominated by Ohmic losses. This is typical for MFC systems (Logan, 2008). Therefore, decreasing the Ohmic losses in the system should greatly improve performance. The low electrochemical efficiencies (<50%) corroborate the need to reduce the losses occurring in the MFC system.

Ohmic losses are produced by the resistance towards the flow of electrons through the circuit and the resistance of ions through the membrane (Logan et al., 2006). A different system architecture, such as a single-chamber reactor, should result in improved power densities for all the catalysts. A previous study using wastewater as an inoculum source and biodiesel waste as a fuel reported a power density of 2100 mW m^{-2} using a single-chambered system (Feng et al., 2011). This is significantly higher than the power densities obtained in this study. Other measures that should improve performance include limiting the flow of other ions through the membrane and improving the circuit connections (Kim et al., 2007a). Epoxy resins could be used to secure the wire to the electrodes to provide more solid connections.

Although power densities values were low, they still allowed for a comparison between the strains. The best performance was exhibited using *P. freudenreichii* ssp. *shermanii* as an anodic catalyst, and its use should be further explored using an optimized MFC design. The use of AR2 as a catalyst is also promising due to the benefits of mixed culture use on an industrial scale.

Chapter 7. Conclusions and overall discussion

7.1 Summary

This thesis was split into three distinct sections: biocatalyst selection (Chapter 4), anaerobic glycerol conversion in pure and mixed cultures (Chapter 5), and screening potential biocatalysts in a glycerol-oxidizing MFC (Chapter 6). The most significant conclusions of each section are summarized below.

7.1.1 Biocatalyst selection

Both pure and mixed cultures of bacteria were selected for screening as anodic catalysts in a glycerol-oxidizing MFC. Four strains of *E. coli*, W3110, TG1, DH5α, BL21, and two strains of *P. freudenreichii*, subspecies *freudenreichii* and *shermanii*, were selected as pure strain catalysts. Mixed cultures (AR1, AR2, AR3) were enriched from compost samples for communities with a high anaerobic oxidative capacity towards glycerol. All selected pure strains and mixed cultures were non-pathogenic and non-stringent anaerobes, thereby making them appropriate for any future scale-up applications.

7.2.2 Anaerobic glycerol conversion

Anaerobic batch fermentation experiments were used to determine the oxidative capacity of the strains towards glycerol. All strains were able to grow and consume glycerol anaerobically, which is an important quality for use in any future MFC applications. Using M9 minimal medium with 10 g/L tryptone as a rich nutrient additive, the highest anaerobic glycerol conversion of the pure strains was observed in *E. coli* W3110 and DH5α with values of 4.1 ± 0.3 g/L and 3.9 ± 0.4 g/L, respectively. This was significantly lower than the mixed culture AR2 which achieved a glycerol conversion of 20 g/L using the same medium.

Using 5 g/L yeast extract instead of 10 g/L tryptone as a rich nutrient additive in the medium enhanced the anaerobic glycerol conversion capabilities of the *P. freudenreichii* strains. Both subspecies were able to metabolize 10 g/L of glycerol using yeast extract

compared to 3.6 ± 0.3 g/L and 3.0 ± 0.6 g/L using tryptone for the *shermanii* and *freudenreichii* subspecies, respectively.

Analysis of glycerol conversion products demonstrated that three different glycerol bioconversion pathways were being utilized by the three catalyst groups. The mixed cultures produced both 1,3-PDO and CO_2 as metabolites while the *E. coli* strains produced only CO_2, and the *P. freudenreichii* strains produced neither metabolite.

Based on anaerobic glycerol conversion results, one catalyst from each group was selected for MFC testing: *E. coli* W3110, *P. freudenreichii* ssp. *shermanii* and AR2 (mixed cultures).

7.2.3 MFC performance

E. coli W3110, *P. freudenreichii* ssp. *shermanii*, and AR2 were tested as biocatalysts in both resazurin-mediated and mediatorless MFC systems using pure glycerol as a fuel. Using the electron-mediated system, the best performance was observed by AR2, followed by *P. freudenreichii* ssp. *shermanii*, and finally *E. coli* W3110. All three catalysts demonstrated exoelectrogenic activity. In the mediatorless system, the highest power density was demonstrated by *P. freudenreichii* ssp. *shermanii* (14.7 mW m^{-2}), followed by AR2 (11.7 mW m^{-2}), and finally *E. coli* W3110 (9.8 mW m^{-2}). Although the *P. freudenreichii* catalyst exhibited the best performance in the mediatorless system, it was concluded that the use of mixed cultures should also be further explored for glycerol-oxidizing MFC systems due to the benefits of using mixed cultures on an industrial scale.

7.2 Overall discussion and future directions

This research has identified two novel exoelectrogenic catalysts for use in a glycerol-oxidizing MFC: *P. freudenreichii* ssp. *shermanii* and AR2 (mixed culture enriched from compost). A previously known exoelectrogen, *E. coli*, has also been shown to work as an anodic catalyst in an MFC using glycerol as a novel fuel but produced lower power densities compared to the other two catalysts. For this reason, the use of AR2 and *P. freudenreichii* ssp. *shermanii* should be focused on in future research.

Power is produced in an MFC by the anodic biocatalyst metabolizing the fuel. Production of metabolites and biomass can divert electrons from completing the MFC circuit. Therefore, there is a balance between metabolite and power production. Complete degradation of the fuel and the production of valuable by-products, along with electricity generation, are attractive characteristics of an MFC catalyst. In the case of the AR2 mixed culture, complete consumption of high concentrations of glycerol (29 g/L throughout two glycerol injections) was demonstrated using a batch MFC system. The production of both 1,3-PDO and voltage occurred in the MFC. The generation of 1,3-PDO from glycerol is desirable since it is a value-added product used as a monomer for the synthesis of several polyesters (Witt et al., 1994).

No glycerol metabolites were identified using *P. freudenreichii* ssp. *shermanii* as a catalyst; however, it is suspected based on the literature review that propionic acid is produced as a major product (Barbirato et al., 1997). Propionic acid is of similar value to pure glycerol (Table 2.1). A complete metabolite analysis should be performed using both AR2 and *P. freudenreichii* ssp. *shermanii* as catalysts to determine whether or not product purification is economical and also the value of using each catalyst based on the combination of electricity production and metabolite production.

Now that the exoelectrogenic activity of the catalysts has been confirmed, additional research can be performed on enhancing electricity generation from glycerol. The use of *P. freudenreichii* ssp. *shermanii* produced the highest power density of all the catalysts and also demonstrated strong exoelectrogenic activity. In the mediatorless MFC, the catalyst only consumed about 5 g/L of the initial 10 g/L present in the medium, however, it was still able to achieve a higher power density than AR2, which was able to consume 29 g/L glycerol. It was shown in Chapter 5 that *P. freudenreichii* ssp. *shermanii* can consume at least 10 g/L of glycerol under the same growth conditions used in the MFC under 200 hours. In the MFC containing resazurin, it also took approximately 200 hours to consume the 10 g/L of glycerol; however, in the mediatorless MFC, within the same time frame, only 5 g/L glycerol was consumed. In literature, it has previously been shown that *P. freudenreichii* ssp. *shermanii* can convert 20 g/L glycerol using minimal medium enriched with yeast extract and tryptic

soy broth (Himmi et al., 2000). Comparison of glycerol conversion values in the mediatorless MFC to both literature values and the previously performed batch fermentation and electron-mediated MFC experiments show that there is the potential to enhance glycerol conversion in order to increase power production. This could be done through further medium optimization or simply by ensuring the use of a more active pre-culture.

Conversion rates were similar using AR2 between the fermentation and MFC experiments (~ 20 g/L in 50 hours). Electricity generation using AR2 could perhaps be enhanced if the mixed culture was enriched in the fuel cell environment for a community that can use the electrode as a final electron acceptor. This could increase the overall exoelectrogenicity of the mixed culture.

In terms of increasing the performance of both catalysts, the MFC architecture and operation can both be re-designed. Reducing the internal resistance inherent in the MFC should greatly increase power production. The polarization curves shown in Chapter 6 demonstrate that the resistance is dominated by Ohmic losses that can be reduced through better system design. This includes reducing the resistance towards the flow of electrons through the circuit and the resistance of ions through the membrane (Logan et al., 2006). Improving electrical contacts and switching to a membraneless MFC are some potential improvements in design that could be explored to decrease the Ohmic resistance. To reduce any activation losses, electrode surface area can be greatly increased. The use of a fed-batch or continuous operation should also enhance performance. Glycerol conversion in all the catalysts should be improved when there is a continuous supply of all nutrients necessary for growth. Further research into medium selection should also be considered to make sure glycerol conversion is optimized for the selected biocatalysts.

The global objective of this research is to find a novel and environmentally friendly platform for the surplus of glycerol generated through biodiesel production. The ultimate goal is to both make biodiesel production a more economically viable alternative to conventional diesel and to develop a novel renewable energy technology for glycerol. In order to achieve this goal, the crude glycerol streams from biodiesel production must be used directly as an MFC fuel. The biocatalyst must therefore be able tolerate any impurities

present in the crude glycerol. *P. freudenreichii* ssp. *shermanii* has been shown to ferment crude glycerol from biodiesel production corroborating its choice as a promising catalyst (Kośmider et al., 2010). Experiments should be performed to test the tolerance of AR2 to crude glycerol from biodiesel production, and further enrichment of mixed cultures could be performed to improve this tolerance.

The benefits of using mixed cultures on an industrial scale have been emphasized throughout this thesis. Pure culture research however is still necessary for the identification of active strains and even genes. The addition of active pure cultures to mixed cultures is also an avenue to consider. For example, the addition of *P. aeruginosa*, a known exoelectrogen, to mixed cultures was shown to enhance the electron transfer rate in an MFC (Rabaey et al., 2005). *P. freudenreichii* ssp. *shermanii* has demonstrated a strong exoelectrogenic nature and perhaps its addition to AR2 would enhance the performance of the mixed culture. Further research using both catalysts should be performed for the development of a glycerol-oxidizing MFC.

7.3 Concluding remarks

Three bacterial cultures, *E. coli* W3110, *P. freudenreichii* ssp. *shermanii*, and mixed culture AR2, were shown to function as catalysts in a mediatorless MFC using glycerol as a fuel. The most significant results include the exoelectrogenicity of *P. freudenreichii* ssp. *shermanii* and AR2 and their enhanced performance based on power production compared to *E. coli* W3110. Further development of the MFC should be explored using both AR2 and *P. freudenreichii* ssp. *shermanii* in order to improve power densities. The development of an MFC that can use crude glycerol from biodiesel production as a fuel will greatly increase the economic viability of current biodiesel production processes.

Chapter 8. References

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Appendix A. Standard curves

A1. Standard curves for correlation of protein concentrations to OD_{600}

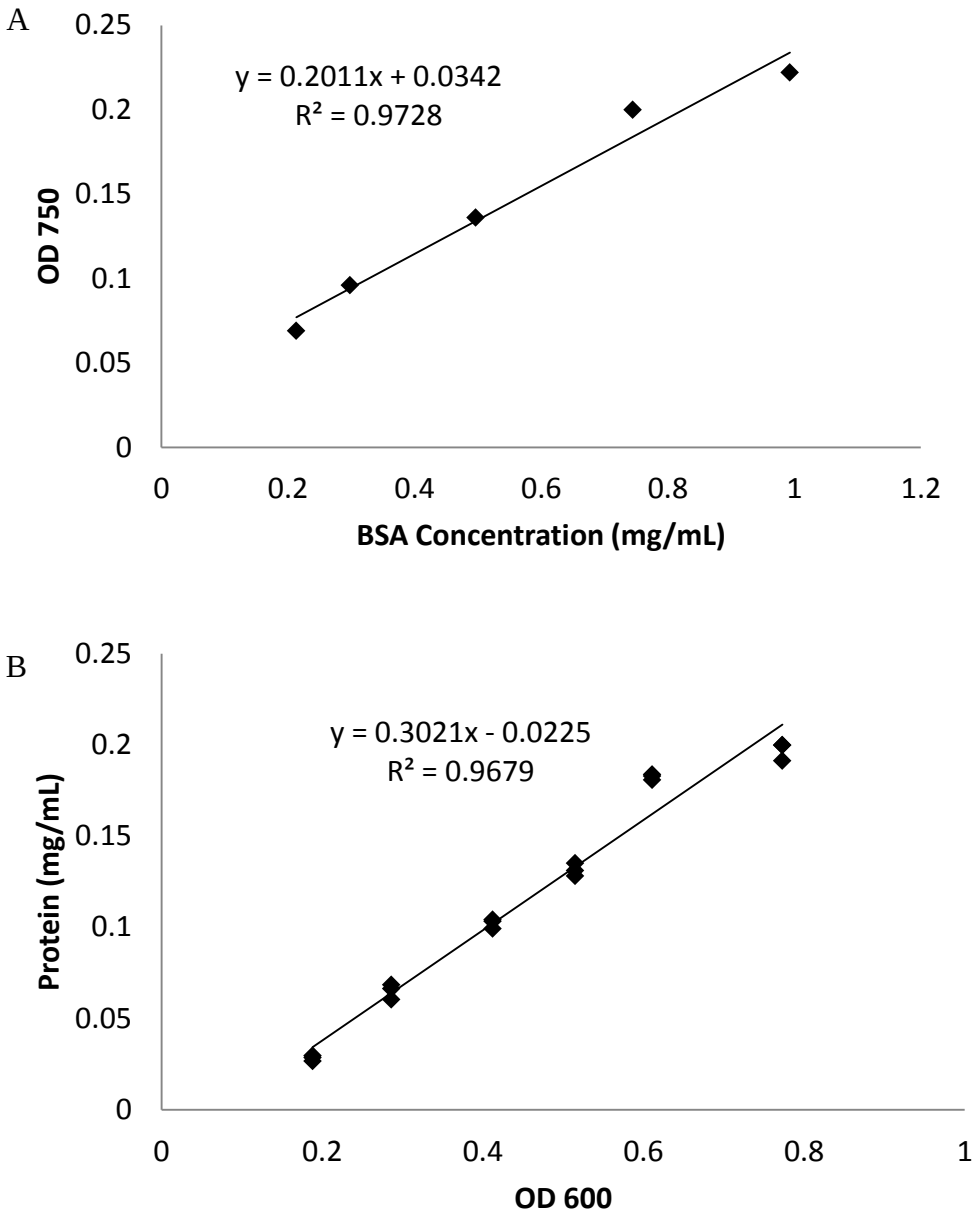

Figure A.1. A. BSA standard curve for *E. coli* W3110 protein assay. B. Protein concentration as a function of OD_{600} for *E. coli* W3110 anaerobic growth curve.

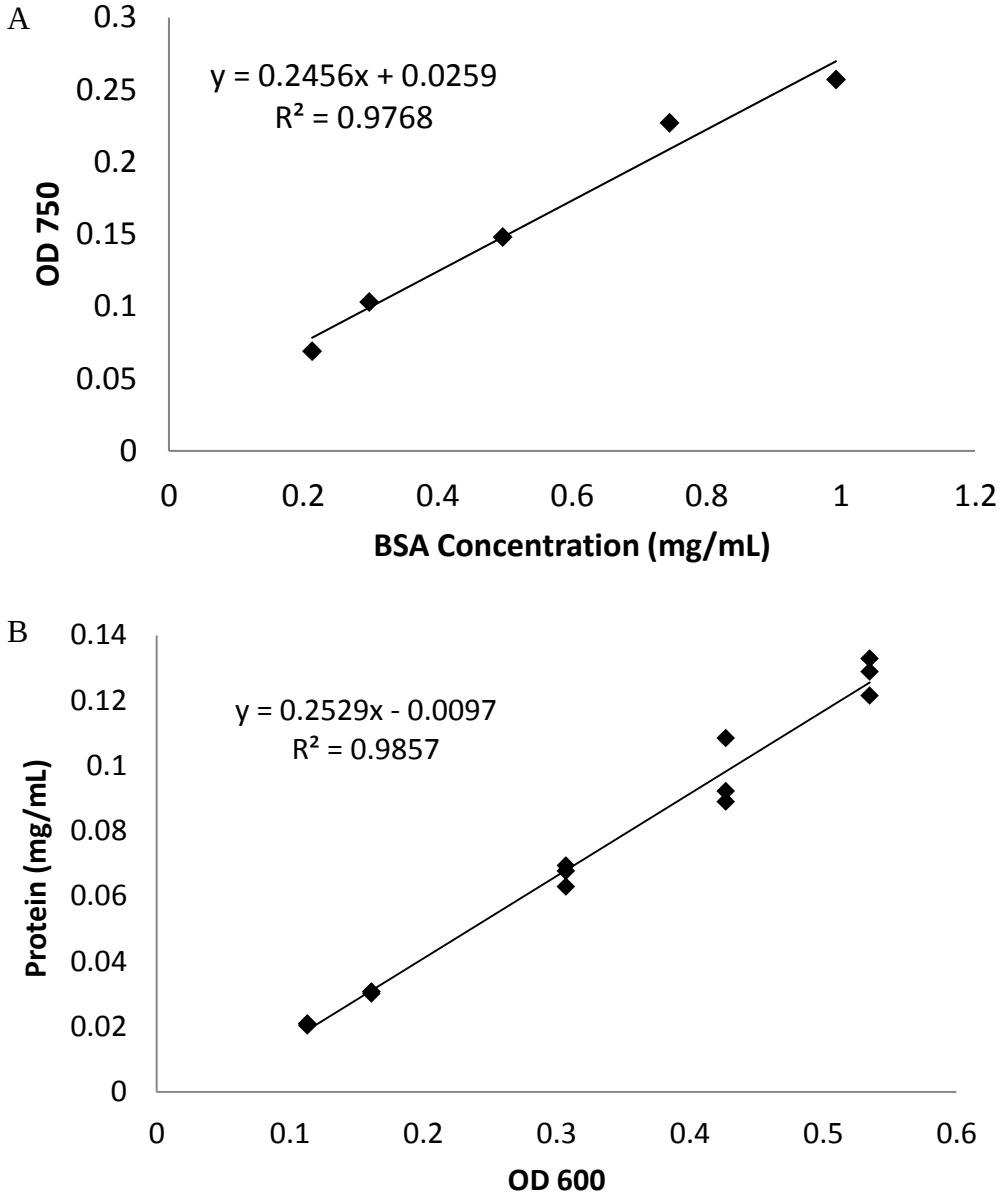

Figure A.2. A. BSA standard curve for *E. coli* TG1 protein assay. B. Protein concentration as a function of OD_{600} for *E. coli* TG1 anaerobic growth curve.

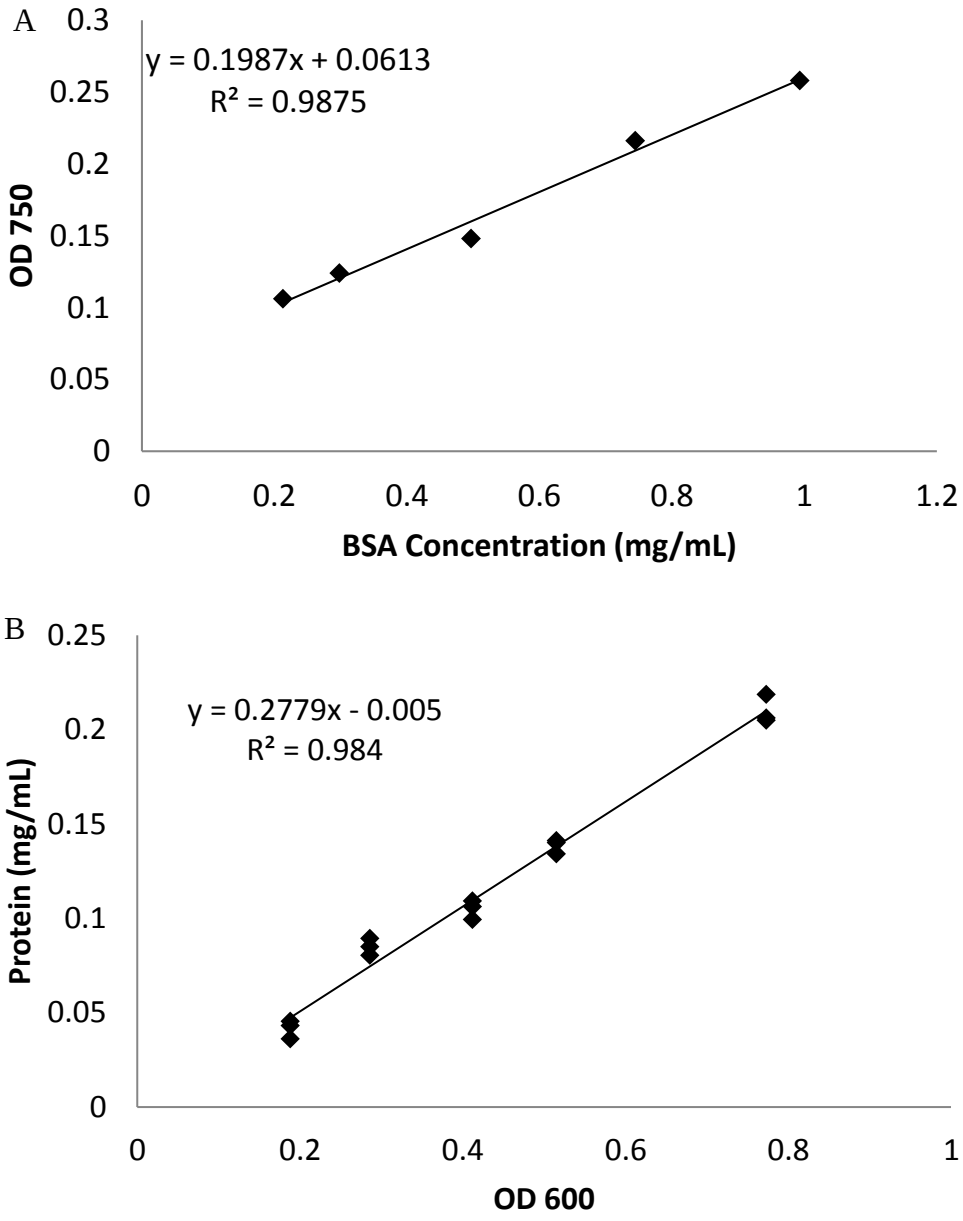

Figure A.3. A. BSA standard curve for *E. coli* BL21 protein assay. B. Protein concentration as a function of OD_{600} for *E. coli* BL21 anaerobic growth curve.

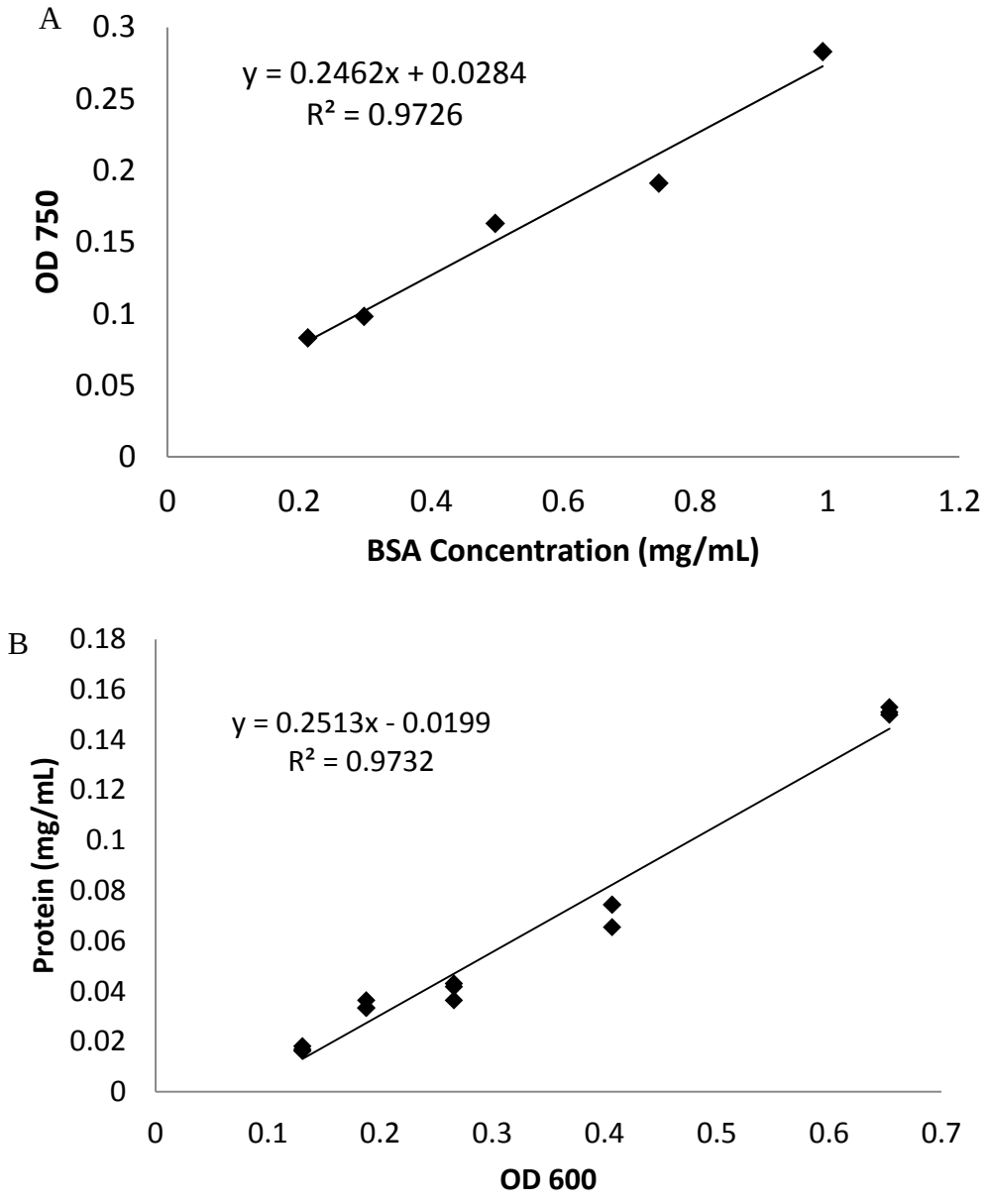

Figure A.4. A. BSA standard curve for *E. coli* DH5α protein assay. B. Protein concentration as a function of OD_{600} for *E. coli* DH5α anaerobic growth curve.

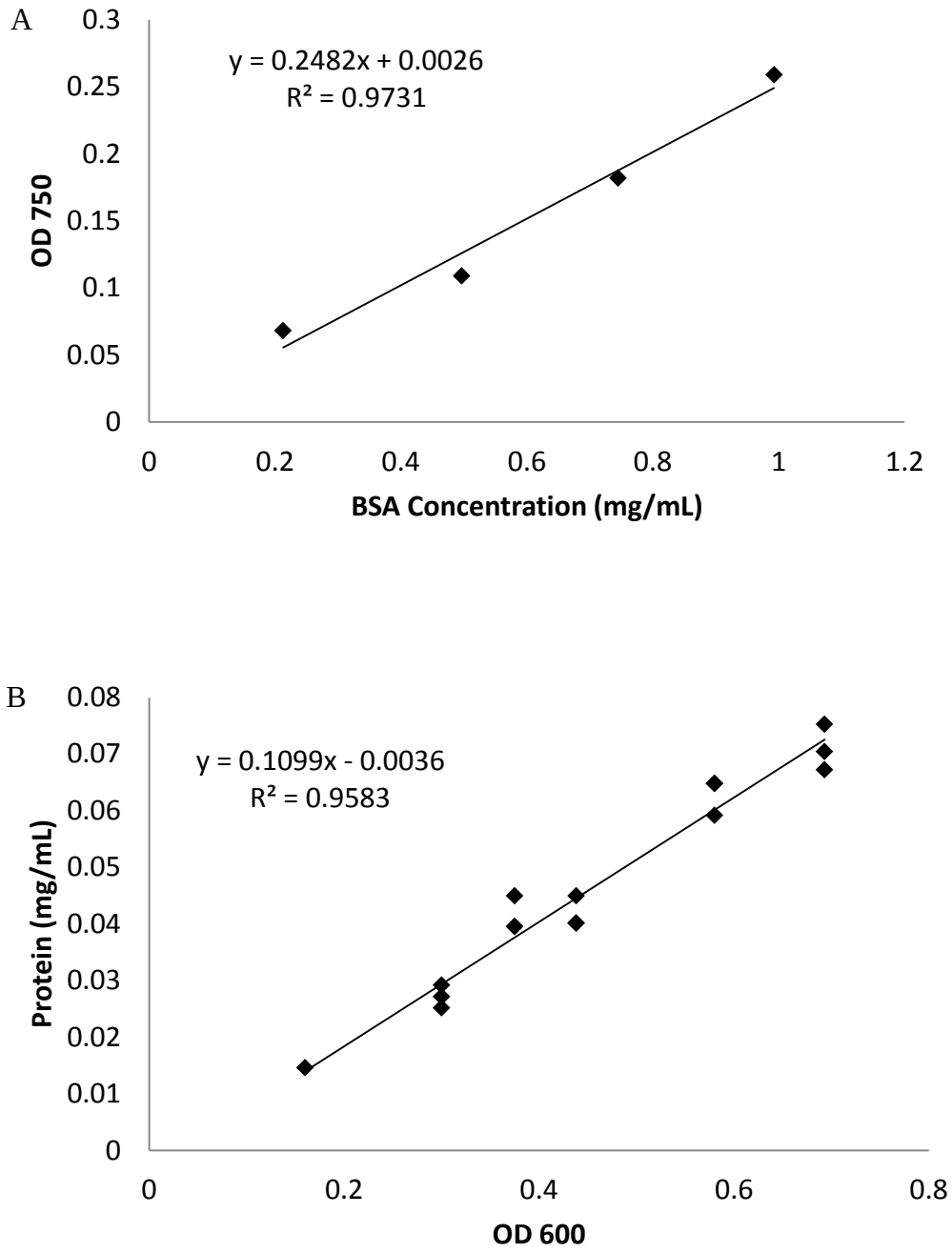

Figure A.5. A. BSA standard curve for *P. freudenreichii* ssp. *freudenreichii* protein assay. B. Protein concentration as a function of OD_{600} for *P. freudenreichii* ssp. *freudenreichii* anaerobic growth curve.

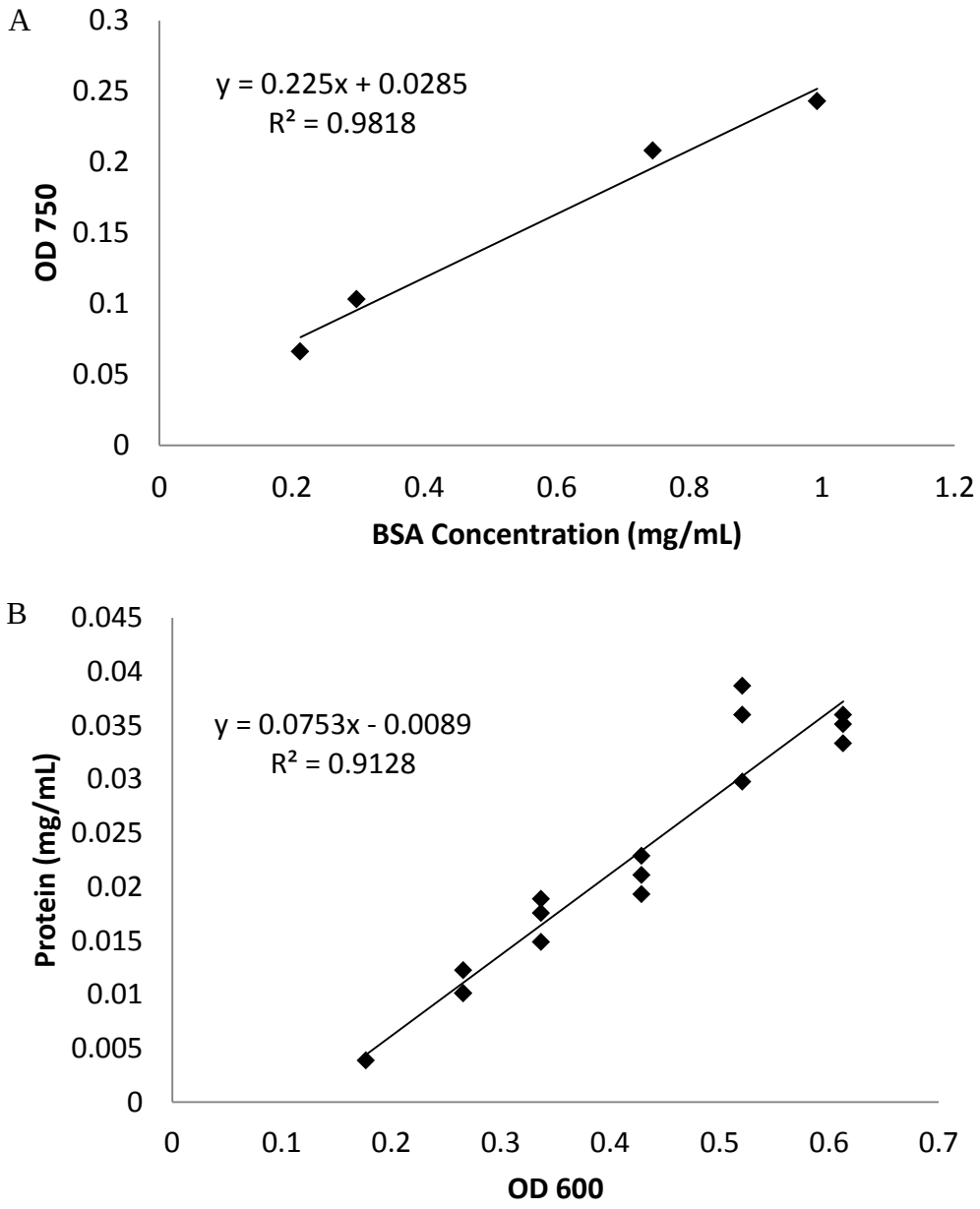

Figure A.6. A. BSA standard curve for *P. freudenreichii* ssp. *shermanii* protein assay. B. Protein concentration as a function of OD_{600} for *P. freudenreichii* ssp. *shermanii* anaerobic growth curve.

A2. Standard curve for quantification of glycerol concentration by HPLC

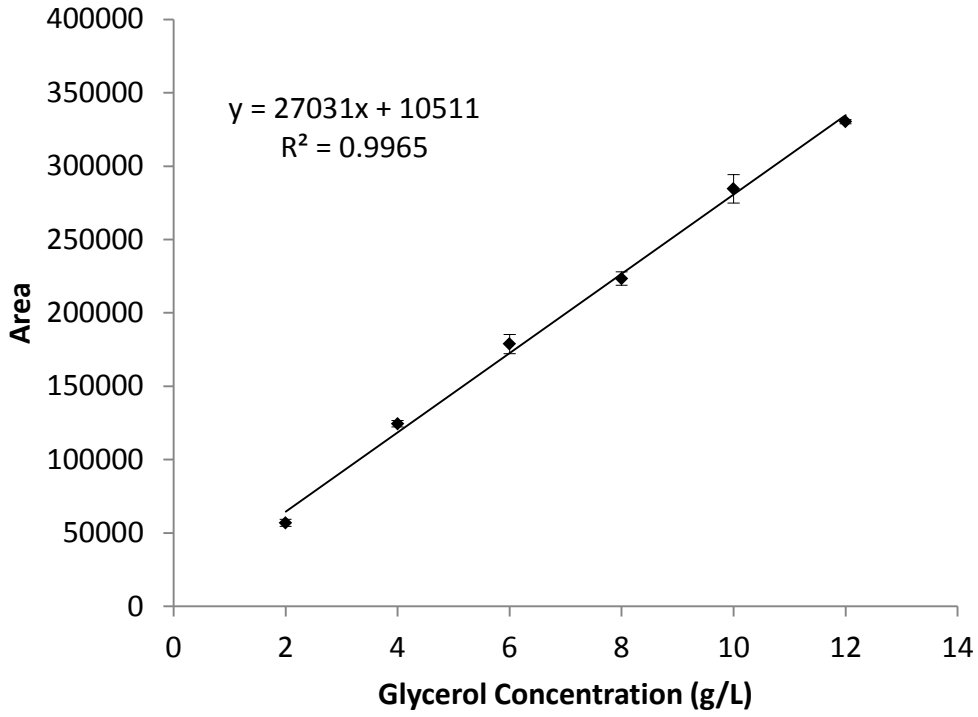

Figure A.7. Standard curve for glycerol quantification by HPLC. Average values are presented (n = 3, ± standard deviation).

Appendix B. Calculations

Growth and Glycerol Conversion

Protein Yield (Y)

Protein concentrations (y_i) were calculated based on optical density values read at 600 nm (OD_{600}). Calibration curves for protein concentration as a function of OD_{600} are presented in Appendix A.

Each pure strain of bacteria was grown anaerobically, and samples were taken throughout the growth curve at various optical densities. Pellets were resuspended in 1 N NaOH and were heated at 90°C for 10 minutes in a water bath. BSA standards were prepared using 1 N NaOH and were subjected to the same pretreatment as the bacterial samples. The standards and samples were then used to perform the RC DC™ protein assay as described by the manufacturer.

Using *E. coli* W3110 as an example, the standard curve presented in Fig. A1.A was used to correlate the absorbance values read at 750 nm (ABS_{750}) of the BSA standards to protein concentration. ABS_{750} values were then taken for the bacterial samples and used to find their protein concentration (Equation 2).

$$ABS_{750,i} = 0.2011 * y_{s,i} + 00342 \quad [1]$$

$$y_{s,i} = \frac{ABS_{750,i} - 0.00342}{0.2011} \quad [2]$$

where, $y_{s,i}$ is the protein concentration of the bacterial standard i in mg/mL
$ABS_{750,i}$ is the absorbance read at 750 nm for sample i

The calculated $y_{s,i}$ values were then plotted against OD_{600} to obtain the following equation used to calculated the protein concentration of bacterial samples (Fig. A1.B):

$$y_i = 0.2011 * OD_{600,i} + 0.0342 \quad [3]$$

where, y_i is the protein concentration of the bacterial sample i in mg/mL
$OD_{600,i}$ is the optical density of sample i read at 600 nm

Glycerol concentrations (g_i) were calculated based on the area under the curve ($AREA_i$) of the peak eluting at 2.3 minutes on the HPLC chromatogram. A standard curve was used to correlate glycerol concentration to the AREA and is presented in Fig. A.7. Glycerol concentration was calculated using Equation 4.

$$g_i = 27031 * AREA_i + 10511 \quad [4]$$

where, g_i is the glycerol concentration of sample i in g/L
AREA is the area under the curve of the HPLC chromatogram for the peak eluted at 2.3 minutes

Protein yield (Y) was then calculated one day after stationary phase was reached at time, t.

$$Y = \frac{y_t}{g_o - g_t} \quad [5]$$

where, Y is the protein yield in g protein/g glycerol
y_t is the protein concentration at time t in g/L
g_o is the initial glycerol concentration in g/L
g_t is the glycerol concentration at time t in g/L

Glycerol Conversion (C_g)

Glycerol conversion was calculated at the end of the fermentation time (f).

$$C_g = g_o - g_f \quad [6]$$

where, C_g is the glycerol conversion in g/L
g_o is the initial glycerol concentration in g/L
g_f is the final glycerol concentration in g/L

Growth Rate (μ)

Growth rate (μ) was calculated throughout the exponential portion of the growth curve as follows:

$$\mu = \frac{\ln(y_2) - \ln(y_1)}{t_2 - t_1} \quad [7]$$

where, μ is the growth rate in h^{-1}
y_1 is the protein concentration at t_1 in mg/mL
y_2 is the protein concentration at t_2 in mg/mL
t_1 is time point 1 in hours
t_2 is time point 2 in hours

MFC Calculations

Power density (P_{An})

For calculations of power density (P_{An}), the current (I) was first calculated based on the measured cell voltage (E_{cell}) and the external resistance (R_{ext}):

$$I = \frac{E_{cell}}{R_{ext}} \quad [8]$$

where, I is the current in units of A
E_{cell} is the voltage in units of V
R_{ext} is the external resistance in units of Ω

The power (P) could then be calculated:

$$P = I * E_{cell} \quad [9]$$

where, P is the power in units of W
I is the current in units of A
E_{cell} is the voltage in units of V

Power density (P_{An}) was calculated by normalizing the calculated power (P) with the anode surface area (A_{An}):

$$P_{An} = \frac{P}{A_{An}} \qquad [10]$$

where, P_{An} is the power density in units of mW m^{-2}
P is the power in units of mW
A_{An} is the anode surface area in units of m^{-2}

Internal resistance (R_{int})

The internal resistance [Ω] was determined by finding the external resistance (R_{ext}) at the maximum power density (P_{max}). At P_{max}, the internal and external resistances are equal.

Electrochemical Efficiency (η)

The electrochemical efficiency (η) is calculated by first determining the theoretical maximum open circuit voltage (E^o).

In order to determine E^o, the Gibbs energy for the reaction (G) must be determined.

The overall fuel cell reaction assuming all glycerol is converted to carbon dioxide is:

$C_3H_8O_3 + 7/2\ O_2 \rightarrow 3CO_2 + 4H_2O$

The operating temperature (T) of the fuel cells had to be accounted for. For MFCs using mixed culture or *P. freudenreichii* catalysts, the fuel cells were operated at 30ºC or 303 K. The *E. coli* fuel cells were operated at 37ºC or 310 K. The value of G was then calculated using the following standard enthalpies of formation (H_f^o) and entropies of formation (S_f^o).

For glycerol: H_f^o = -665.9 kJ/mol; S_f^o = -638.9 J/molK

For carbon dioxide: H_f^o = -393.5 kJ/mol; S_f^o = 2.9 J/molK

For water: H_f^o = -285.84 kJ/mol; S_f^o = -163.3 J/molK

The Gibbs energy for the products and reactants (G_i) were then calculated using the following formula:

$$G_i = : H_f^o - T * S_f^o \quad [11]$$

The Gibbs energy for the reaction (G) was then calculated by:

$$G = \sum G_{products} - \sum G_{reactants} \quad [12]$$

From G, the theoretical OCV (E^o) is calculated by:

$$E^\circ = \frac{-G}{nF} \quad [13]$$

where F is Faraday's constant and n is the number of moles of electrons produced per moles of glycerol consumed (n = 14).

For both temperatures, E^o = 1.23 V. To determine the electrochemical efficiency (η), the measured OCV is divided by E^o.

$$\eta = \frac{OCV}{E^\circ} \quad [14]$$