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Understanding Fermentative Glycerol Metabolism and its Application for the Production of Fuels and Chemicals

by

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Abstract

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Due to its availability, low-price, and higher degree of reduction than lignocellulosic sugars, glycerol has become an attractive carbon source for the production of fuels and reduced chemicals. However, this high degree of reduction of carbon atoms in glycerol also results in significant challenges in regard to its utilization under fermentative conditions. Therefore, in order to unlock the full potential of microorganisms for the fermentative conversion of glycerol into fuels and chemicals, a detailed understanding of the anaerobic fermentation of glycerol is required. The work presented here highlights a comprehensive experimental investigation into fermentative glycerol metabolism in *Escherichia coli*, which has elucidated several key pathways and mechanisms. The activity of both the fermentative and respiratory glycerol dissimilation pathways was found to be important for maximum glycerol utilization, a consequence of the metabolic cycle and downstream effects created by the essential involvement of PEP-dependent dihydroxyacetone kinase (DHAK) in the fermentative glycerol dissimilation pathway. The decoupling of this cycle is of central importance during fermentative glycerol metabolism, and while multiple decoupling mechanisms were identified, their relative inefficiencies dictated not only their level of involvement, but also

implicated the activity of other pathways/enzymes, including fumarate reductase and pyruvate kinase. The central role of the PEP-dependent DHAK, an enzyme whose transcription was found to be regulated by the cyclic adenosine monophosphate (cAMP) receptor protein (CRP)-cAMP complex, was also tied to the importance of multiple fructose 1,6-bisphosphotases (FBPases) encoded by *fbp*, *glpX*, and *yggF*. The activity of these FBPases, and as a result the levels of fructose 1,6-bisphosphate, a key regulatory compound, appear to also play a role in the involvement of several other enzymes during fermentative glycerol metabolism including PEP carboxykinase. Using this improved understanding of fermentative glycerol metabolism as a platform, *E. coli* has been engineered to produce high yields and titers of ethanol (19.8 g/L, 0.46 g/g), co-produced along with hydrogen, and 1,2-propanediol (5.6 g/L, 0.21 g/g) from glycerol, demonstrating its potential as a carbon source for the production of fuels and reduced chemicals.

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Chapter 1

Introduction

The rising concerns related to the cost, sustained availability, and environmental impact of use of fossil fuels has led to the search for new technologies that generate alternative fuels from renewable carbon sources (Hirsch et al., 2006; Kerr, 2007). In light of these concerns, interest and investment in the production of biofuels from renewable resources has grown significantly in recent years (Schubert, 2006). The availability of diverse biomass resources, such as agricultural lignocellulosic residues and edible and non-edible crops, has resulted in significant advancements in the conversion of biomass into renewable fuels and chemicals through the implementation of different technologies capable of utilizing specific biomass feedstock constituents (Clomburg and Gonzalez, 2010; Dellomonaco et al., 2010; Fischer et al., 2008). At present, bioethanol production through microbial fermentation of the sugars derived from sources such as sugar cane or corn and biodiesel production via the transesterification of vegetable oil or

animal fats with an alcohol to produces esters have been the two most successful technological platforms for the industrial scale conversion of biomass into fuels (Schubert, 2006). In addition, several developing technologies capable of making a wide array of fuels and chemicals from biomass, including direct gasoline replacements, have had initial success, and continued research in the area will optimize and add to the current portfolio of available technologies (Clomburg and Gonzalez, 2010; Conner and Liao, 2009; Rude and Schirmer, 2009).

Despite the initial success of these technologies, their long term economic viability remains a concern. While the production of biofuels such as bioethanol from the most commonly used feedstocks on an industrial scale (starches and simple sugars derived from sources such as sugar cane and corn) is highly efficient (Fischer et al., 2008), these feedstocks are expensive and non-sustainable due to their concurrent integration as an essential part of the food-feed chain. Lignocellulosic crops, in contrast to sugar cane and corn, have the benefits of higher productivities and their use as a feedstock for biofuel production is considered sustainable and renewable, however their conversion into sugars is more difficult than that of corn or sugarcane which leads to higher feedstock and operating costs (Dellomonaco et al., 2010; Fischer et al., 2008). In addition, similar economic and political factors have led to an increase in the feedstock and operating costs for biodiesel production that at times, result in feedstock and operating costs that nearly match the market price of biodiesel (Yazdani and Gonzalez, 2007). Thus, despite the promise of biofuel production from biomass, unless the future

economics of the processes can be sustained, many doubts as to their viability will remain.

In order to increase the economic viability for the future production of biofuels, the concept of a biorefinery has been proposed (Kamm and Kamm, 2007). The concept of a biorefinery is analogous to the way a petroleum refinery produces multiple fuels and products from a single feedstock. However, unlike current petroleum refineries, in which petroleum is the main feedstock, biomass would be used as the main feedstock in a biorefinery. By using both by-product streams and a small portion of the feedstock to produce higher value, small market chemicals along with the biofuels, the value derived from the biomass feedstock can be maximized. This concept would vastly improve the economic viability of future biofuel production and theoretically allow for their use as alternative energy sources. However, before this concept can be implemented, processes must be developed that can produce these chemicals from the biomass feedstock as well as by-product waste streams generated during biofuel production.

One promising avenue for the coupling of processes within a biorefinery is the utilization of glycerol as a substrate for the production of biochemicals and biofuels. Glycerol is an inevitable by-product generated during both bioethanol and biodiesel production processes, and the tremendous growth of these industries has led to a dramatic decrease in crude glycerol prices over the past few years (Khanal et al, 2008; Rausch and Belyea, 2006; Yazdani and Gonzalez, 2007) (Figure 1.1).

During biodiesel production, the transesterification of fats and oils with an alcohol, results in 10 lbs of crude glycerol for every 100 lbs of biodiesel produced.

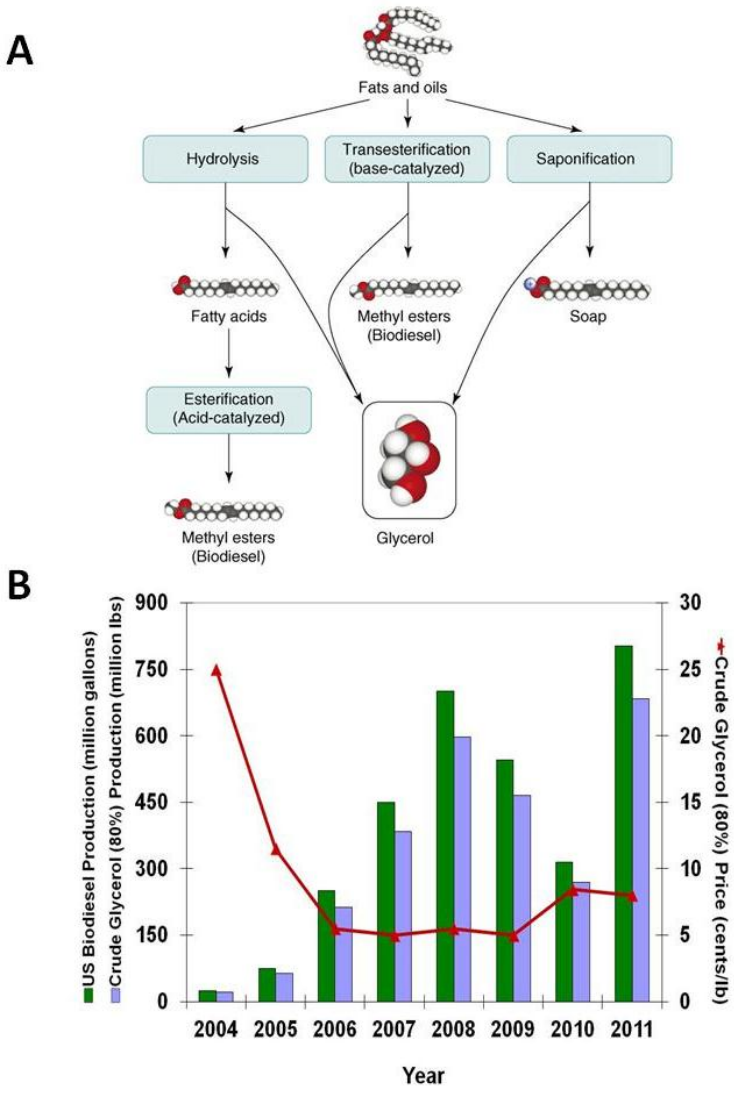

Figure 1.1 Oils, fats, biodiesel, and glycerol. A: Biodiesel production and its relationship with primary oleochemical platform pathways (Yazdani and Gonzalez, 2007). B: US biodiesel production and crude glycerol price (www.thejacaobsen.com)

Furthermore, regardless of the feedstock used (i.e. sugar cane or corn), bioethanol produced through the fermentation of sugars by yeasts is accompanied with the generation of significant amounts of glycerol as a fermentation by-product of these

microorganisms. During a typical industrial bioethanol process, the ethanol is separated via distillation, and the remaining material (often called the column bottoms, vinasse, or whole stillage, depending on the feedstock) may be further processed to recover value from the by-products (Rausch and Belyea, 2006). The liquid fraction of the whole stillage (i.e. thin stillage) contains a complex mixture of chemicals, including up to 2% glycerol (Rausch and Belyea, 2006). With the tremendous growth of the bioethanol industry, current by-product glycerol generation from all U.S. bioethanol plants is comparable to the current world production of this compound (Claude et al., 2000; Zeng and Biebl, 2002). In addition to the large amounts produced by these two industries, waste streams containing high levels of glycerol are generated in almost every industry that uses animal fats and vegetable oils as the starting material (Figure 1.1). For example, waste streams of 55-90% glycerol are readily generated by the oleochemical industry and this additional surplus may further negatively affect glycerol prices (Yazdani and Gonzalez, 2007). Also of note is the fact that future opportunities for the availability of even larger amounts of glycerol also exist due to the intracellular accumulation of up to 7.8 M glycerol (equivalent to 718 g/L glycerol in water) in certain species of algae such as those in the *Dunaliella* genus (Oren, 2005). These facts, combined with the overall growth of the biofuels industry in recent years highlight the need and potential for efficient, cost effective processes for the conversion of glycerol into higher value fuels and chemicals.

The current methods for converting glycerol into more valuable products can be grouped into two main platforms: chemical and biological. The chemical

platform takes advantage of traditional chemical catalytic methods while the biological platform utilizes the biocatalytic activities of microorganisms to convert glycerol into desired products. Current chemical catalytic methods enable the conversion of glycerol into several products including hydrogen and synthesis gas (Soares et al., 2006) as well as propanediols and lower polyols (Miyazawa et al., 2007). While these chemical conversion techniques are able to produce valuable products, several disadvantages exist that make these processes industrially undesirable. Low product specificity, high operating temperatures and pressures, and the inability to use crude glycerol with high levels of contaminants are just some of the disadvantages associated with current chemical conversion techniques (Yazdani and Gonzalez, 2007). These problems lead to higher operating and production costs as well as lower product yields that effectively minimize the margins of profit for these processes. While a significant amount of research is aimed at improving the efficiency of chemical glycerol conversion (Simonetti et al., 2007), biological techniques avoid many of these disadvantages directly and provide a means of synthesizing a wide array of products and functionalities. Additionally, the current price (Figure 1.1) allows the microbial fermentation of glycerol to be very competitive with sugars (~15-25 cents/lb) used for the production of chemicals and fuels (Yazdani and Gonzalez, 2007).

In addition to the availability and low-prices of glycerol, the highly reduced nature of carbon atoms in glycerol confers an additional advantage for the microbial production of fuels and reduced chemicals when compared to the use of sugars. During microbial fermentation, the requirement of an overall redox balance within

the cell is a key factor determining the overall yields and productivities that can be achieved during the synthesis of a specific product from a given carbon source. Considering this key constraint and the fact that the conversion of glycerol into the metabolic intermediate pyruvate generates twice the number of reducing equivalents produced during the metabolism of lignocellulosic sugars such as glucose or xylose, glycerol provides the natural advantage of higher theoretical product yields for reduced chemicals and fuels (Figure 1.2).

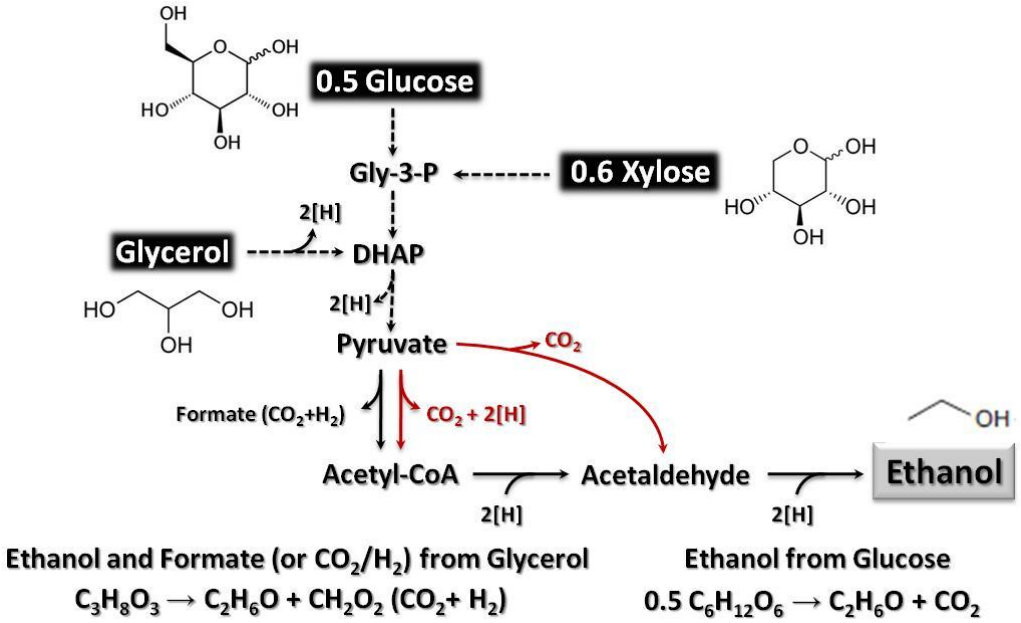

Figure 1.2 Ethanol production from glycerol, glucose, and xylose. The additional reducing equivalents generated from the conversion of glycerol into pyruvate permit the co-production of ethanol and formate (or H_2 and CO_2). The oxidation state of glucose and xylose dictates the conversion of pyruvate into ethanol must take place through the the pathways indicated in red, leading to the loss of carbon as CO_2.

For example, the additional reducing equivalents generated from the conversion of glycerol to pyruvate permits the co-production of ethanol and formic acid (or ethanol and hydrogen), the former would result in doubling the overall product

yield compared with glucose fermentation to ethanol, a process in which half of the sugar in lost as CO_2 due to the higher oxidation state of glucose (Figure 1.2). In order to fully realize these advantages, the use of fermentative (i.e. in the lack of external electron acceptors) conditions is highly desirable. In the absence of external electron acceptors, redox poise must be maintained through the terminal transfer of electrons to internally generated organic compounds (Neidhardt et al., 1996) and as such, the natural shift toward reduced products during anaerobic fermentation of glycerol provides a means of maximizing the production of reduced chemicals and fuels (Murarka et al., 2008). An additional advantage is conferred from the fact that anaerobic fermentation provides lower operating and capital costs than their aerobic counterparts, as anaerobic fermenters are less expensive to build and operate and use less energy (Yazdani and Gonzalez, 2007).

While the highly reduced nature of carbon atoms in glycerol conveys many advantages for the production of fuels and reduced chemicals via microbial fermentation, these advantages also come with considerable challenges, as the fermentative utilization of glycerol requires microorganisms with key metabolic characteristics that enable the metabolism of such a reduced carbon source. Unlike traditional carbon sources, the degree of reduction per carbon (κ, Nielsen et al., 2003), a measure of the number of available electrons per unit of carbon, of glycerol ($\kappa = 4.67$) is greater than that of cell mass ($\kappa = 4.3$), illustrating that the formation of cell mass from glycerol will result in the generation of reducing equivalents (i.e. electrons). The ability of a microorganism to maintain overall redox poise, and hence possess the capability of utilizing glycerol as a carbon source under

fermentative conditions, is directly tied to the ability to produce a product more reduced than glycerol, whose formation serves a sink for the excess reducing equivalents generated during the formation of cell mass. Therefore, while numerous microorganisms are able to utilize glycerol under respiratory conditions, few are capable of utilizing glycerol in the absence of external electron acceptors. Fermentative metabolism of glycerol has been reported for several species of the *Enterobacteriaceae* family, including *Citrobacter freundii* and *Klebsiella pneumoniae*, as well as species from the genera *Enterobacter*, *Clostridium*, *Lactobacillus*, *Bacillus*, *Propionibacterium*, and *Anaerobiospirillum* (Yazdani and Gonzalez, 2007). However, the potential for using these organisms at the industrial level could be limited due to issues that include pathogenicity, requirement of strict anaerobic conditions, need of supplementation with rich nutrients, and unavailability of the genetic tools and physiological knowledge necessary for their effective manipulation.

On the other hand, *Escherichia coli*, the workhorse of modern biotechnology, has become a promising host organism for the microbial production of renewable fuels and chemicals (Clomburg and Gonzalez, 2010). The availability of genetic tools and the large genomic, metabolic, and physiological knowledge base for *E. coli* has facilitated the ease at which this organism can be engineered as an efficient biocatalyst. In addition, *E. coli* possesses several advantageous traits for industrial biofuel and biochemical production, including showing efficient growth at industrially relevant conditions, the ability to grow in mineral salts medium with inexpensive components and in the absence of oxygen, as well as having the capability of utilizing a wide variety of substrates including main biomass

constituents. High productivities and yields of biofuels can also be attained with *E. coli* in part due to its high growth and metabolic rates, and tolerance to high concentrations of substrate and products. While both the number and productivity of compounds naturally produced by *E. coli* are limited, advances in synthetic biology, metabolic engineering, and systems biology have resulted in the ability to engineer *E. coli* to produce a wide variety of biofuels far beyond the scope of what any single organism can naturally produce (Clomburg and Gonzalez, 2010).

With this in mind, the recent discovery that *E. coli*, which was long thought to require the presence of external electron acceptors in order to utilize glycerol, is able to utilize glycerol in a fermentative manner (Dharmadi et al., 2006; Gonzalez et al., 2008; Murarka et al., 2008) holds great promise for the industrial scale conversion of glycerol into a wide variety of value added fuels and reduced chemicals. However, the complexities of cellular metabolism in general and the specific challenges associated with the fermentative utilization of glycerol necessitate a large knowledge base on the essential and important cellular pathways, mechanisms, and regulatory aspects required for the efficient utilization of glycerol as a carbon source under fermentative conditions before these promising goals can be achieved.

The overall aim of this work encompasses two main objectives, namely the understanding and harnessing of the fermentative metabolism of glycerol in *E. coli*, both essential aspects required for the use of *E. coli* with glycerol as a carbon source for the production of fuels and reduced chemicals. Specifically, the main goal of the

understanding portion of the work is the establishment of a comprehensive fundamental knowledge on the important and essential pathways, mechanisms, and regulatory aspects of fermentative glycerol metabolism in *E. coli*. Once this knowledge base and improved fundamental understanding of glycerol fermentation by *E. coli* is established, the principles and key findings will be used as a platform for rational metabolic engineering and synthetic biology strategies aimed at increasing the yields and titers of target fuels and reduced chemicals from glycerol.

The study of fermentative glycerol metabolism in *E. coli* makes use of both conceptual and mathematical modeling tools (such as *in silico* Metabolic Flux Analysis) as well as conventional experimental approaches (entailing the use of genetic and biochemical techniques to stimulate or repress the activities of postulated pathways and mechanisms) in order to analyze the importance of certain genes/enzymes/pathways on both a singular and network scale. This comprehensive investigation into the fermentative utilization of glycerol by *E. coli* elucidated several key pathways and mechanisms, including the activity of both the fermentative and respiratory glycerol dissimilation pathways, the involvement of several FBPases for the production of 6-carbon intermediates, and the CRP-cAMP regulation of the fermentative glycerol dissimilation pathway. Of central influence to many of these findings is the metabolic cycle created by the coupling of glycerol dissimilation with pyruvate synthesis through the action of PEP-dependent dihydroxyacetone kinase, the understanding of which is essential for the design of any metabolic engineering strategy geared toward fuel and chemical production from glycerol. The establishment of this fundamental understanding and knowledge

base was then applied as a platform to design and demonstrate effective strategies for the efficient production of 1,2-propanediol, ethanol, and hydrogen, showing the potential use of glycerol waste streams as a carbon source for the industrial scale production of value added fuels and reduced chemicals.

Chapter 2

Advantages and Challenges of the Use of Glycerol as a Carbon Source for the Fermentative Production of Fuels and Chemicals

While the abundance and low price of glycerol highlight the need and opportunity of its use as a carbon source, the chemical structure and composition of glycerol provides both unique advantages and challenges for its conversion into fuels and chemicals. From a biological standpoint, the high degree of reduction of carbon atoms in glycerol confers the ability to produce fuels and reduced chemicals at higher yields when compared to the use of carbohydrate based feedstocks. This advantage comes with a considerable burden however, as the fermentative utilization of glycerol requires microorganisms with key metabolic characteristics resulting in the ability to maintain redox poise during the metabolism of such a reduced carbon source. A detailed background on the basis of the advantageous nature of glycerol as well as detailed knowledge of the key traits and characteristics

enabling fermentative utilization of glycerol is critical to fully capitalize on the use of glycerol as a carbon source for fuel and chemical production and are provided in next sections.

2.1. Efficient production of reduced chemicals and fuels

The aforementioned advantages glycerol provides for the production of reduced chemicals and fuels compared to traditional carbon sources is best illustrated through the calculation of the maximum theoretical yields of a reduced product, in this case 1,2-propanediol (1,2-PDO), that can be obtained from both glycerol and glucose (Figure 2.1).

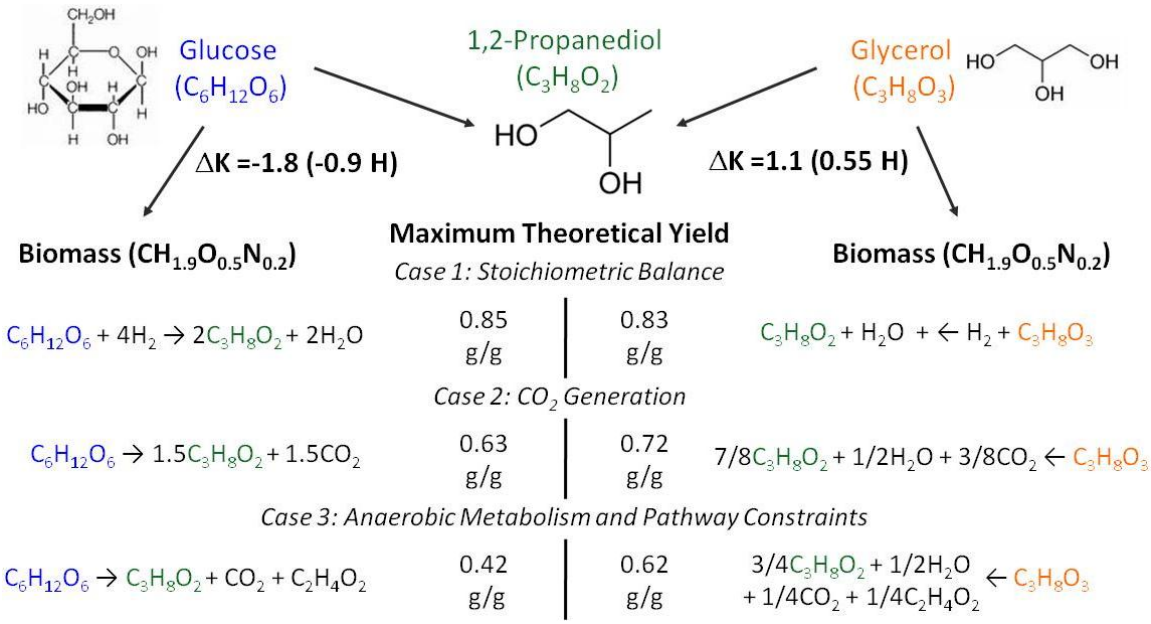

Figure 2.1 Efficient synthesis of reduced products: 1,2-propanediol yields from glycerol and glucose. The reduced nature of carbon atoms in glycerol confers the advantage of allowing higher theoretical yields than glcuose under metabolic constraints. A degree of reduction balance to biomass from each carbon source is also shown.

The best possible yield of 1,2-PDO is obtained from a simple stoichiometric balance without consideration of metabolic pathways or constraints (Figure 2.1, Case 1). In this case, the maximum yields for 1,2-PDO from both glucose and glycerol are very similar, however it is important to note that 1,2-PDO production from glucose would require the input of twice the amount of hydrogen as that needed with glycerol to achieve equal production of 1,2-PDO on a molar basis (chemical hydrogenation). However, when one considers the biological conversion of these carbon sources to 1,2-PDO the advantageous nature of glycerol is clearly seen. Under the assumption that no external source of energy is available other than the given carbon source, the maximum theoretical yield of 1,2-PDO from glycerol is clearly higher than that from glucose (Figure 2.1, Case 2). In this case, a portion of the carbon source must be converted to a more oxidized co-product, CO_2, which can provide the excess reducing equivalents and energy needed. While this balance is theoretically possible in a biological context, in reality the co-production of a highly reduced product and CO_2 would likely be a difficult task, as it would require high activity of the tricarboxylic acid (TCA) cycle for CO_2 production, a process which necessitates respiratory conditions making the production of fermentative products difficult. In the context of anaerobic metabolism and pathway constraints, which represent the most constrained process and in all likelihood the most feasible conditions for reduced product synthesis, the use of glycerol enables a 50% higher maximum theoretical yield of 1,2-PDO than that obtained from the use of glucose (Figure 2.1, Case 3). In this case, 1,2-PDO is co-produced with acetic acid and CO_2, generated from the conversion of the carbon source to acetic acid, which provide a redox

balanced process in the absence of external electron acceptors required under fermentative metabolism. Overall, this analysis illustrates the advantageous potential for the use of glycerol as a carbon source for the production of reduced chemicals and fuels at higher yields than can be obtained from traditional carbohydrate feedstocks. While this advantage is a direct result of the high degree of reduction of glycerol, this trait also results in considerable challenges for the microbial metabolism of glycerol under fermentative conditions, the understanding of which is essential for the efficient conversion of glycerol into fuels and reduced chemicals.

2.2. Fermentative utilization of glycerol by microorganisms

Although many microorganisms are able to metabolize glycerol in the presence of external electron acceptors (respiratory metabolism), few are capable of doing so under fermentative conditions (i.e. in the absence of electron acceptors) (Yazdani and Gonzalez, 2007). The main constraint for the utilization of glycerol under fermentative conditions is the highly reduced nature of carbon atoms in glycerol relative to the cell mass synthesized from the carbon source and the consequences this has on the metabolic network.

This relative degree of reduction can be quantified through a degree of reduction analysis, which is based on the fact that all carbon containing substrates can be oxidized to CO_2, and therefore all substrates and carbon containing metabolic products are in a reduced state (Nielsen et al, 2003). A particular route through the

metabolic network of a microorganism will convert the substrate to a product that may be either reduced or oxidized relative to the substrate. Since all feasible pathways must be redox neutral in an organism working at steady state in a constant environment, the net-reaction from substrate to product must be accompanied by consumption or production of a separate chemical species in which redox power is stored. Several compounds can be used to transfer redox power from one pathway to another, including NADH, NADPH, and $FADH_2$. For subsequent discussions the unit of redox power (H) will be defined as one H atom and all redox-carrying cofactors are equivalent to H_2 and carry 2 redox equivalents (H = NADH = NAPDH = $FADH_2$ = "H_2" = 2[H]). The degree of reduction per carbon (κ_i) can be calculated by defining neutral compounds for each element of interest and using these compounds to obtain the redox levels of each element (i.e O, C, N, S, and P). Selecting H_2O, CO_2, NH_3, H_2SO_4, and H_3PO_4 as redox neutral results in the elemental redox levels of O=-2, C=4, N=-3, S=6, and P=5 with a unit of redox H=1. These elemental redox levels can then be used to calculate the degree of reduction of any carbon containing compound on a 1 C-atom basis by simply determining the sum of the number of atoms of each element multiplied by their respective elemental redox level.

Using this definition and the calculated degrees of reduction of glycerol (κ = 4.67), glucose (κ = 4), and cell mass (κ = 4.3; based on average molecular formula for an *E. coli* cell of $CH_{1.9}O_{0.5}N_{0.2,}$ Nielsen et al., 2003), a quantitative comparison of the synthesis of cell mass from glycerol or glucose through a degree of reduction balance reveals the distinct differences in the formation of cell mass from either

substrate. The formation of cell mass from glucose results in a ΔK value of -1.8, indicating the net consumption of 0.9 reducing equivalents per mole of glucose converted to cell mass, while the reduced nature of glycerol results in the generation of 0.55 reducing equivalents per mole of glycerol converted to cell mass (ΔK = 1.1, Figure 2.1). It is important to note that this analysis neglects the carbon loss as 1-C metabolites during the conversion of glycerol to cell mass and as a consequence, the degree of reduction balance in this case represents the minimum amount of redox units generated.

As previously discussed, all the metabolic pathways within a microbial system must balance to form an overall redox balance. Therefore, in the lack of external electron acceptors (negating the transfer of these electrons from reducing equivalents to an externally provided acceptor, such as oxygen) any pathway that produces redox units must be accompanied by pathway(s) that consume redox units in order for the net production of redox units to equal zero (as in the steady state operation of the cell). In the case of glycerol utilization, a sink for the consumption of the reducing equivalents generated during the conversion of glycerol to cell mass must be available to maintain reox balance and allow for steady state operation of the cell. However, in order to consume these excess reducing equivalents, pathways enabling the formation of a product with a higher degree of reduction than glycerol (hence a product whose conversion from glycerol results in a net consumption of reducing equivalents) are required (Figure 2.2).

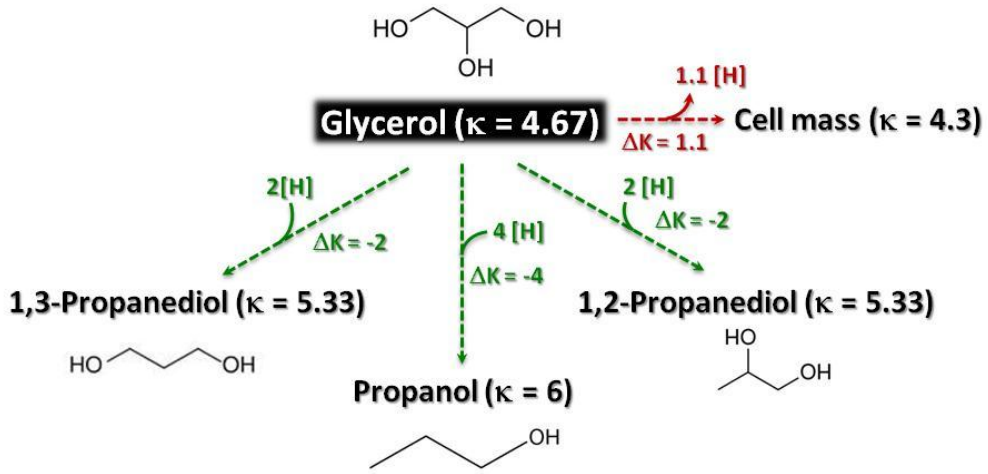

Figure 2.2 Fermentative utilization of glycerol by microorganisms The reducing equivalents generated during the formation of cell mass from glycerol (red line) must be consumed by the formation of a metabolic product more reduced than glycerol (green lines). ΔK value represents degree of reduction balance of given conversion. 2[H] = NADH/NADPH/$FADH_2$.

The capability of microorganisms to utilize glycerol as a carbon source is thus directly related to the formation of highly reduced products whose formation from glycerol result in the consumption of excess reducing equivalents.

2.3. Metabolic models for the fermentative utilization of glycerol in microorganisms

The characterization of the underlying metabolic models for the production of highly reduced products and the subsequent ability to utilize glycerol is vital for the overall understanding of glycerol metabolism in microorganisms. Models for the fermentative metabolism of glycerol encompass the genes, enzymes, and metabolic pathways that are directly linked to the ability of the microorganism to produce a highly reduced compound and utilize glycerol as a carbon source. The

current knowledge of glycerol utilization by microorganisms is very limited and few models have been previously characterized.

2.3.1. 1,3-Propanediol dependent model

The most well studied and characterized metabolic model for the fermentative utilization of glycerol is the 1,3-propanediol-dependent model in which the ability to utilize glycerol is directly linked to the synthesis of the highly reduced compound 1,3-propanediol (1,3-PDO, κ= 5.33) (Figure 2.3). The conversion of glycerol into 1,3-PDO results in the net consumption of 1 mole of reducing equivalents per mole of glycerol converted to 1,3-PDO (ΔK = -2), a pathway which serves as a sink for the excess reducing equivalents generated during the formation of cell mass.

Fermentative metabolism of glycerol in several species of the *Enterobacteriaceae* family, such as *Klebsiella* and *Citrobacter*, has been extensively studied and the fermentative utilization of glycerol has been found to be linked to the 1,3-PDO-dependent model (Bouvet et al., 1995). In these organisms, two parallel pathways, an oxidative branch and reductive branch, are responsible for the dissimilation of glycerol into glycolytic intermediates and the synthesis of 1,3-PDO in a two step conversion from glycerol (Figure 2.3) (Booth, 2005; Sun et al., 2003). The oxidative pathway dehydrogenates glycerol to dihydroxyacetone (DHA) through the action of a NAD-linked glycerol dehydrogenase, which is then phosphorylated by a PEP (phosphoenolpyruvate)- or ATP-dependent DHA kinase (Bouvet et al., 1994; Daniel et al., 1995; Sun et al., 2003). The phosphorylated

product, DHA-phosphate, can then enter glycolysis enabling the formation of essential metabolic intermediates and major fermentation products. The parallel reductive pathway dehydrates glycerol through the action of a coenzyme B12-dependent glycerol dehydratase to form 3-hydroxypropionaldehyde. 1,3-propanediol is then formed by the reduction of 3-hydroxypropionaldehyde through NADH-linked 1,3-PDO dehydrogenase (Bouvet et al., 1994; Sun et al., 2003).

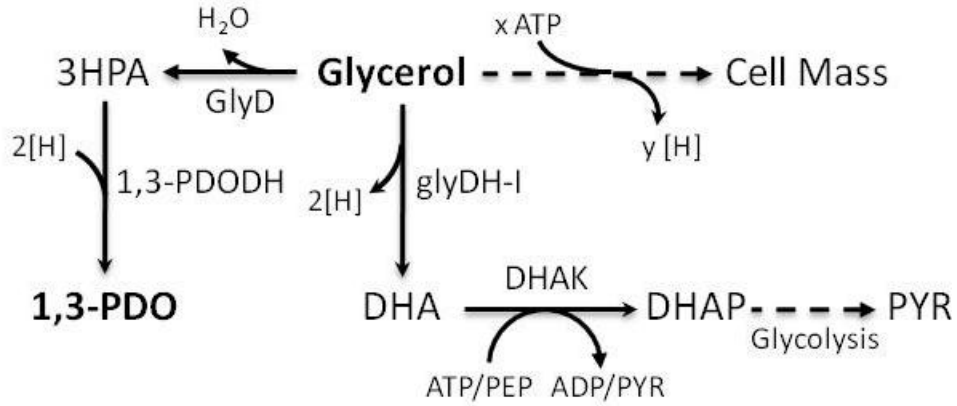

Figure 2.3 Current model for 1,3-PDO-dependent fermentative metabolism of glycerol in species of the *Enterobacteriaceae* family. Abbreviations: DHA, dihydroxyacetone; DHAK, DHA kinase; DHAP, DHA phosphate; GlyD, glycerol dehydratase; glyDH-I, glycerol dehydrogenase type I; PEP, phosphoenolpyruvate: PYR, pyruvate; 1,3-PDO, 1,3-propanediol; 1,3-PDODH, 1,3-PDO dehydrogenase; 3HPA, 3-hydroxypropionaldehyde; 2[H] = NADH/NADPH/$FADH_2$**.**

The reductive branch therefore consumes NADH released by the formation of cell mass and regenerates NAD^+, thus providing a means for the cell to achieve a redox balance in the absence of external electron acceptors. The four key enzymes in this pathway are encoded by the *dha* regulon, the expression of which is induced when DHA or glycerol is present (Forage and Lin, 1982). It has been reported that only eight taxa of the *Enterobacteriaceae* (out of 1123 strains from 128 taxa tested) grow

fermentatively on glycerol and all of these produce 1,3-PDO and possess both type I glycerol dehydrogenase and 1,3 propanediol dehydrogenase (Bouvet et al., 1995). This provides further evidence that the ability to produce 1,3-PDO, a product more reduced than glycerol, enables the utilization of glycerol as a carbon source under fermentative conditions. In addition to *Klebsiella* and *Citrobacter*, species from the genera *Enterobacter* (Bouvet et al., 1995), *Clostridium* (Luers et al., 1997; Macis et al., 1998), *Lactobacillus* (Talarico et al., 1990), and *Bacillus* (Volsenet, 1914, 1918) have also been reported to ferment glycerol in a 1,3-PDO-dependent manner.

2.3.2. 1,2-Propanediol-ethanol dependent model

The absence of 1,3-PDO producing capability was previously thought to confine microorganisms to the use of glycerol only in the presence of external electron acceptors (Booth, 2005, Bouvet et al., 1995). However, recent studies have reported fermentative glycerol utilization in *Escherichia coli* (Dharmadi et al., 2006) and *Paenibacillus macerans* (Gupta et al., 2009), microorganisms for which no 1,3-PDO production capability has been found. In these organisms, the ability to utilize glycerol under fermentative conditions is linked to the synthesis of 1,2-propanediol (1,2-PDO) and ethanol as a means of facilitating redox balance and ATP generation, respectively (Figure 2.4) (Gonzalez et al., 2008; Gupta et al., 2009). In this model, glycerol fermentation is enabled by the production of 1,2-PDO which provides the capacity to dispose of excess reducing equivalents generated during the incorporation of glycerol into cell mass, as the conversion of glycerol to 1,2-PDO results in the net consumption of 1 mole of reducing equivalents per mole of

glycerol converted to 1,2-PDO (ΔK = -2). In addition, ethanol production is required as a means of ATP generation, as the pathway from glycerol to ethanol (and formate) represents a redox balanced ATP generating pathway.

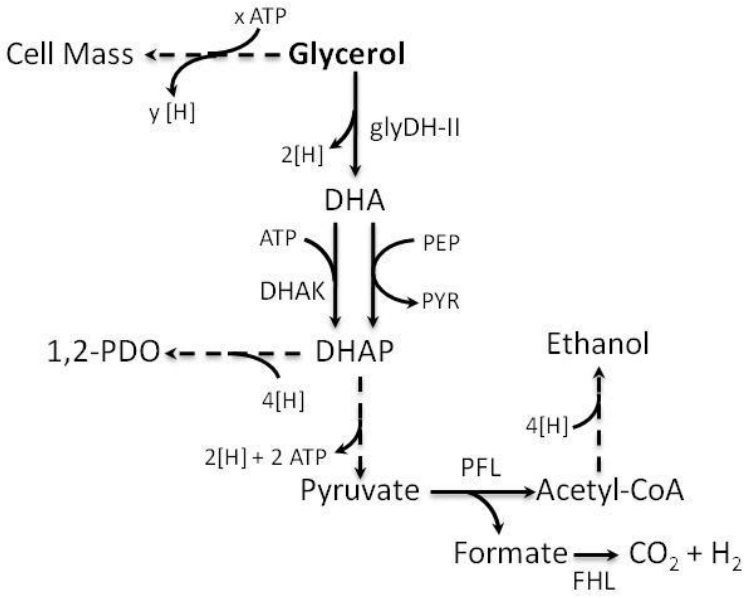

Figure 2.4 1,2-PDO-ethanol dependent model for fermentative glycerol utilization. Dashed lines indicate multiple steps. Abbreviations: DHA, dihydroxyacetone; DHAK, dihydroxyacetone kinase; DHAP, dihydroxyacetone phosphate; FHL, formate hydrogen lyase; glyDH-II, type II glycerol dehydrogenase; PEP, phosphoenolpyruvate; PFL, pyruvate formate-lyase; PEP, phosphoenolpyruvate; PYR, pyruvate; 2[H] = NADH/NADPH/$FADH_2$.

This newly characterized model consists of an oxidative, type II glycerol dehydrogenase (glyDH-II) and PEP- or ATP-dependent dihydroxyacetone kinase (DHAK) mediated pathway working in partnership with a reductive 1,2-PDO-producing pathway (Figure 2.4) (Gonzalez et al., 2008; Gupta et al., 2009). The oxidative branch results in the conversion of glycerol into the glycolytic intermediate DHAP, a key node for both the reductive branch and ethanol synthesis (Figure 2.4). The reductive 1,2-PDO pathway mediates the reduction of DHAP into

1,2-PDO through pathways associated with methylglyoxal synthesis and detoxification (Booth, 2005), while common glycolytic enzymes can also convert DHAP into pyruvate (Romeo and Snoep, 2005), for subsequent conversion to ethanol. Glycerol fermentation by both *Escherichia coli* (Gonzalez et al., 2008) and *Paenibacillus macerans* (Gupta et al., 2009) have been shown to proceed via this model, and both show near homoethanologenic fermentation of glycerol with 1,2-PDO also identified as a required fermentation product.

2.3.3. Other models for the fermentative utilization of glycerol

In addition to microorganisms whose ability to ferment glycerol has been linked to the two previously discussed models, a few other organisms have been shown to possess the ability to utilize glycerol fermentatively with no reported propanediol producing capability. *Propionibacteria acidipropionici* and *Propionibacteria freudenreichii* ssp. *shermanii* were used in the effective production of propionic acid from glycerol (Bories et al., 2004) and the conversion of glycerol into succinic acid using *Anaerobiospirillum succiniproducens* was also reported (Lee et al., 2001). These microorganisms are strict anaerobes (i.e. require anaerobic conditions for growth) for which no 1,3- or 1,2-PDO producing capability has been reported. Despite the fact that these organisms have been shown to utilize glycerol under anaerobic conditions, the mechanisms and pathways mediating the dissimilation of glycerol have not been investigated. However, the fact that glycerol can be utilized fermentatively with no 1,3- or 1,2-PDO production provides evidence that other models enabling the glycerol fermentation exist. While the reduced

product(s) and pathways for its formation remain unclear in the case of *Anaerobiospirillum succiniproducens,* the formation of propanol by *Propionibacteria* during glycerol fermentation has been reported (Barbirato et al., 1997b; Bories et al., 2004), providing some insight in the metabolic model by which these organisms are able ferment glycerol (Figure 2.5).

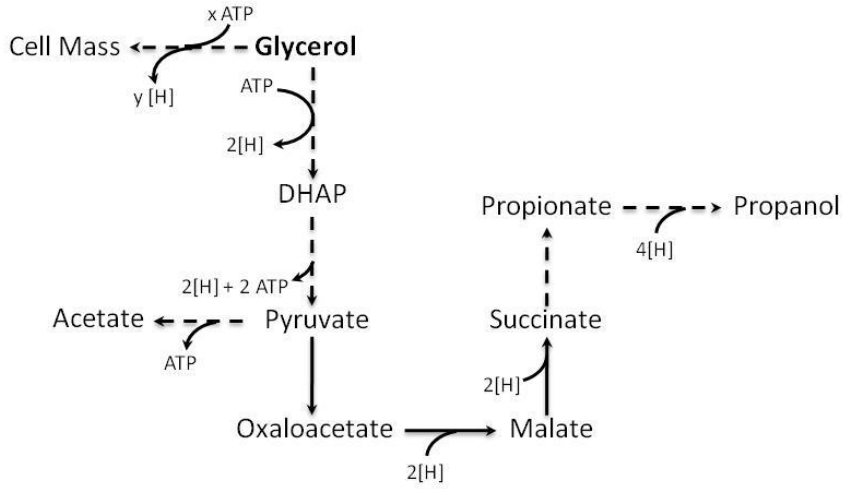

Figure 2.5 Proposed propanol-dependent model for fermentative glycerol utilization Dashed lines indicate multiple or proposed steps. Abbreviations: DHAP, dihydroxyacetone phosphate; 2[H] = NADH/NADPH/$FADH_2$.

In this proposed metabolic model, the formation of the highly produced compound propanol, whose synthesis from glycerol results in the net consumption of 2 mole of reducing equivalents per mole of glycerol converted to 1,2-PDO ($\Delta K = -4$), through the reduction of the major fermentation product propionic acid would enable glycerol fermentation by providing a sink for the excess reducing equivalents generated during the formation of cell mass from glycerol (Figure 2.5). Evidence of enabling nature of propanol production for glycerol fermentation in *Propionibacteria* is provided by the fact that while propionic acid was the major

fermentation product of *Propionibacterium acidipropionici* with glycerol, glucose, and lactic acid as carbon sources, propanol was only observed when glycerol was used as the carbon source (Barbirato et al., 1997b; Bories et al., 2004).

2.4. Glycerol metabolism in *Escherichia coli*

Glycerol utilization in *E. coli* was long thought to be limited to respiratory conditions requiring the presence of external electron acceptors (Booth, 2005; Lin, 1976; Quastel et al., 1925; Quastel and Stephenson, 1925). As a result, the majority of knowledge of glycerol metabolism is restricted to pathways and enzymes required under respiratory conditions, which may or may not play an important role under fermentative conditions. The discovery that *E. coli* is indeed able to ferment glycerol via the 1,2-PDO-ethanol model (Gonzalez et al., 2008; Murarka et al., 2008) represents a significant breakthrough, as *E. coli* has been used extensively for the production of numerous fuels and chemicals and the familiarity with the organism allows for efficient metabolic engineering strategies to be implemented with ease. Thus these studies created a starting point for the study of fermentative glycerol metabolism and established key environmental conditions and several important pathways essential for glycerol fermentation (Gonzalez et al., 2008; Murarka et al., 2008).

2.4.1. Respiratory metabolism of glycerol

Utilization of glycerol in *E. coli* was long thought to require the presence of external electron acceptors, and respiratory glycerol metabolism has been

extensively studied (Booth, 2005; Lin, 1976; Quastel et al., 1925; Quastel and Stephenson, 1925). Glycerol enters the cytoplasm through the glycerol diffusion factor (GlpF, coded by *glpF*) at which point an ATP-dependent glycerol kinase (GK, encoded by *glpK*) catalyzes the transfer of a phosphate group to form glycerol-3-phosphate (G3P) (Figure 2.6). Two respiratory, membrane-bound G3P dehydrogenases (G3PDH) are then available to convert G3P into dihydroxyacetone phosphate (DHAP) with the generation of a reducing equivalent. Aerobic G3PDH (encoded by *glpD*) accepts either oxygen or nitrate as the electron acceptor, while anaerobic G3PDH is coupled to electron acceptors other than oxygen. DHAP can then enter glycolysis and proceed down the well documented pathway to form pyruvate.

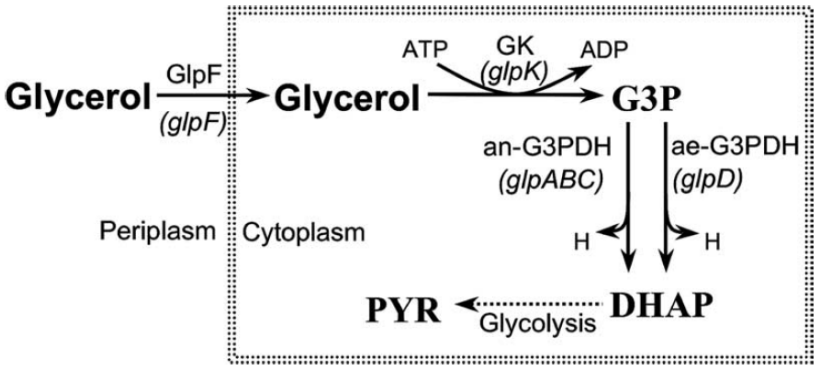

Figure 2.6 Respiratory metabolism of glycerol in *E. coli* (Murarka et al., 2008). Glycerol dissimilation in the presence of electron acceptors is mediated by an ATP-dependent glycerol kinase (GK, encoded by *glpK*)and two respiratory glycerol 3-phosphate dehydrogenases (aerobic and anaerobic enzymes, encoded by *glpD* and *glpABC*, respectively). Abbreviations: DHAP, dihydroxyacetone phosphate; GK, glycerol kinase; ae-G3PDH, aerobic G3PDH; an-G3PDH, anaerobic G3PDH; H, reducing equivalents (H=NADH/NADPH/$FADH_2$); PYR, pyruvate.

Glycerol kinase is known to be the rate-limiting step in glycerol utilization by *E. coli*, and its activity is controlled through feedback inhibition by fructose-1,6-bisphosphate (Lin, 1976). Previous studies have also shown the existence of an alternative pathway for glycerol dissimilation (Jin et al., 1983). This pathway is composed of an NAD-linked type II glycerol dehydrogenase (glyDH-II, encoded by *gldA*) that converts glycerol to dihydroxyacetone (DHA) and a PEP-dependent dihydroxyacetone kinase (DHAK) which converts DHA into DHA-phosphate. This alternative pathway was cryptic in wild-type *E. coli* and activated by the disruption of *glpK*, *glpR*, and *glpD* genes, followed by a mutagenesis and selection process (Jin et al., 1983). The metabolism of glycerol in the resulting strain still required oxygen as an electron acceptor. Additionally, *gldA* is thought to be cryptic in wild-type cells and is only activated through mutation. It is also important to note that neither glyDH-II nor DHAK has been previously shown to be involved in glycerol metabolism in wild-type *E. coli* and their true physiological role is uncertain.

2.4.2. Fermentative metabolism of glycerol

In comparison to respiratory glycerol metabolism, the understanding of the ability for *E. coli* to utilize glycerol in the absence of external electron acceptors, a metabolic capability only recently discovered, and the associated complexities of fermentative glycerol metabolism are still in their relative infancy. However, in addition to determining the model by which *E. coli* ferments glycerol (1,2-PDO-ethanol dependent model), recent studies have established both key environmental conditions that enable glycerol fermentation as well as several pathways and

mechanisms essential during fermentative glycerol metabolism in *E. coli* (Dharmadi et al., 2006; Gonzalez et al., 2008; Murarka et al., 2008). The findings of these studies highlight the narrow range of conditions which enable glycerol fermentation in *E. coli*, which are attributed to the sensitivities of key pathways and mechanisms to environmental factors.

2.4.2.1. Environmental factors influencing glycerol fermentation

Due to the fact that the discovery of the metabolic capability for *E. coli* to utilize glycerol under fermentative conditions was in direct contradiction to previous reports, the establishment of the key differences in the environmental factors and their influence on glycerol fermentation in *E. coli* was critical to understanding why the potential for glycerol fermentation in *E. coli* had been undiscovered. Previous studies into glycerol metabolism in *E. coli* used media containing high levels of Na^+, K^+, and PO_4^{3-} (Bouvet et al., 1995; Freedberg et al., 1971; Richey and Lin, 1972; Sprenger et al., 1989; St Martin et al., 1977; Tanaka et al., 1967; Tang et al., 1982a; Tang et al., 1982b; Zwaig et al., 1970). For example, the medium reported by Tanaka et al. (1967) contained 34 mM Na_2HPO_4 and 64 mM K_2HPO_4 whereas the minimal media for *Enterobacteriaceae* defined by Neidhardt et al. (1974) contains only 1.32 mM Na_2HPO_4. In these prior studies, PO_4^{3-} was used to control the pH of the culture, which ranged from 7 to 7.5. Gonzalez et al. (2008) showed that supplementation of the Neidhardt et al. (1974) media with similar levels of Na^+, K^+, and PO_4^{3-} severely impaired glycerol fermentation at a pH of 7.5. These effects were found to be caused by K^+ and PO_4^{3-}, as supplementation with only

high levels of Na^+ did not impair glycerol fermentation, while supplementation with high levels of either K^+ or PO_4^{3-} had significant effects. Additionally, glycerol fermentation was found to be significantly diminished at alkaline pH (such as the pH of previous studies) even at low K^+ and PO_4^{3-} concentrations, and a detailed study of the effect of pH showed that glycerol fermentation proceeded within a narrow pH range, with an optimal pH of 6.3 (Murarka et al., 2008).

Another critical factor that may have impaired glycerol fermentation in previous studies was the use of low initial concentrations of glycerol in the media, which was typically 20-30 mM. The use of initial glycerol concentrations in this range with high levels of K^+ and PO_4^{3-} did not support cell growth or glycerol fermentation at pH 7.5 (Gonzalez et al., 2008). Further evidence that low initial concentrations of glycerol do not support glycerol fermentation is the characteristic that significant amounts of glycerol (typically 10-30 mM) are left unmetabolized in the medium after stationary phase had been reached, which suggests a threshold concentration limiting the process (Gonzalez et al., 2008).

In addition to the critical elements of the media formulation, an additional environmental factor found to be significant during glycerol fermentation was the detrimental impact of the accumulation of fermentation gases. The accumulation of H_2, co-produced along with CO_2 during the oxidation of formate by the formate hydrogen lyase (FHL) complex, was shown to significantly impair glycerol fermentation (Murarka et al., 2008). This detrimental impact is related to the fact that under fermentative conditions, the hydrogenase isoenzyme 1 (Hyd-1) or 2

(Hyd-2) can serve the function of recycling the hydrogen evolved by the FHL complex (Clark, 1989; Sawers and Boxer, 1986; Sawers et al., 1985; Sawers et al., 2004). In the absence of external electron acceptors, the hydrogen utilized through the action of these hydrogenases can be channeled to internally generated fumarate via the quinone pool and then readily used as an electron donor in the reduction of fumarate to succinate by furmarate reductase (FRD, encoded by *frdABCD*) (Sawers et al., 2004). Considering that the conversion of glycerol to succinate is a redox balanced process, the use of hydrogen as an electron donor creates an excess of reducing equivalents which cannot be consumed by any other pathway, creating a redox imbalance that severely impairs glycerol fermentation (Murarka et al., 2008). Further evidence to this point was provided by the fact that a Δ*frdA* mutant, lacking a functional fumurate reductase and the ability to reduce fumarate to succinate, exhibited cell growth and glycerol fermentation levels in the presence of hydrogen similar to that of wild-type cells in the presence of argon with continuous sparging (Murarka et al., 2008). Similar behavior was also observed during growth of the Δ*frdA* mutant in closed vessels (Murarka et al. 2008). These findings were especially pertinent considering the fact that previous studies into glycerol fermentation utilized closed vessels in which the accumulation of fermentation gases, specifically hydrogen, could also have been a major environmental factor resulting in the inability for *E. coli* to ferment glycerol.

2.4.2.2. Proposed model for glycerol fermentation by *Escherichia coli*

The fermentative metabolism of glycerol in *Enterobacteriaceae* was long thought to require the ability to produce 1,3-propanediol. However, as previously discussed, the ability for *E. coli* (which has no 1,3-PDO producing capability) to ferment glycerol was recently established and found to be linked to the 1,2-PDO-ethanol metabolic model for fermentative glycerol utilization (Gonzalez et al., 2008). In addition, several important pathways and mechanisms were determined (Murarka et al., 2008; Gonzalez et al., 2008), which serve as the initial knowledge base into the overall understanding of fermentative glycerol metabolism in *E. coli*. Several of these pathways and mechanisms are closely tied to the established key environmental factors, as several of these factors have been shown to negatively influence the pathways and mechanisms in the context of glycerol fermentation.

The aforementioned 1,2-PDO-ethanol dependent metabolic model enabling glycerol fermentation in *E. coli* consists of an oxidative, type II glycerol dehydrogenase (glyDH, encoded by *gldA*) and a PEP-dependent dihydroxyacetone kinase (DHAK, encoded by *dhaKLM*) mediated pathway working in partnership with a reductive 1,2-PDO-producing pathway (Figure 2.7) (Gonzalez et al., 2008). In addition to the oxidative and reductive branches required for glycerol fermentation, an active formate hydrogen lyase (FHL) complex was essential and ethanol production during glycerol fermentation was required, as a Δ*adhE* mutant lacking the alcohol dehydrogenase (ADH) responsible for ethanol production resulted in the inability to ferment glycerol (Murarka et al., 2008).

The oxidative pathway results in the conversion of glycerol into the glycolytic intermediate dihydroxyacetone phosphate (DHAP), in a two-step pathway mediated by glyDH and DHAK with dihydroxyacetone (DHA) as the pathway intermediate (Figure 2.7). The central role of the glyDH-DHAK pathway mediating the generation of the glycolytic intermediate DHAP is supported by several direct and indirect experimental findings. The inability for glyDH-deficient mutants (Δ*gldA*) to ferment glycerol and the impairment and subsequent plasmid complemented restoration of glycerol fermentation in DHAK-deficient strains (Δ*dhaK*) provide direct evidence of the requirement of this proposed pathway (Gonzalez et al., 2008).

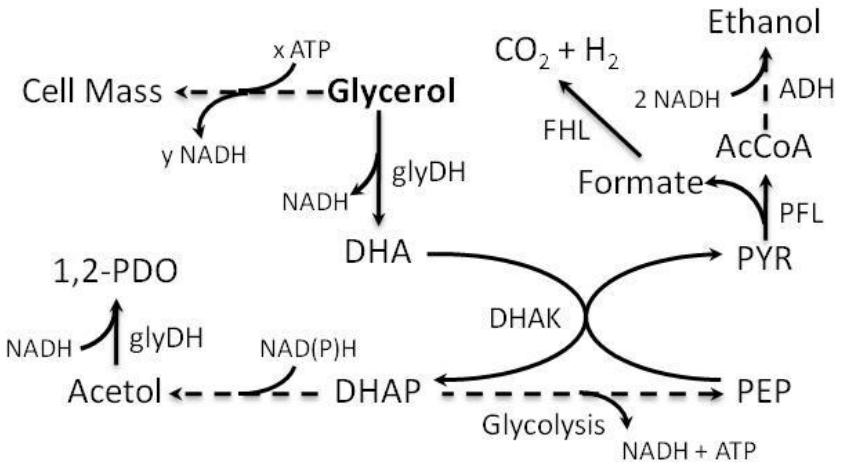

Figure 2.7 Current state of knowledge of glycerol fermentation by *Escherichia coli*. In the absence of external electron acceptors, glycerol utilization is mediated by an oxidative glycerol dehydrogenase (glyDH, encoded by *gldA*)-PEP-dependent dihydroxyacetone kinase (DHAK, encoded by *dhaKLM*) pathway working in combination with a reductive 1,2-propandeiol (1,2-PDO) producing pathway. An active formate hydrogen-lyase (FHL) complex and ethanol production are also required for glycerol fermentation. Abbreviations: AcCoA, acetyl-CoA; ADH, aldehyde/alochol dehydrogenase; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; PFL, pyruvate formate-lyase; PYR, pyruvate.

Additionally, the existence of a threshold concentration of glycerol limiting its fermentation provides inferred evidence of the activity of glyDH for the conversion of glycerol into DHA. The existence of this threshold concentration indicates the involvement of a low affinity pathway such as that mediated by the *gldA* encoded glyDH which exhibits K_M values for glycerol ranging from 3-40 mM (Truniger and Boos, 1994). One intriguing aspect of the essential activity of this pathway in *E. coli* is the fact that while these two enzymes have been characterized (Gutknecht et al., 2001; Paulsen et al., 2000; Truniger and Boos, 1994), their physiological roles are uncertain, and in the case of the glyDH encoded by *gldA*, was thought be cryptic in wild-type cells and not essential to any function (Liyanage et al., 2001; Truniger and Boos, 1994).

The reductive pathway of the model provides a means of consuming excess reducing equivalents generated by the formation of cell mass through the synthesis of the highly reduced compound 1,2-PDO. This process consists of several pathways that are also associated with methylglyoxal (MG) synthesis and detoxification in *E. coli* (Booth, 2005). The glycolytic intermediate DHAP is first converted into MG by MG synthase, which is then converted into hydroxyacetone (HA, also known as acetol) by an aldo-keto reductase (AKR). The final step is the conversion of HA into 1,2-PDO mediated by a glycerol dehydrogenase (encoded by *gldA*) (Figure 2.7). Several key pieces of evidence support this pathway as both the enabling factor for acquiring redox balance during glycerol fermentation and the preferred route from MG to 1,2-PDO (Multiple routes from MG to 1,2-PDO exist, Booth, 2005; Subedi et al., 2008). The decrease in both cell growth and glycerol fermented accompanied by

the decrease in 1,2-PDO production in mutants deficient in *mgsA*, as well as those deficient in the AKRs encoded by the *yafB*, *yeaE*, and *yghZ* genes, provides evidence to the hypothesis that 1,2-PDO production is essential for fermentative glycerol utilization (Gonzalez et al., 2008). The identification of activities for both an aldo-keto reductase (AKR) converting MG to HA and glycerol dehydrogenase converting HA to 1,2-PDO as well as the lack of a MG reductase activity (which catalyzes the conversion of MG into lactaldehyde) provided direct evidence that the HA serves as the intermediate between MG and 1,2-PDO (Gonzalez et al., 2008). This was further supported by the decrease of 1,2-PDO production seen by Δ*yafB*, Δ*yeaE*, and Δ*yghZ* strains and the wild-type 1,2-PDO production levels seen in Δ*fucO* (converts lactaldehyde to 1,2-PDO) strains (Gonzalez et al., 2008).

In addition to the oxidative and reductive pathways required for the fermentative utilization of glycerol, several other important mechanisms were found to play a critical role in the ability for *E. coli* to ferment glycerol. The FHL mediated conversion of formate into CO_2 and H_2 and the activity of the F_0F_1-ATPase system, two systems inherently linked due to the ATPase requirement for FHL activity (Bagramyan et al., 2002; Bagramyan and Trchounian, 2003; Hakobyan et al., 2005), are also required for glycerol fermentation (Gonzalez et al., 2008). These systems may be important to supply CO_2 for cell growth and maintain pH homeostasis. Under fermentative conditions the disproportionation of formate into CO_2 and H_2, catalyzed by FHL, is the main source of CO_2 (Dharmadi et al., 2006). The generation of CO_2 by dehydrogenases, such as the pyruvate dehydrogenase complex (PDH), 2-oxoglutarate dehydrogenase, isocitrate dehydrogenase, and 6-

phosphogluconate dehydrogenase, is constrained by the need to recycle the NADH that is also produced by these enzymes. This is especially relevant considering that the large number of reducing equivalents generated due to the highly reduced nature of glycerol is already a constraint for the fermentative utilization of glycerol. Therefore, any CO_2 required for cell growth needs to come from a source which minimizes the generation of reducing equivalents, such as that provided by the FHL complex. Transcription of FHL occurs only during fermentative growth and is dependent on acidic pH and formate availability (Sawers et al., 2004). This is supported by the fact that glycerol fermentation was impaired at alkaline pH under which negligible FHL activity would be expected and also impaired in FHL-deficient strains (Gonzalez et al., 2008). Glycerol fermentation was improved in both acidic and basic pH for both wild-type and FHL-deficient strains when the atmosphere was CO_2 enriched, providing evidence that CO_2 needed for cell growth is a key component of the active FHL requirement (Gonzalez et al., 2008). These systems may also play an important role in maintaining pH homeostasis which is vital for the ability to fermentatively utilize glycerol in *E. coli*.

Another key component of glycerol fermentation in *E. coli* was the requirement for ethanol production, as an aldehyde/alcohol dehydrogenase (encoded by *adhE*) deficient mutant (Δ*adhE*) was unable to ferment glycerol and complementation of this strain with an *adhE* expressing plasmid restored the ability to grow on glycerol under fermentative conditions (Murarka et al., 2008). The requirement for ethanol production, as well as the near homoethanologenic nature of glycerol fermentation (Dharmadi et al., 2006), is a result of the highly reduced

nature of carbon atoms of glycerol. The conversion of glycerol to ethanol and formate (or CO_2/H_2) represents a redox balanced pathway that generates ATP. While the production of succinate from glycerol also represents a redox balanced pathway, its contribution to ATP generation is very limited (Unden and Kleefeld, 2004), and the production of all other major fermentation products (acetate, lactate) from glycerol result in the generation of reducing equivalents. Therefore, in the lack of external electron acceptors, ethanol is the only major fermentation product whose production from glycerol does not generate reducing equivalents and generates ATP via substrate level phosphorylation, satisfying two of the main constraints under fermentative conditions.

Overall, the key pathways and mechanisms for fermentative glycerol utilization in *E. coli* that have been elucidated thus far fit nicely in the context of the environmental factors influencing glycerol fermentation (Section 2.4.2.1). The observed changes in glycerol fermentation upon alterations in pH and concentrations of potassium, phosphate, and glycerol are linked to the sensitivity of several key components to these environmental factors. High levels of phosphate promote decomposition of both DHA and HA and negatively affect glyDH activity as well as its inducibility by HA (Truniger and Boos, 1994). Additionally, MG synthase, a key enzyme for 1,2-PDO synthesis, is inhibited by high phosphate levels (Cooper, 1984; Hopper and Cooper, 1971; Zhu et al., 2001). High concentrations of potassium, especially at alkaline pH, increase the toxicity of MG, a key intermediate in the synthesis of 1,2-PDO (Booth, 2005). The central role of glyDH and its low affinity for glycerol agree with both the requirement of high concentration of

glycerol and the observation that 10-30 mM glycerol remain unmetabolized in the medium. glyDH also exhibits strong pH dependence with higher oxidative activity at very alkaline pH and reductive activity at neutral to alkaline conditions (Gonzalez et al., 2008). Acidic conditions reduce not only the activity of glycerol dehydrogenase, but also MG-reducing activities, which are required for the synthesis of 1,2-PDO (Gonzalez et al., 2008). On the other hand, alkaline conditions increase the toxicity of MG (Booth, 2005). The situation becomes even more complicated by the fact that FHL, which has been shown to be active only at acidic pH (Self et al., 2004), is required for glycerol fermentation. Therefore, it is obvious that the intracellular pH needs to be carefully controlled to avoid MG toxicity and low activities of these key enzymes. This helps to explain the narrow pH range at which glycerol fermentation optimally proceeds. Under alkaline conditions, the F_0F_1-ATPase system may be required for preventing alkalinization of the cytoplasm helping to avoid MG toxicity. The combination of the sensitivities of the key components to environmental factors leads to a complex network of interactions during glycerol fermentation. For example, while an intracellular pH of 6.3 prevents MG toxicity, the cells still require an active FHL system to prevent the pH from falling below levels permissible for glycerol dehydrogenase and MGR/AKR activities. However, FHL activity could generate excess hydrogen that if accumulated would negatively affect glycerol fermentation. These sensitivities of key components to environmental factors result in the narrow range of conditions under which fermentative utilization of glycerol is observed.

This inherent complexity and sensitivity highlights the challenges associated with the use of glycerol as a carbon source under fermentative conditions. While these recent studies demonstrate the potential for the conversion of glycerol into value added products, they also underscore the relative lack of fundamental knowledge of fermentative glycerol metabolism. In order to unlock the full potential for the use of glycerol as carbon source, an improved understanding of fermentative glycerol metabolism in *E. coli* is required, which can provide the key knowledge base required for the design of metabolic engineering strategies for the efficient production of fuels and reduced chemicals from glycerol.

Chapter 3

Materials and Methods

3.1. Strains, plasmids, and genetic methods

For experiments involved in the study of fermentative glycerol metabolism, wild-type *Escherichia coli* K12 strain BW25113 and single gene knockout mutants obtained from the National BioResource Project (NIG, Japan) (Baba et al., 2006) were used. For the implementation of metabolic engineering strategies for reduced chemical and fuel production, wild-type K12 *Escherichia coli* strain MG1655 (Kang et al., 2004) was used as the host. Gene knockouts were introduced in BW25113, MG1655, and their derivatives by P1 phage transduction (Miller, 1972; Yazdani and Gonzalez, 2008). Single gene knockout mutants from the National BioResource Project were used as donors of specific mutations, except for the deletion of the *ackA-pta* operon, which was created by a previously reported method (Metcalf et al., 1996). All wild-type and constructed strains are listed in Table 3.1. All mutations,

both in single gene deletion strains obtained from the National BioResource Project as well as those constructed via P1 phage transduction, were confirmed by polymerase chain reaction using the "verification" primers listed in the Appendix. The disruption of multiple genes in a common host was achieved as previously described (Yazdani and Gonzalez, 2008).

Table 3.1 Wild type and constructed strains used in this study

Strain	Genotype/Description	Source
BW25113	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph*-1	Baba et al. (2006)
BW25113 Δ*glpK*Δ*glpA*	BW25113, Δ*glpK*::*FRT* Δ*glpA FRT-Kan-FRT;* sequential deletion of *glpK* and *glpA* genes	This Study
BW25113 Δ*fsaA*Δ*fsaB*	BW25113, Δ*fsaA*::*FRT* Δ*fsaB FRT-Kan-FRT;* sequential deletion of *fsaA* and *fsaB* genes	This Study
BW25113 Δ*pykA*Δ*pykF*	BW25113, Δ*pykA*::*FRT* Δ*pykF FRT-Kan-FRT;* sequential deletion of *pykA* and *pykF* genes	This Study
BW25113 Δ*glpK*Δ*pps*	BW25113, Δ*glpK*::*FRT* Δ*pps FRT-Kan-FRT;* sequential deletion of *glpK* and *pps* genes	This Study
BW25113 Δ*frdA*Δ*pps*	BW25113, Δ*frdA*:*FRT* Δ*pps FRT-Kan-FRT;* sequential deletion of *frdA* and *pps* genes	This Study
MG1655	F- λ- *ilvG- rfb*-50-*rph*-1	Kang et al. (2004)
MG1655 Δ*dhaK*	MG1655, Δ*dhaK*::*FRT-Km-FRT*; deletion mutant for *dhaK* gene	This Study
MG1655 Δ*gloA*	MG1655, Δ*gloA*::*FRT-Km-FRT*; deletion mutant for *gloA* gene	This Study
MG1655 Δ*aldA*	MG1655, Δ*aldA*::*FRT-Km-FRT*; deletion mutant for *aldA* gene	This Study
MG1655 Δ*frdA*	MG1655, Δ*frdA*::*FRT-Km-FRT*; deletion mutant for *frdA* gene	This Study
MG1655 Δ*ackA-pta*	MG1655 Δ*ackA-pta*::*FRT*; deletion mutant for *ackA-pta* operon	This Study
MG1655 Δ*ldhA*	MG1655, Δ*ldhA*::*FRT-Km-FRT*; deletion mutant for *ldhA* gene	This Study
MG1655 Δ*adhE*	MG1655, Δ*adhE*::*FRT-Km-FRT*; deletion mutant for *adhE* gene	This Study
MG1655 Δ*ackA-pta* Δ*ldhA*Δ*dhaK*	MG1655, Δ*ackA-pta*::*FRT* Δ*ldhA*::*FRT* Δ*dhaK*::*FRT-Kan-FRT;* sequential deletion of *ackA-pta*, *ldhA* and *dhaK*	This Study
MG1655 Δ*ackA-pta* Δ*ldhA*Δ*dhaK*Δ*aceF*	MG1655, Δ*ackA-pta*::*FRT* Δ*ldhA*::*FRT* Δ*dhaK*::*FRT* Δ*aceF*::*FRT-Kan-FRT;* sequential deletion of *ackA-pta*, *ldhA*, *dhaK*, and *aceF*	This Study
MG1655 Δ*ackA-pta* Δ*ldhA*Δ*dhaK*Δ*fdhF*	MG1655, Δ*ackA-pta*::*FRT* Δ*ldhA*::*FRT* Δ*dhaK*::*FRT* Δ*fdhF*::*FRT-Kan-FRT;* sequential deletion of *ackA-pta*, *ldhA*, *dhaK*, and *fdhF*	This Study
MG1655 Δ*ackA-pta* Δ*ldhA*Δ*dhaK* Δ*aceF*Δ*fdhF*	MG1655, Δ*ackA-pta*::*FRT* Δ*ldhA*::*FRT* Δ*dhaK*::*FRT* Δ*aceF*::*FRT* Δ*fdhF*::*FRT-Kan-FRT;* sequential deletion of *ackA-pta*, *ldhA*, *dhaK*, *aceF*, and *fdhF*	This Study
MG1655 Δ*ackA-pta* Δ*ldhA*Δ*dhaK*Δ*frdA*	MG1655, Δ*ackA-pta*::*FRT* Δ*ldhA*::*FRT* Δ*dhaK*::*FRT* Δ*frdA*::*FRT-Kan-FRT;* sequential deletion of *ackA-pta*, *ldhA*, *dhaK*, and *frdA*	This Study

All plasmids used in this study are listed in Table 3.2 and the primers used for their construction are listed in the Appendix. Plasmids pZSBlank (Yazdani and

Gonzalez, 2008), pZSKLMgldA (Yazdani and Gonzalez, 2008), pZSKLM (Bachler et al. 2005), pZSKLcf (Bachler et al. 2005), and pZSgldA (Yazdani and Gonzalez, 2008) have been previously described. Plasmids pZSadhE, pZSglpF, pZSldhA, pZSpflB, pZStpiA, and pZSackApta were constructed by PCR amplification of the *adhE* gene, *glpF* gene, *ldhA* gene, *pflB* gene, *tpiA* gene, and *ackA-pta* operon from MG1655 genomic DNA and cloning the resulting products into pZSKLMgldA (Yazdani and Gonzalez, 2008) digested with *Kpn*I and *Mlu*I using the InFusion PCR cloning kit (Clontech Laboratories, Inc., Mountain View, CA).

For the construction of plasmids involved in 1,2-propanediol production, the *E. coli* genes *yqhD* and *fucO* were PCR amplified with flanking *Xho*I and *Kpn*I sites and each cloned into pTrcHis2A (Invitrogen, Carlsbad, CA) doubly digested with *Xho*I and *Kpn*I to produce pTHyqhD and pTHfucO. To build the plasmid pTHKLcfgldAmgsAyqhD, the gene cluster KLcfgldAmgsA from pZSKLcfgldAmgsA (see below) was PCR amplified and cloned into pTHyqhD digested with *Xho*I, using the InFusion PCR cloning kit. The same approach was used to build pTHKLcfgldAmgsAfucO, inserting the PCR product into pTHfucO, also linearized with *Xho*I. pZSKLcfgldAmgsA was constructed by PCR amplifying the *gldA* gene with flanking *Bam*HI and *Mlu*I sites and then cloning into pZSKLcf (Bachler et al., 2005) doubly digested with *Bam*HI and *Mlu*I to produce pZSKLcfgldA. The *mgsA* gene PCR amplified with *Mlu*I sites on both ends was then cloned into pZSKLcfgldA digested with *Mlu*I and treated with *Sap*I to produce pZSKLcfgldAmgsA. The *dhaKL* genes from *Citrobacter freundii* were removed from pTHKLcfgldAmgsAfucO and pTHKLcfgldAmgsAyqhD by using primers (containing a novel *Bgl*II site overhang)

that flank the *KLcf* genes, thereby amplifying the entire plasmid except for *KLcf* region, followed by *Bgl*II digestion and self ligation.

Table 3.2 Plasmids used in this study

Plasmid	Description	Source
pZSBlank	oriR pSC101*, tetR, *cat,* contains $P_{Ltet0-1}$	Yazdani and Gonzalez (2008)
pZSKLMgldA	*E. coli dhaKLM* and *gldA* genes under control of $P_{Ltet0-1}$(tetR, oriR SC101*, cat)	Yazdani and Gonzalez (2008)
pZSKLM	*E. coli dhaKLM* genes under control of $P_{Ltet0-1}$(tetR, oriR SC101*, cat)	Bachler et al. (2005)
pZSKLcf	*C. freundii dhaKL* gene under control of $P_{Ltet0-1}$ (tetR, oriR SC101*, cat)	Bachler et al. (2005)
pZSgldA	*E. coli gldA* gene under control of $P_{Ltet0-1}$(tetR, oriR SC101*, cat)	Yazdani and Gonzalez (2008)
pZSKLcfgldA	*C. freundii dhaKL* gene and *E. coli gldA* gene under control of $P_{Ltet0-1}$ (tetR, oriR SC101*, cat)	This Study
pZSKLcfgldAmgsA	*C. freundii dhaKL* gene, *E. coli gldA* and *mgsA* genes under control of $P_{Ltet0-1}$ (tetR, oriR SC101*, cat)	This Study
pZSackApta	*E. coli ackA* and *pta* genes under control of $P_{Ltet0-1}$ (tetR, oriR SC101*, cat)	This Study
pZSadhE	*E. coli adhE* gene under control of $P_{Ltet0-1}$(tetR, oriR SC101*, cat)	This Study
pZSglpF	*E. coli glpF* gene under control of $P_{Ltet0-1}$(tetR, oriR SC101*, cat)	This Study
pZSldhA	*E. coli ldhA* gene under control of $P_{Ltet0-1}$(tetR, oriR SC101*, cat)	This Study
pZSpflB	*E. coli pflB* gene under control of $P_{Ltet0-1}$(tetR, oriR SC101*, cat)	This Study
pZStpiA	*E. coli tpiA* gene under control of $P_{Ltet0-1}$(tetR, oriR SC101*, cat)	This Study
pTrcHis2A	pTrcHis2A (pBR322-derived), oriR pMB1, *lacI*^{q}, *bla*	Invitrogen (Carlsbad, CA)
pTHgldAmgsAyqhD	*E. coli gldA, mgsA,* and *yqhD* genes under *trc* promoter and *lacI*^{q} control	This Study
pTHgldAmgsAfucO	*E. coli gldA, mgsA,* and *fucO* genes under *trc* promoter and *lacI*^{q} control	This Study
pTHKLcfgldAmgsAyqhD	*C. freundii dhaKL*, *E. coli gldA, mgsA,* and *yqhD* genes under *trc* promoter and *lacI*^{q} control	This Study
pTHKLcfgldAmgsAfucO	*C. freundii dhaKL*, *E. coli gldA, mgsA,* and *fucO* genes under *trc* promoter and *lacI*^{q} control	This Study
pTHyqhD	*E. coli yqhD* gene under *trc* promoter and *lacI*^{q} control	This Study
pTHfucO	*E. coli fucO* gene under *trc* promoter and *lacI*^{q} control	This Study

Manufacturer protocols and standard methods (Miller, 1972; Sambrook et al., 1989) were followed for DNA purification (Qiagen, Valencia, CA), restriction endonuclease digestion (New England Biolabs, Ipswich, MA), and DNA amplification (Stratagene, La Jolla, CA and Invitrogen, Carlsbad, CA). The strains were kept in 32.5% glycerol stocks at -80°C. Plates were prepared using LB medium containing

1.5% agar, and appropriate antibiotics were included at the following concentrations: ampicillin (100 μg/mL), kanamycin (50 μg/mL), and chloramphenicol (34 μg/mL).

3.2. Culture medium and cultivation conditions

The minimal medium designed by Neidhardt et al. (1974) with Na_2HPO_4 in place of K_2HPO_4 and supplemented with 10 g/L glycerol was used as the base medium for all studies unless otherwise stated. For experiments conducted in closed Hungate tubes (see below), the above media was also supplemented with 2 g/L tryptone and included the oxygen indictor resazurin (1 mg/L) (Ferguson and Cummins, 1978) and reducing agent dithiothreitol (DTT, 1mM). For these studies, antibiotics (50 μg/mL kanamycin, 12.5 μg/mL chlorampheicol) and inducer (100 ng/mL anhydrotetracycline) were included when appropriate. For aerobic shake flask experiments (see below) the base media with no additional supplementation was used, with antibiotics (50 μg/mL kanamycin, 12.5 μg/mL chlorampheicol) and inducer (100 ng/mL anhydrotetracycline) included when appropriate. For experiments conducted in sparged tubes or the SixFors fermentation system (see below), the base medium was supplemented with 10 g/L tryptone and 5 g/L yeast extract and included antibiotic(s) (50 μg/mL kanamycin, 34 μg/mL chlorampheicol, 100 μg/mL ampicillin) and inducer(s) (100 ng/mL anhydrotetracycline, 0.1 mM Isopropyl β-D-1-thiogalactopyranoside) when appropriate. Chemicals were obtained from Fisher Scientific (Pittsburg, PA) and Sigma-Aldrich Co. (St. Louis, MO),

except crude glycerol, which was provided by Renewable Energy Group, Inc. (Ames, IA). Crude glycerol had the following content (w/w%): glycerol (83.3), methanol (0.01), water (10.0), fatty acids (0.04), salt (6.63), and ash (6.6). The pH was 6.38 and the density was 1.26 g/mL.

Closed tube fermentations were conducted in 17-mL Hungate tubes (Bellco Glass, Inc., Vineland, NJ). Prior to inoculation, oxygen in the medium was removed through the addition of reducing agent (DTT) and storage in an oxygen-free environment (5 % CO_2, 5% H_2, 90% N_2) provided within a BACTRON I Anaerobic Chamber (Sheldon Manufacturing Inc., Cornelius, OR) until the medium was void of color (resazurin remains pink in the presence of oxygen). A single colony was used to inoculate 5 mL of medium in each tube, which were then incubated at 37 °C with rotation in a LabQuake rotator (Fisher Scientific, Pittsburgh, PA) for 96 hours in an Isotemp Incubator (Fisher Scientific, Pittsburgh, PA).

Aerobic shake flask experiments were conducted in 25 mL Pyrex Erlenmeyer flasks (Corning Inc., Corning, NY). Pre-cultures for these fermentations were prepared by inoculating 5 mL of LB media with a single colony in Hungate tubes and incubating these tubes with rotation at 37 °C until an optical density of ~0.4 at 550 nm was reached. An appropriate volume of actively growing pre-culture was centrifuged, washed twice with minimal medium and used to inoculate 5 mL of medium in each flask, with a target initial optical density of 0.05 at 550 nm unless otherwise stated. Flasks were capped with a foam plug and incubated at 37 °C and

140 rpm in a Lab Companion SI-600 rotary shaker (Jeiotech Ltd., Seoul, Korea) for 24 hours.

Sparged tube fermentations were conducted in 17-mL Hungate tubes modified by piercing the septa with two Luer lock needles: one for oxygen-free argon sparging (20G × 8 in.; Hamilton Compoany-USA, Reno, NV) and one for gas efflux (20G × 2 in.; Hamilton Compoany-USA, Reno, NV). Prior to inoculation, oxygen in the medium was removed by autoclaving and storage in an oxygen-free environment provided within the BACTRON I Anaerobic Chamber. A single colony was used to inoculate 10 ml of medium in each tube, which were then incubated (37 °C) for 96 hours in an Isotemp Incubator (Fisher Scientific, Pittsburgh, PA) and sparged with ultra-high purity argon (Matheson Tri-Gas, Houston, TX). To maintain sterile conditions, the inlet gas was passed through a 0.2 μm HEPA filter (Millipore, Billerica, CA) and the outlet gas line immersed in a 1 M $CuSO_4$ solution.

Additional fermentations were conducted in a SixFors multi-fermentation system (Infors HT, Bottmingen, Switzerland) equipped with six 500 mL working volume vessels and independent control of temperature (37 °C), pH (controlled to 6.3 or 7.5 with NaOH and H_2SO_4), and stirrer speed (200 rpm). The system was fully equipped and computer controlled using manufacturer IRIS NT software. Each vessel was fitted with a condenser operated at 4 °C cooling ethylene glycol-water supply to minimize evaporation. Anaerobic conditions were maintained by sparging the medium with ultra-high purity argon both before and after inoculation. To maintain sterile conditions, the inlet gas was passed through a 0.2 μm HEPA filter

and the outlet gas line immersed in a 1 M $CuSO_4$ solution. Pre-cultures for these fermentations were prepared by inoculating Hungate tubes completely filled with medium with a single colony and incubating these tubes with rotation at 37 °C until an optical density of ~0.4 at 550 nm was reached. An appropriate volume of actively growing pre-culture was centrifuged, washed twice with fresh medium and used to inoculate 400 mL of medium in each fermenter, with a target initial optical density of 0.05 at 550 nm unless otherwise stated.

3.3. Analytical methods

Optical density was measured at a wavelength of 550 nm and used as an estimate of cell mass (1 OD_{550} = 0.34 g dry weight/L). For all closed Hungate tube experiments, optical density was measured at 600 nm using a WPA BioWave CO8000 Cell Density Meter (Biochrom Ltd, Cambridge, England). After centrifugation of the sample, the supernatant was stored at -20 °C for high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) analysis. Quantification of the concentration of glycerol, organic acids, ethanol, and other compounds was accomplished with ion-exclusion HPLC using a Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments Inc., Columbia, MD) equipped with an HPX-87H organic acid column (Bio-Rad, Hercules, CA) with previously determined operating conditions to optimize peak separation (30 mM H_2SO_4 mobile phase, 0.3 mL/min flowrate, 42 °C column temperature) (Dharmadi and Gonzalez, 2005).

When required, the identity of metabolic products was determined through one-dimensional (1D) proton nuclear magnetic resonance (NMR) spectroscopy. 60 µL of D_2O and 1 µL of 600 mM NMR internal standard TSP [3-(trimethylsilyl) propionic acid-D4, sodium salt] were added to 540 µL of the sample (culture supernatant). The resulting solution was then transferred to a 5 mm-NMR tube, and 1D proton NMR spectroscopy was performed at 25°C in a Varian 500-MHz Inova spectrometer equipped with a Penta probe (Varian, Inc., Palo Alto, CA) using the following parameters: 8,000-Hz sweep width, 2.8-s acquisition time, 256 acquisitions, 6.3-µs pulse width, 1.2-s pulse repetition delay, and presaturation for 2 s. The resulting spectrum was analyzed using FELIX 2001 software (Accelrys Software Inc., Burlington, MA). Peaks were identified by their chemical shifts and J-coupling values, which were obtained in separate experiments in which samples were spiked with metabolite standards (2 mM final concentration).

3.4. Enzyme assays

Escherichia coli cells from anaerobic cultures were harvested by centrifugation (10 min, 10,000 × g, 4 °C), washed twice with 9 g/L NaCl and stored as cell pellets at -20 °C. For enzyme assays, cells were resuspended to an optical density of ~10 of the buffer used in the specific assay and permeabilized by vortex mixing with chloroform unless otherwise stated (Murarka et al., 2008; Osman et al., 1987; Tao et al., 2001). Absorbance changes for all assays were monitored in a Biomate 5 spectrophotometer (Thermo Scientific, MA, USA). The linearity of reactions (protein concentration and time) was established for all assays and the

nonenzymatic rates were subtracted from the observed initial reaction rates. Enzymatic activities are reported as μmol of substrate per minute per mg of cell protein and represent averages for at least three cell preparations.

The activity of glycerol dehydrogenase in the oxidation of glycerol was assayed by measuring the change in absorbance at 340 nm and 25 °C in a 1.5 mL reaction mixture containing 500 μM NAD^+, 100 mM glycerol, 100mM potassium carbonate buffer (pH 9.5), 30 mM ammonium sulfate, and 50 μL crude cell extract (Truniger and Boos, 1994). Glycerol kinase activity was assayed using a 1 mL reaction mixture containing 0.15 M glycine (pH 9.5), 11 mM $MgCl_2$, 0.27 M hydrazine, 1.2 mM NAD^+, 5 mM ATP, 2 mM glycerol, 20 U α-glycerophosphate dehydrogenase, and 50 μL crude cell extract by monitoring the absorbance changes at 340 nm and 25 °C (Pettigrew et al., 1997).

Aerobic and anaerobic glycerol-3-phosphate dehydrogenases were assayed by monitoring absorbance changes at 570 nm and 25 °C. The 1 mL reaction mixture for the aerobic G3P dehydrogenase assay consisted of 75 μM 3-(4,5-dimethylthiazdyl-2)-2,5 diphenyltetrazolim bromide (MTT), 600 μM phenazine methosulfate (PMS), 10 mM glycerol 3-phosphate, 0.2% Triton-X-100, 10 μM NaCN, and 50 μL crude cell extract (Murarka et al., 2008). For the anaerobic G3P dehydrogenase assay, the NaCN was removed and replaced with 10 μM FAD and 1 mM FMN. The anaerobic G3P dehydrogenase assay measures both aerobic and anaerobic G3P dehydrogenase activities, therefore the true anaerobic activity is

calculated by subtracting the measured aerobic activity from the measured activity of anaerobic G3P dehydrogenase.

PEP-dependent dihydroxyacteone kinase activity was assayed using the method reported by Kornberg and Reeves (1972) with minor modifications. Decryptification buffer (0.1 M-sodium-potassium phosphate, pH 7.2, and 5 mM $MgCl_2$) was used for cell washing in place of 9 g/L NaCl mentioned above. Cells were resuspended in decryptification buffer to obtain ~1 mg dry cell weight/mL. A portion of the ice chilled cell suspension was placed in a test tube and vigorously mixed for 1 minute at which point 0.2 volume of a toluene-ethanol mixture (1:9 v/v) was added and the suspension mixed for an additional 1 min. The assay was conducted in a 1 mL reaction mixture containing 1 mM phosphoenolpyruvate, 0.1 mM NADH, 2U lactate dehydrogenase, and the toluene-ethanol treated cells (~50 μg dry weight). This assay mixture was incubated at 30 °C for 15 minutes after which dihydroxacetone was added to a concentration of 1 mM and the decrease in absorbance followed at 340 nm. ATP-dependent dihydroxyacetone kinase activity was followed in a coupled system in which the NADH-dependent reduction of the reaction product, dihydroxyacetone phosphate (DHAP), to glycerol 3-phosphate was measured at 340 nm (Barbirato et al., 1997a). The reaction mixture contained 50 mM potassium bicarbonate (pH 9.0), 2.5 mM ATP, 0.4 mM NADH, 15 mM $MgCl_2$, 2 mM DTT, 18 U glycerophosphate dehydrogenase, and 10 mM DHA. To prevent any secondary reaction (with GlyDH), 15 mM α,α'-dipyridyl was systematically added to the assay mixture.

The activity of pyruvate dehydrogenase (toward pyruvate) was monitored spectrophotometrically by following the reduction of NAD^+ at 340 nm (Murarka et al., 2010). The reaction mixture (1 ml, final pH 7.0) contained 50 mM potassium phosphate (pH 8.0), 2.0 mM sodium pyruvate, 2.5 mM NAD^+, 1.0 mM $MgCl_2$, 2.6 mM cystine-HCl, 0.13 mM CoA, 0.2 mM thiamine pyrophosphate and 50 μl crude cell extract.

The activity of methylglyoxal synthase was determined using a colorimetric assay, which consisted of a reaction step that produced methylglyoxal followed by a detection step (Berrios-Rivera et al., 2003). Methylglyoxal reducing activities (i.e its conversion to acetol or lacaldehyde) were assayed by measuring the absorbance change at 340 nm of a 1 mL reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 10 mM MG, 0.1 mM NAD(P)H, and 50 μL crude cell extract (Ko et al., 2005). 1,2-PDO reductase activities were measured in a 1 mL reaction mixture containing 100 mM Tris-HCl buffer (pH 9.0), 1 mM $MnCl_2$, 10 mM 1,2-PDO, 5 mM NAD^+, and 50 μL crude cell extract by monitoring the absorbance change at 340 nm (Oude Elferink et al., 2001). Reduction of acetol to 1,2-PDO was assayed by measuring the absorbance change at 340 nm of a 1 mL reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 10 mM acetol, 0.1 mM NADH, and 50 μL crude cell extract (Gupta et al., 2009).

3.5. Calculation of fermentation parameters

Growth and product yields (mmol/mmol glycerol or g/g glycerol) were calculated as the amount of cell mass or product synthesized per amount of glycerol consumed at the end of the fermentation time (96 hr unless otherwise stated). Data for cell growth, glycerol consumption, and product synthesis were used to calculate volumetric rates/productivities (mmoles/L/h). Specific rates (mmoles/g cell/h) were calculated by dividing the volumetric rates by the time average concentration of cells. In these calculations, an average molecular weight for an *E. coli* cell of 24.7 was used, which corresponds to an average cell of molecular formula $CH_{1.9}O_{0.5}N_{0.2}$ (Nielsen et al., 2003).

3.6. *in silico* Metabolic Flux Analysis

Metabolic flux analysis (MFA) was conducted using *in silico* modeling. Flux balance analysis (referred to here as *in silico* MFA) is a method based on the mass balance constraints in a metabolic network (Edwards and Palsson, 2000), which can be mathematically represented as $\mathbf{S} \bullet \mathbf{v} = \mathbf{0}$. "**S**" is the *m* x *n* stoichiometric matrix, *m* and *n* being the number of metabolites and reactions in the network, respectively. "**v**" is a vector containing all the fluxes in the metabolic network. The *E. coli* model used in this work is based on the pathways known to be active in *E. coli* during fermentative metabolism in general (Keseler et al., 2005; Neidhardt et al., 1990, 1996; Pramanik and Keasling, 1997; Sawers and Clark, 2004; Stryer, 1995), as well as those specific to glycerol utilization (Gonzalez et al., 2008; Lin, 1976; Murarka et

al., 2008) (see Reaction Network included in Appendix). The number of fluxes in the model was 80 and the number of mass balances 78. A unique distribution of metabolic fluxes was obtained using linear programming (MetaFluxNet®) (Lee et al., 2003) whereby the biomass flux was maximized. A list of the reactions included in this analysis along with the details about the metabolic network and metabolites considered, and the values of estimated fluxes are provided in the Appendix.

Chapter 4

On the Pathways and Regulation of Fermentative Glycerol Metabolism in *Escherichia coli*

Glycerol utilization by *Escherichia coli* was long thought to require the presence of external electron acceptors (Booth, 2005; Lin, 1976; Quastel et al., 1925; Quastel and Stephenson, 1925), a metabolic mode that limits the ability to capitalize on the advantages of glycerol for the production of fuels and reduced chemicals. The recent discovery of the ability of *E. coli* to utilize glycerol under fermentative conditions (Dharmadi et al., 2006) and the elucidation of key environmental factors, pathways, and mechanisms (Gonzalez et al., 2008; Murarka et al., 2008) have demonstrated the potential for the use of *E. coli* as a biocatalyst for the conversion of glycerol to higher value products. While these studies have provided an initial platform, due to the system complexities of cellular metabolism and the specific challenges associated with the use of glycerol as carbon source, an improved

understanding of fermentative glycerol metabolism is needed in order to provide the detailed knowledge base required for the design of metabolic engineering strategies aimed at efficient fuel and chemical production from glycerol.

While the high degree of reduction of carbon atoms in glycerol provides a distinct advantage for the production of fuels and reduced chemicals compared to traditional carbon sources, it also results in significant challenges in regard to the utilization of glycerol under fermentative conditions. The generation of reducing equivalents during the synthesis of cell mass from glycerol results in the need for pathway(s) that act as a sink for the consumption of these excess reducing equivalents. While the ability to utilize glycerol under fermentative conditions was long thought to be linked to the ability to produce the highly reduced compound 1,3-propanediol, fermentative glycerol utilization in *E. coli* was determined to be enabled by the production of 1,2-propanediol (Gonzalez et al., 2008). This newly characterized model in *E. coli* consists of the reductive 1,2-PDO-producing pathway working in partnership with an oxidative, glycerol dehydrogenase (glyDH, encoded by *gldA*)-dihydroxyacetone kinase (DHAK, encoded by *dhaKLM*) mediated pathway (Gonzalez et al., 2008). In addition, an active formate hydrogen-lyase complex was essential and ethanol production was a requirement due to the fact that the glycerol to ethanol (and formate or CO_2/H_2) pathway is a redox balanced, ATP generating pathway (Figure 2.7) (Gonzalez et al., 2008; Murarka et al., 2008).

The establishment of this 1,2-PDO-ethanol dependent metabolic model enabling the fermentative utilization of glycerol by *E. coli* serves as the starting point

for the understanding of fermentative glycerol metabolism in *E. coli*. However, at present, no studies on key areas of fermentative metabolism have been investigated. With the aforementioned metabolic challenges associated with the high degree of reduction of glycerol, and the intrinsic changes in the metabolic state this causes, fermentative glycerol metabolism may require significant divergence from the fermentative metabolism of more traditional carbon sources and result in the activity and involvement of unique pathways and mechanisms. Furthermore, the fact that glycerol is a 3-carbon compound entering glycolysis as dihydroxyacetone phosphate results in the need for both glycolytic and gluconeogenic pathways for the generation of precursor metabolites for biosynthesis, a unique characteristic compared to traditional carbon sources such as 6-carbon glucose. These underlying complexities highlight the need for a more comprehensive study into the key pathways, mechanisms, and regulatory aspects involved in the fermentative metabolism of glycerol by *E. coli*, which can both increase the fundamental knowledge of cellular metabolism as well as provide the detailed platform needed to engineer *E. coli* for the production of a wide array of fuels and chemicals from this abundant and inexpensive carbon source. For this purpose, both stoichiometric modeling (*in silico* Metabolic Flux Analysis) and an extensive experimental investigation into key areas of glycerol utilization and fermentative metabolism, as well as the kinetics of glycerol utilization were conducted in order to provide a comprehensive picture of important and essential elements of fermentative glycerol metabolism in *E. coli*.

4.1. *in silico* Metabolic Flux Analysis of fermentative glycerol metabolism

in silico Metabolic Flux Analysis (or Flux Balance Analysis, FBA) was used as an initial modeling tool to obtain a quantitative prediction of the effects the stoichiometric, energetic, and redox constraints associated with the use of glycerol as a carbon source have on the overall metabolic network. FBA utilizes linear programming and constraint-based modeling in order to simulate the predicted fluxes within a network with few or even no experimental data. With this approach, it is possible to obtain a unique solution for the intracellular fluxes by optimizing a given objective function subject to the constraints of the metabolite balances (Edwards and Palsson, 2000; Stephanopoulos et al., 1998). The assumption of this method is that the metabolic model reaches a steady state constrained by the stoichiometry of the model. These stoichiometric constraints define a bounded solution space (region of feasible fluxes). In order to restrict the solution space, other constraints such as the reversibility of the reactions are added into the model.

The *E. coli* model developed for fermentative glycerol metabolism is based on the pathways known to be active in *E. coli* during fermentative metabolism in general, as well as those specific to glycerol utilization (see Materials and Methods and Reaction Network included in the Appendix). With this model in place, a unique distribution of metabolic fluxes was obtained using linear programming whereby the objective function was chosen as the maximization of biomass flux, which has been shown to the most biologically meaningful objective function as unicellular

organisms have evolved toward maximal growth performance (Segre et al., 2002). A summary of relevant fluxes with the metabolic network during fermentative glycerol metabolism in shown in Figure 4.1 and the complete flux distribution is provided in the Appendix.

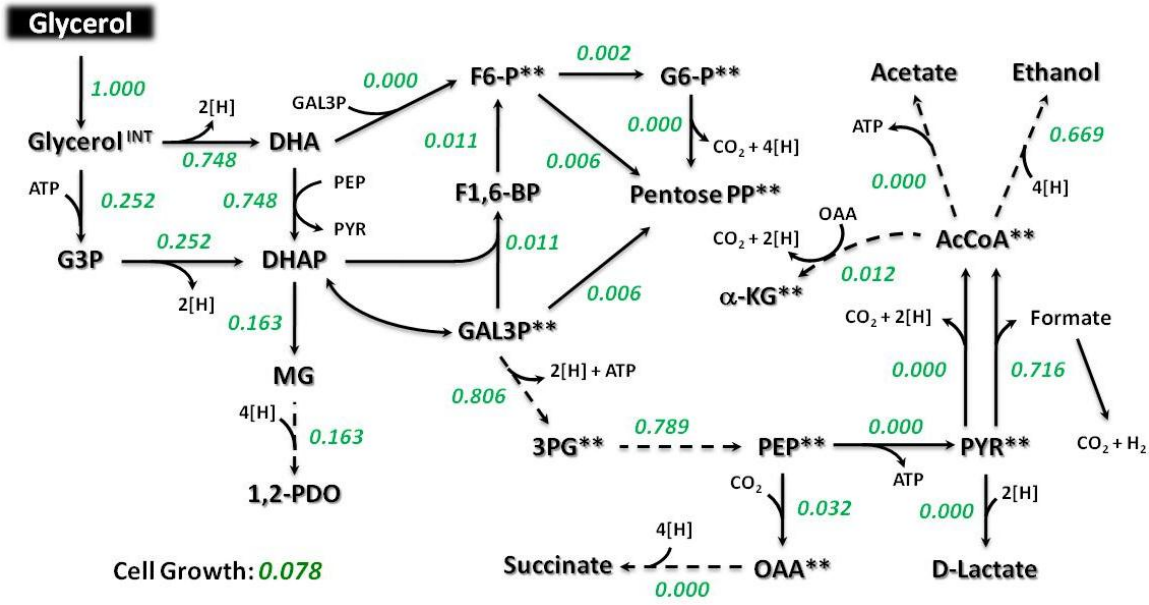

Figure 4.1 Simulated flux distribution during fermentative glycerol metabolism. Reported fluxes are normilized with respect to glycerol transport flux. Broken lines illustrate multiple steps. Abbreviations: 1,2-PDO, 1,2-propanediol; 3PG, 3-phospoglycerate; α-KG, α-ketoglutarate; AcCoA, acetyl-CoA; DHA, dihydroxyacetone; DHAP, DHA phosphate; F1,6-BP, fructose-1,6-bisphosphate; F6-P, fructose-6-phosphate; G3P, glycerol-3-phosphate; G6-P, glucose-6-phosphate; GAL3P, glyceraldehyde-3-phosphate; INT, intracellular; MG, methylglyoxal; OAA, oxaloacetate; Pentose PP, pentose phosphate pathway; PEP, phosphoenolpyruvate; PYR, pyruvate; 2[H], NADH/NADPH/$FADH_2$/QH_2. ** denotes precursor metabolite required for cell biosynthesis.

Several interesting characteristics are evident in the simulated flux distribution, which reflect both key characteristics of the proposed model as well as areas in which further experimental investigation can help elucidate additional essential mechanisms. In agreement with the product profiles seen during fermentative

glycerol metabolism (Dharmadi et al., 2006), ethanol represents the main product, with minimal amounts of acetate, succinate, and lactate (Figure 4.1). The relatively large flux to 1,2-PDO (0.163) reflects this pathways ability to serve as the sink for the excess of reducing equivalents generated during the formation of cell mass from glycerol, in agreement with proposed 1,2-PDO-ethanol dependent model for fermentative glycerol utilization (Gonzalez et al., 2008). The enabling nature of this pathway for glycerol fermentation is also evident from the fact that the removal of the 1,2-PDO production pathway from the reaction network results in the inability to find a solution to the flux distribution with a non-zero flux toward cell growth (i.e. no cell growth possible under these conditions) (Appendix, Table A5).

Examination of individual fluxes also reveals some interesting facets related to pathway activity during fermentative glycerol metabolism. As would be expected under anaerobic conditions, all flux from pyruvate to acetyl-CoA takes place through pyruvate formate lyase (as indicted by the generation of formate in Figure 4.1), with no predicted activity of the pyruvate dehydrogenase (PDH) complex. From a mathematical standpoint, this is a result of the generation of an extra reducing equivalent by the PDH complex, however *in vivo* it is also likely that PDH complex activity will be minimal, as PDH is typically active under respiratory conditions and the absence of activity has been explained by its sensitivity to NADH inhibition (de Graef et al., 1999; Sawers and Clark, 2004). In addition, while PDH has recently been reported to play an important during the fermentative metabolism of glucose (Murarka et al., 2010a) and glucuronate (Murarka et al., 2010b), these studies demonstrated that the role of PDH is more significant with more oxidized carbon

sources and as a result, PDH activity should be negligible with a substrate as reduced as glycerol. No direct conversion of PEP to pyruvate is predicted, as the majority of PEP produced by glycolysis is directly utilized as the phosphate group donor in the phosphorylation of DHA, while small amounts are also converted to the precursor metabolite oxaloacetate needed for biosynthesis (Figure 4.1). Perhaps the most intriguing aspect of the simulated flux distribution is the predicted activity of the both the fermentative (through DHA as an intermediate) and respiratory (through G3P as an intermediate) glycerol dissimilation pathways (Figure 4.1). Even more interesting is the fact that the removal of the respiratory glycerol dissimilation route from the reaction network results in no non-zero cell growth flux distributions, reflecting an important role this pathway may have in fermentative glycerol metabolism (Appendix, Table A5). While this could be a mathematical result of the need for PEP formation for the activity of the fermentative glycerol dissimilation pathway, this could also reflect a consequence of the coupling between PEP synthesis and glycerol dissimilation in the fermentative pathway, which may limit the availability of PEP as well as precursor metabolites needed for the synthesis of cell mass.

Overall, these results reflect the key characteristics of the previously established 1,2-PDO-ethanol dependent model enabling glycerol fermentation in *E. coli*, as both 1,2-PDO and ethanol production (data not shown) are required in order enable cell growth in the stoichiometric model. These results are a consequence of the ability for these pathways to provide a sink for excess reducing equivalents generated in the formation of cell mass from glycerol, and provide ATP generation

in a redox balanced pathway, respectively. Confirmation of these pathway characteristics as the enabling factor for glycerol fermentation in the model was provided through the addition of arbitrary redox sink (i.e. NADH → redox sink) and ATP generation (ATPsupply → ATP) pathways, whose activity resulted in cell growth in the absence of 1,2-PDO and ethanol production, respectively (data not shown). In addition to complementing the previous findings on fermentative glycerol metabolism, the simulated flux distribution provided by *in silico* Metabolic Flux Analysis also provides some intriguing areas to begin the comprehensive experimental investigation, with the predicted activity of both the fermentative and respiratory glycerol dissimilation pathways.

4.2. Experimental investigation into fermentative glycerol metabolism in *Escherichia coli*

An experimental investigation into key areas of fermentative metabolism and glycerol utilization was conducted through the use of genetic and biochemical techniques to stimulate or repress the activities of postulated pathways, and probe mechanisms and cellular functions, thus enabling the assessment of their role in fermentative glycerol metabolism. For these studies, wild-type *Escherichia coli* K12 strain BW25113 and single gene knockout mutants obtained from the National BioResource Project (NIG, Japan) (Baba et al., 2006) were used, with additional gene deletions introduced into the knockout mutants via P1 phage transduction as needed (See Materials and Methods). Environmental conditions were selected

based on the findings of previous studies (Dharmadi et al., 2006; Gonzalez et al., 2008; Murarka et al., 2008) to ensure these factors did not impair glycerol fermentation. In addition to the previously defined media, the redox indicator resazurin (Ferguson and Cummins, 1978) and reducing agent dithiothreitol (DTT) were included as a means to both detect (resazurin is pink in the presence of oxygen) and eliminate oxygen from the media to ensure that strict anaerobic conditions were kept throughout the course of fermentations.

In order to assess the effects of genetic perturbations on the ability to utilize glycerol as a carbon source, levels of glycerol consumption during the fermentation were used as the main metric, with ethanol production used as a secondary metric due the requirement for its production during glycerol fermentation by *E. coli* (Section 2.4.2). The reliance on cell growth as a key metric was avoided due to the inclusion of 2 g/L tryptone in the media, which has been found to be required for fermentative glycerol utilization in the absence of hydroxyacetone supplementation (Gonzalez et al., 2008; Murarka et al., 2008). While it has been shown that the inclusion of tryptone does not compromise the fermentative nature of glycerol utilization (Murarka et al., 2008), comparison of cell growth levels due to genetic perturbations can be somewhat misleading as tryptone can directly contribute to cell growth. A detailed summary of all measured values, including cell growth and all external metabolite levels, for the experiments detailed in subsequent sections can be found in the Appendix (Table A6).

The experimental investigation into fermentative glycerol metabolism was divided into several key areas of central carbon metabolism, both glycerol- and non-glycerol-specific. Initial investigation focused on areas including postulated pathways and mechanisms involved in glycerol transport and dissimilation, the formation of 6-carbon intermediates, the PEP/pyruvate node and carboxylating enzymes, in addition to more global mechanisms such as global regulation of fermentative and glycerol metabolism. The establishment of important and essential elements in these areas then enabled the elucidation of how these key pathways and mechanisms fit in overall context of fermentative glycerol metabolism.

4.2.1. Glycerol transport and dissimilation

Glycerol transport involves a glycerol facilitator (GlpF, encoded by *glpF*) allowing the facilitated diffusion of glycerol across the inner membrane for utilization inside the cell, at which point glycerol dissimilation was long thought to begin by glycerol phosphorylation by an ATP-dependent glycerol kinase (GK, encoded by *glpK*) to form glycerol 3-phosphate (G3P) (Lin, 1976). Two respiratory, membrane bound glycerol 3-phosphate dehydrogenases (G3PDH) can then oxidize glycerol 3-phosphate to the glycolytic intermediate dihydroxyacetone phosphate (DHAP) with electrons tied to the quinone pool and the electron transport chain. Aerobic G3PDH (encoded by *glpD*) accepts either oxygen or nitrate as the terminal electron acceptor, while anaerobic G3PDH (encoded by *glpABC*) is coupled to electron acceptors other than oxygen. Recent studies have shown that under

fermentative conditions a glycerol dehydrogenase (glyDH, encoded by *gldA*) and a PEP-dependent dihydroxyacetone kinase (DHAK, encoded by *dhaKLM*) are essential for fermentative glycerol utilization (Gonzalez et al., 2008). This alternative fermentative glycerol dissimilation pathway functions through the NAD^+ dependent oxidation of intracellular glycerol to dihydroxyacetone (DHA), mediated by glyDH, and the subsequent phosphorylation of DHA to DHAP through the action of DHAK, which utilizes PEP as the phosphate group donor (Figure 4.2).

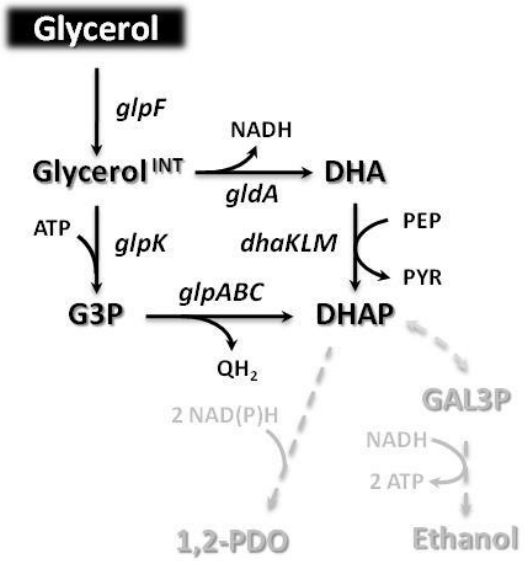

Figure 4.2 Pathways involved in glycerol transport and dissimilation. Broken lines illustrate multiple steps. Relevant reactions are represented by the gene(s) coding for the enzymes. See text for description. Abbreviations: DHA, dihydroxyacetone; DHAP, DHA phosphate; GAL3P, glyceraldehyde-3-phosphate; G3P, glycerol-3-phosphate; INT, intracellular; PEP, phosphoenolpyruvate; PYR, pyruvate.

In light of the *in silico* MFA prediction of flux through both the fermentative and respiratory glycerol dissimilation pathways, measurement of the enzyme activities and assessment of the effect of gene disruption and overexpression of these key

enzymes is essential for the determination of the *in vivo* activities and roles of these pathways during fermentative glycerol utilization.

In order to assess the involvement of each glycerol dissimilation pathway during fermentative glycerol metabolism, enzyme assays were conducted to measure the activities of each of these enzymes in wild-type BW25113 (Table 4.1).

Table 4.1 Measured activities of enzymes involved in glycerol dissimilation

Enzyme Assayed (*Gene*)	Activity [a,b] (μmol/mg protein/min)
glycerol dehydrogenase (*gldA*)	0.132 ± 0.008
PEP-dependent dihydroxyacetone kinase (*dhaKLM*)	0.051 ± 0.002
glycerol kinase (*glpK*)	0.102 ± 0.008
anaerobic glycerol-3-phosphate dehydrogenase (*glpABC*)	0.120 ± 0.007
aerobic glycerol-3-phosphate dehydrogenase (*glpD*)	ND[c]

[a]All activities measured as described in Materials and Methods and values are reported as average ± standard deviation of triplicate assays
[b]Activities measured in wild-type BW25113 after 72 hours growth
[c]ND: Not detectable (minimum detectable activity was 0.001 μmol/mg protein/min)

These measurements reveal detectable activity of enzymes in both the fermentative (glyDH and PEP-dependent DHAK) and respiratory (GK and G3PDH) glycerol dissimilation pathways, indicating that both routes to DHAP are active during fermentative glycerol utilization. Similar levels are seen for all detectable activities, with PEP-dependent DHAK activity the exception as the measured activity is less than half of the other values. This is likely the result of the fact that PEP-dependent DHAK is a membrane bound protein (Erni et al., 2006) requiring an alternative cell disruption method for activity measurement (See Materials and Methods). It is also noteworthy that no detectable levels of aerobic G3PDH (encoded by *glpD*) were measured, which would be expected due to the fact this enzyme utilizes oxygen as

the terminal electron acceptor. While these activity measurements indicate the possible involvement of both glycerol dissimilation pathways, it is important to note that these measurements are *in vitro* activities and additional evidence supporting their contribution during fermentative glycerol metabolism is needed to validate these findings.

Further assessment of the involvement of these pathways for glycerol dissimilation, as well as the importance of the glycerol facilitator GlpF, was conducted through the deletion of genes encoding for these specific enzymes/proteins and analysis of their impact on the ability of *E. coli* to fermentatively utilize glycerol (Figure 4.3). The deletion of the *glpF* gene (encoding for GlpF) lead to only a minimal reduction in the concentration of glycerol consumed as compared to wild-type BW25113. This is not entirely surprising, as glycerol, like other small uncharged molecules, can cross the cytoplasmic membrane by passive diffusion, with cells limited to passive uptake at a growth disadvantage only at low glycerol concentrations (Richey and Lin, 1972). Consistent with previous studies (Gonzalez et al., 2008), the deletion of *gldA* or *dhaK*, leading to strains lacking glyDH or a functional PEP-dependent DHAK respectively, resulted in severe impairment in the ability to utilize glycerol and no ethanol production (Figure 4.3). These experiments also confirmed the importance of the respiratory glycerol dissimilation pathway, as strains lacking GK (Δ*glpK*) or a functional anaerobic G3PDH (Δ*glpA*) consumed significantly less glycerol when compared to wild-type BW25113 (Figure 4.3).

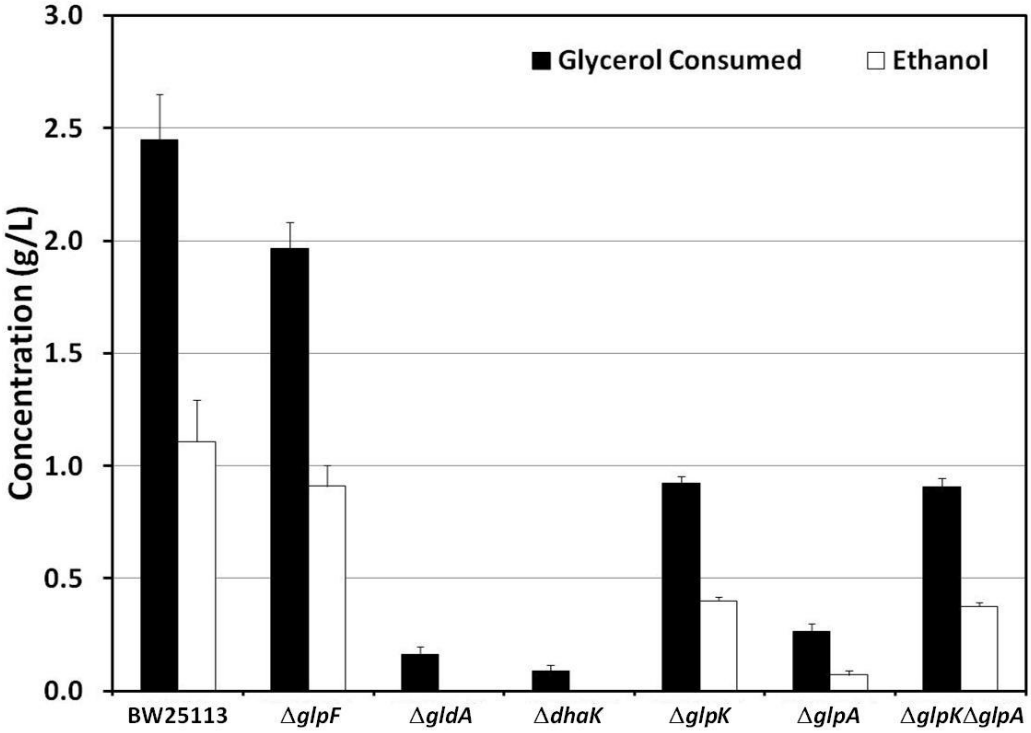

Figure 4.3 Effect of the disruption of glycerol transport and dissimilation pathways on glycerol consumption and ethanol production. Fermentations conducted in 96 hr closed Hungate tube experiments. Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

These results indicate that while only the fermentative glycerol dissimilation pathway is required during fermentative glycerol metabolism, activity of the respiratory dissimilation route in combination with the fermentative pathway provides a means for increased glycerol utilization. It is also important to note that the deletion of other subunits of anaerobic G3PDH (i.e. Δ*glpB* and Δ*glpC*) resulted in nearly identical glycerol consumption and ethanol production as the *glpA* deletion (Appendix, Table A6), indicating a lack of functionally of anaerobic G3PDH with all deletions. Surprisingly, the deletion of *glpA* resulted in lower amounts of glycerol consumption that the deletion of *glpK*, initially indicating that anaerobic G3PDH may

play a more important role than GK. However, the deletion of *glpA* with an active GK could also result in the accumulation of glycerol-3-phosphate (Figure 4.2), a toxic intermediate even at low concentrations (Cozzarelli et al., 1965; Zhu et al., 2002), which could significantly impair cell growth and glycerol consumption. This was tested through the construction of a strain with both *glpK* and *glpA* deleted, which behaved identically to the *glpK* deletion strain (Figure 4.3), suggesting that the increased impairment of glycerol consumption with the deletion of *glpA* compared to the *glpK* deletion was likely a result of the accumulation of glycerol-3-phosphate as opposed to an additional function of anaerobic G3PDH.

With the importance of both glycerol dissimilation pathways established, the next area of investigation within glycerol dissimilation was in the involvement of phosphotransferase system (PTS) enzymes, due to the essential nature of the PEP-dependent DHAK, a dihydroxyacetone PTS enzyme of *E. coli*, which mediates the phosphotransfer from PEP to DHA in seven steps involving multiple other PTS enzymes (Deutscher et al., 2006) (Figure 4.4). Phosphotransfer from PEP to DHA is mediated by five distinct proteins (EI, encoded by *ptsI*; HPr, encoded by *ptsH*; DhaM, encoded by *dhaM*; DhaL, encoded by *dhaL*; DhaK, encoded by *dhaK*). DhaM itself is composed of three PTS domains, a truncated EI, HPr, and an EIIA of the mannose class PTS. From the phosphorylated EIIA domain of DhaM, the phosphoryl group is transferred to an ADP molecule tightly bound to DhaL and from there is transferred to a DHA molecule bound to DhaK (Deutscher et al., 2006).

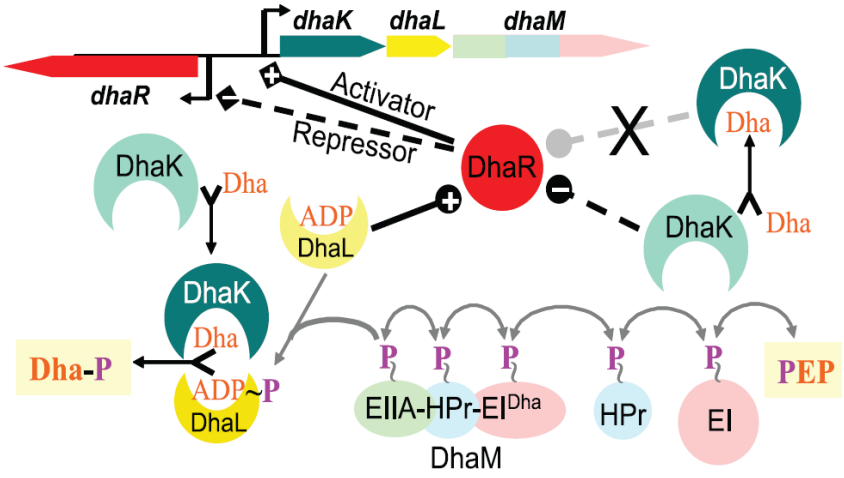

Figure 4.4 Dihydroxyacetone phosphotransferase system of *E. coli*. Phosphotransfer from PEP to DHA is mediated by five distinct proteins (EI, HPr, DhaM, DhaL, and DhaK) (Deutscher et al., 2006).

With the essential involvement of PEP-dependent DHAK during fermentative glycerol metabolism, it is also critical to determine the importance of the other key enzymes involved in the phosphotransfer from PEP to DHA to ensure the indispensable nature of the PEP-dependent DHAK is related to the phosphorylation of DHA. In addition, the link between glycerol dissimilation and the phosphotransferase system warrants testing the effects of some additional PTS enzymes involved in other phosphotransfer mechanisms, such as the glucose specific PtsG (encoded by *ptsG*) and Crr (encoded by *crr*), the two subunits the glucose specific PTS permease as well as subunits of EII^{Glc}, an enzyme required for PEP-dependent glucose phosphorylation (Deutscher et al., 2006), in order to determine any possible involvement of other common PTS enzymes during fermentative glycerol metabolism.

The deletion of either *ptsH* or *ptsI* resulted in severe impairment in glycerol consumption, on par with the deletion of *dhaK*, indicating their involvement in the phosphorylation of DHA (Figure 4.5). This point is further proven by the fact that the overexpression of the fermentative glycerol dissimilation in a *ptsH* deletion strain (i.e. Δ*ptsH* (pZSKLMgldA)) does not lead to increased glycerol consumption, a result which is to be expected with the deletion of a key enzyme in the phosphotransfer chain.

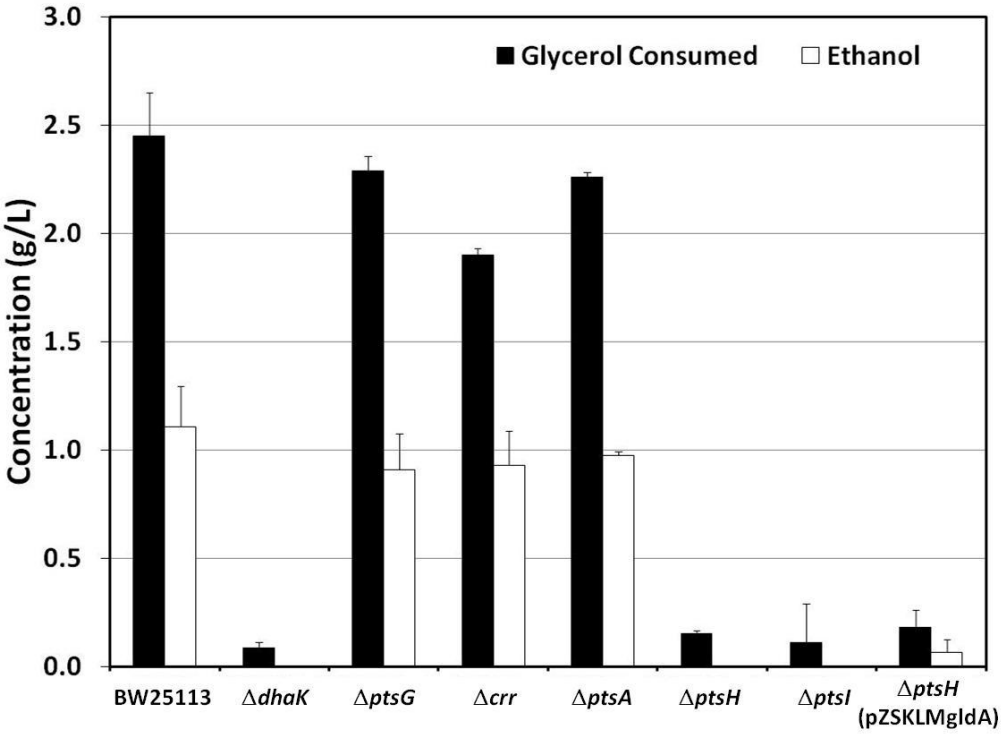

Figure 4.5 Effect of gene deletions of the phosphotransferase system on glycerol consumption and ethanol production. Fermentations conducted in 96 hr closed Hungate tube experiments. Δ"*gene*" represents gene deletion. Gene overexpressions are indicated by the plasmid name in parenthesis (i.e. pZSKLMgldA expressing *E. coli* PEP-dependent DHAK and *E. coli* glycerol dehydrogenase). Error bars represent standard deviations for a minimum of triplicate measurements.

The deletion of glucose specific PTS enzymes had little effect on glycerol consumption, while the deletion of *ptsA*, encoding a protein with similarity to sugar phosphotransferase system (PTS) components as the N-terminal domain is similar to Enzymes I of the PTS, while the C-terminal domain is similar to the Enzyme IIA^{Fru} and DhaM (Reizer et al., 1995), also had little effect (Figure 4.5). The latter is an interesting result, as in addition to the structure similarity to required PTS components (i.e. DhaM), the *ptsA* gene is part of the same operon as *gldA* (i.e. *ptsA-fsaB-gldA* operon) indicating a possible link between the genes. However, these results point to a limited role of PtsA in fermentative glycerol metabolism.

4.2.2. Conversion of 3-carbon intermediates into 6-carbon intermediates

Another unique aspect of glycerol metabolism compared to more traditional carbon sources such as glucose, is the required activity of both glycolytic and gluconeogenic pathways as a result of the 3-carbon composition of glycerol and glycerol dissimilation pathways which convert glycerol into the glycolytic intermediate DHAP (Figure 4.2). While DHAP can subsequently be converted into PEP through common glycolytic pathways, gluconeogenic pathways are required for the formation of 6-carbon intermediates required for biosynthesis, such as glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) among others (Figure 4.6). Triose phosphate isomerase (encoded by *tpiA*) catalyzes the interconversion of DHAP and glyceraldehyde 3-phosphate (GAL3P), metabolites that feed the 6 and 3-carbon parts of the Embden-Meyerhof-Parnas pathway. The conversion of GAL3P into other 3 and 2-carbon glycolytic intermediates is well established, however, the

synthesis of 6-carbon intermediates from 3-carbon compounds could proceed through multiple routes and in some cases through enzymes whose role is not well understood.

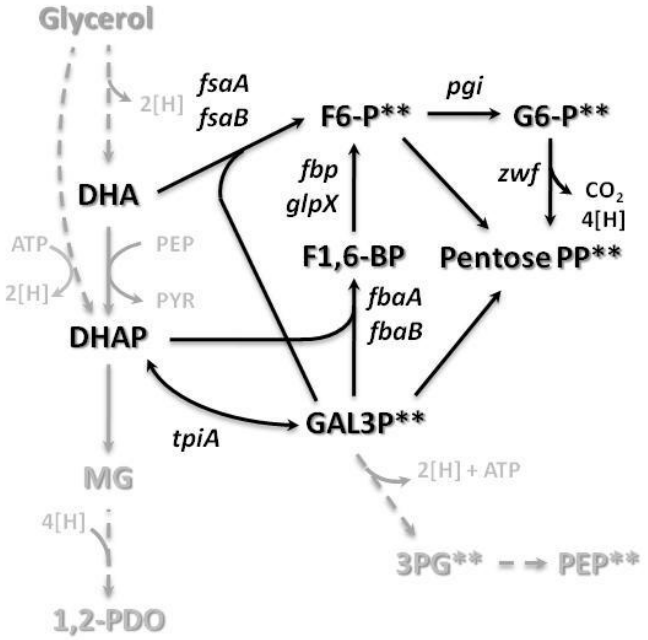

Figure 4.6 Pathways for the conversion of 3-carbon intermediates into 6-carbon intermediates. Broken lines illustrate multiple steps. Relevant reactions are represented by the gene(s) coding for the enzymes. See text for description. Abbreviations: 3PG, 3-phospoglycerate; DHA, dihydroxyacetone; DHAP, DHA phosphate; F1,6-BP, fructose-1,6-bisphosphate; F6-P, fructose-6-phosphate; G6-P, glucose-6-phosphate; GAL3P, glyceraldehyde-3-phosphate; MG, methylglyoxal; Pentose PP, pentose phosphate pathway; PEP, phosphoenolpyruvate; 2[H], NADH/NADPH/$FADH_2$/QH_2. ** denotes precursor metabolite required for cell biosynthesis.

The more established route involves the conversion of DHAP and GAL3P into fructose-1,6-bisphosphate (F1,6BP) by the action of two F1,6BP aldolases, FbaA (Class II) and FbaB (Class I) (encoded by *fbaA* and *fbaB* respectively), with the latter considered to be the gluconeogenic aldolase (Scamuffa and Caprioli, 1980). F1,6BP can then be converted into F6P by the action of fructose 1,6-bisphophatase (FBPase,

encoded by *fbp*), an enzyme considered necessary for growth on glycerol (Babul and Guixe, 1983). A second FBPase, GlpX (encoded by *glpX*), has also been discovered, however its role is still uncertain (Donahue et al., 2000). It has also been proposed that F6P can be generated in a one step reaction from DHA and GAL3P, by recently identified F6P aldolases (FSA: FsaA encoded by *fsaA* and FsaB encoded by *fsaB*) (Schurmann and Sprenger, 2001). It is important to note that the reaction catalyzed by the FSAs is feasible only when DHA is available as an intermediate, indicating a possible link to the newly proposed glyDH-DHAK fermentative glycerol dissimilation pathway. F6P can then be converted into G6P or directly enter the non-oxidative branch of the pentose phosphate pathway. G6P can enter the oxidative branch of the pentose phosphate pathway through the action of G6P-dehydrogenase (encoded by *zwf*) with the ultimate generation of CO_2 and 2 NADPH before entering the non-oxidative branch of the pentose phosphate pathway as ribulose-5-phosphate. With the ambiguity created through the existence of multiple routes for the formation of 6-carbon intermediates and the limited understanding in relation to physiological roles and importance of these enzymes, the determination of their importance for fermentative glycerol metabolism is essential. For this purpose, strains in which the genes encoding for these enzymes were deleted were tested for their ability to utilize glycerol under fermentative conditions (Figure 4.7).

The deletion of *tpiA* (encoding triose phosphate isomerase) significantly impaired the ability to utilize glycerol under fermentative conditions. This is expected as triose phosphate isomerase serves as the main link between glycerol dissimilation and glycolysis, with the deletion of *tpiA* resulting in the inability for

DHAP to enter glycolysis (Figure 4.6). Interestingly, the deletion of one or both of the FBP aldolases (encoded by *fsaA* and *fsaB*) had minimal effects on glycerol consumption levels indicating that the proposed formation of F6P through DHA and GAL3P has a negligible role in fermentative glycerol metabolism (Figure 4.7).

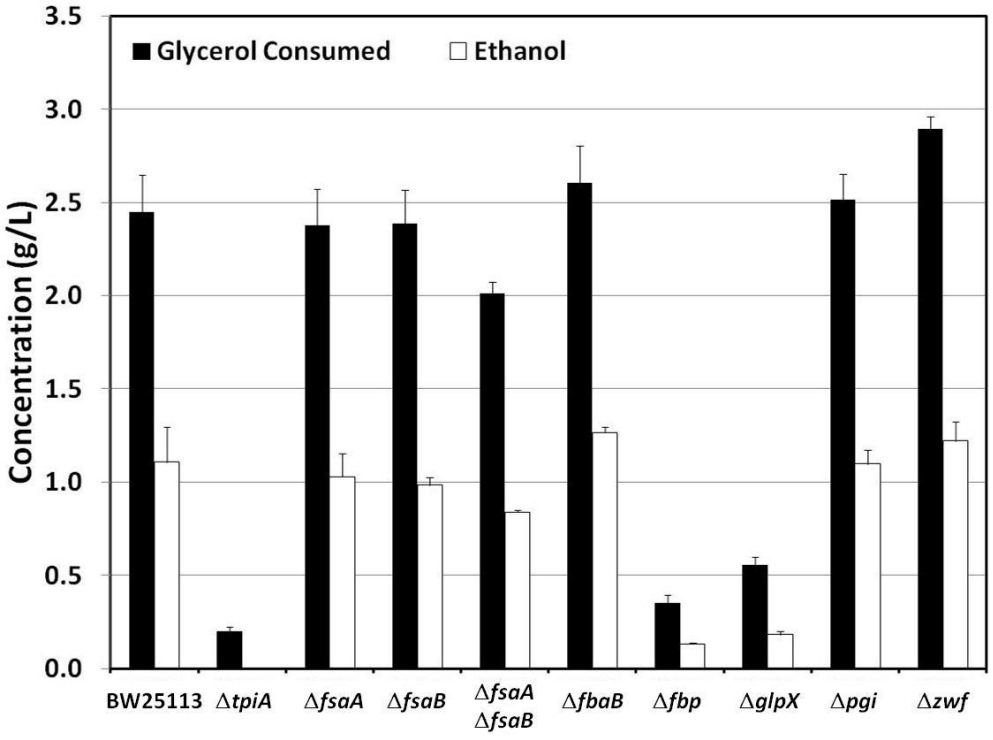

Figure 4.7 Glycerol consumption and ethanol production in *E. coli* strains with gene deletions for pathways involved in 6-carbon intermediate formation. Fermentations conducted in 96 hr closed Hungate tube experiments. Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

As a result, the formation of F1,6BP through either FbaA or FbaB and the subsequent conversion to F6P should be critical for the formation of required 6-carbon intermediates (Figure 4.6). While this is not directly evident from the deletion mutants, as the deletion of *fbaB* had little effect on glycerol consumption, the importance of the conversion of DHAP and GAL3P to F1,6BP can be inferred

from the impact of FBPase deletions (Δ*fbp* and Δ*glpX*) and the essential nature of the class II F1,6BP aldolase (FbaA, encoded by *fbaA*). FbaA was found to be one of 303 essential gene candidates in the *E. coli* genome due to the inability to obtain viable mutants when the deletion of these genes are attempted (Baba et al., 2006), and for this reason a gene deletion mutant for *fbaA* could not be tested. However, with the lack of an impact on glycerol consumption with a *fbaB* deletion and the importance of FBPase activity (Figure 4.7), which would require F1,6BP as a substrate (Figure 4.6), it stands to reason that FbaA likely performs the conversion of DHAP and GAL3P to F1,6BP during fermentative glycerol metabolism. This is a surprising finding however, as the class II F1,6BP aldolase is thought be involved in glycolysis, while the class I enzyme has been shown to be present only when *E. coli* is grown on 3-C carbon sources inferring gluconeogenic activity (Scamuffa and Caprioli, 1980). Another surprising finding is negligible change in glycerol consumption in a strain lacking phosphoglucose isomerase (PGI, encoded by *pgi*), an enzyme which catalyzes the interconversion of F6P and G6P, an essential step in both glycolysis and gluconeogensis pathways (Canonaco et al., 2001; Romeo and Snoep, 2005).

Perhaps the most intriguing finding in this area is the importance of several FBPases during fermentative glycerol metabolism, including GlpX, a type II FBPase whose physiological role has yet to be determined (Donahue et al., 2000). In light of the finding that both *fbp* and *glpX* deletions have a significant impact on glycerol utilization (Figure 4.7) validation of these enzymes involvement is critical for understanding fermentative glycerol metabolism. Under respiratory conditions, the type I FBPase encoded by *fbp* has been found to be the only required FBPase for

growth on gluconeogenic substrates (including glycerol), as *glpX* mutants grow normally when *fbp* is present (Donahue et al., 2000). To confirm the importance of *glpX* only under fermentative conditions, FBPase deletion mutants, including two additional known FBPases (*yggF* and *ybhA*), were tested under both fermentative and respiratory conditions (Figure 4.8).

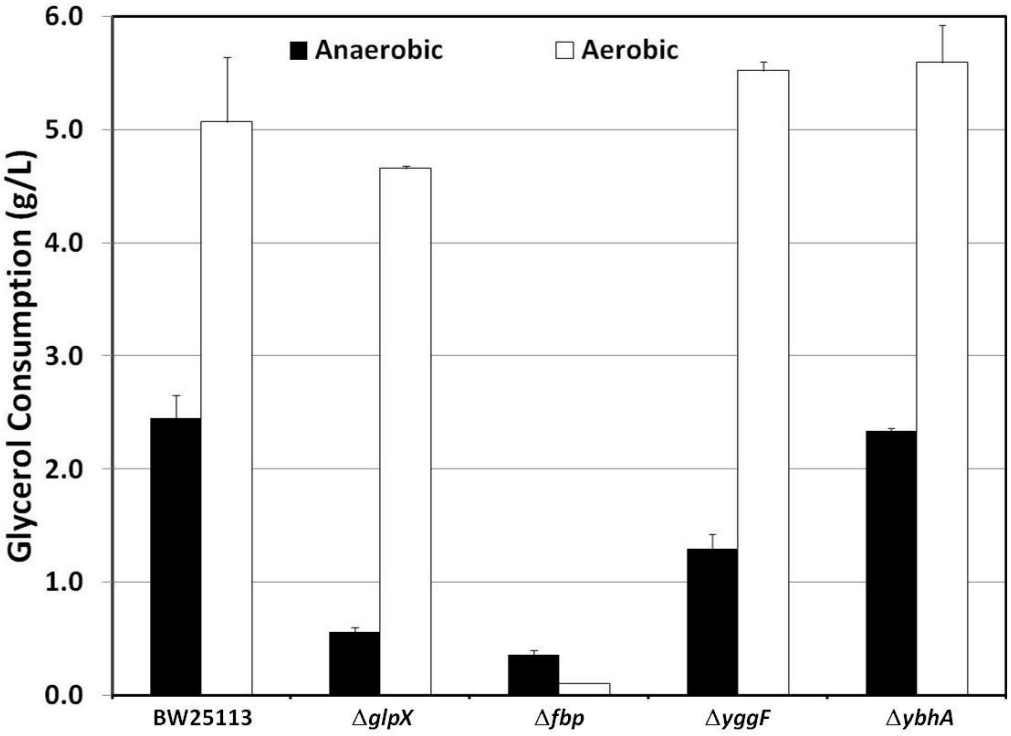

Figure 4.8 FBPase involvement during glycerol metabolism Anaerobic fermentations conducted in 96 hr closed Hungate tube experiments while aerobic experiments run in 24 hr shake flasks. Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

Consistent with previous studies, the deletion of the *glpX* gene had minimal impact on glycerol utilization under respiratory conditions, while *fbp* was required under both respiratory and fermentative conditions (Figure 4.8). In addition, while the FBPase encoded by *fbp* was the only critical enzyme under respiratory

conditions, those encoded by *fbp*, *glpX*, and *yggF* all appear to significantly impact glycerol utilization under fermentative conditions (Figure 4.8). It is also important to note that the minimal impact of the deletion of *glpX* under respiratory conditions proves that there was no upstream disruption of *glpK* during the deletion of the *glpX* gene, which could have resulted due to the fact both genes are part of the same operon (*glpFKX*). If this were to be the case, the Δ*glpX* strain would have shown no growth on glycerol under respiratory conditions, as *glpK* disruption leads to this phenotype (data not shown; Durnin et al., 2009). These findings represent a critical distinction between fermentative and respiratory glycerol metabolism, and demonstrate a possible physiological role for two type II FBPases (*glpX* and *yggF*), whose functions in *E. coli* have yet to be determined.

While difficult to elucidate the exact mechanisms behind the importance of multiple FBPases during fermentative glycerol metabolism, the tight regulation of gluconeogenesis and its link to other critical areas of fermentative glycerol metabolism may provide some basis for these findings. Although the explicit reasons why *E. coli* maintains two distinct classes of FBPases with varying catalytic properties (Brown et al., 2009) is not well understood, the potential for a futile cycle and the need for tight regulation to avoid such a cycle has been proposed as a possible explanation (Brown et al., 2009; Donahue et al., 2000; Hines et al., 2006). FBPase and 6-phosphofructokinase are constitutive enzymes in *E. coli*, and in the absence of some kind of coordinate regulation, define a futile cycle (Hines et al., 2006). However, futile cycling is minimal under glycolytic and gluconeogenic conditions, and hence FBPase must be under metabolic control (Hines et al., 2006).

FBPases can modulate the concentration of F1,6BP and F6P, two regulatory hexoses that affect glycolytic enzymes 6-phosphofructokinase I and II (encoded by *pfkA* and *pfkB*, respectively), pyruvate kinase I (encoded by *pykF*), and PEP carboxylase (encoded by *ppc*), as well as the glycogen synthesis enzyme ADP-glucose pyrophosphorlyase (encoded by *glgC*) and carbon source import pathway enzymes glycerol kinase (encoded by *glpK*) and 1-phosphofructokinase (encoded by *fruK*) (Donahue et al., 2000). Flux through the Embden-Meyerhof pathway in the direction of glycolysis or gluconeogenesis can be allosterically controlled at the enzyme level by other metabolites as well: PEP, ATP, ADP or AMP (Romeo and Snoep, 2005).

Specific to FBPases, PEP has been proposed to be the physiological regulator (activator) of the type I FBPase encoded by *fbp* under gluconeogenic growth conditions with AMP also believed to be a primary physiological regulator (noncompetitive inhibitor) (Hines et al., 2006). This enzyme is exquisitely sensitive to inhibition by AMP, which is alleviated by PEP, although higher concentrations of PEP are inhibitory (Donahue et al., 2000). Kinetic studies have also shown that PEP is an activator and ADP an inhibitor of the type II FBPase encoded by *glpX* (Donahue et al., 2000), although more recent studies saw no effect of AMP, ADP, and PEP on enzymatic activity (Brown et al., 2009). Little is known about the type II FBPase encoded by *yggF*, however AMP, ADP, and PEP had no effect on enzymatic activity while ATP reduced activity (Brown et al., 2009). Further complicating matters are the varying substrate affinities and catalytic efficiencies, as the type I FBPase has showed higher affinity for F1,6BP than either type II FBPase (K_M for F1,6BP: *fbp*,

15.4 μM; *glpX*, 35 μM; *yggF*, 100 μM) with a higher catalytic efficiency (Brown et al., 2009).

In the context of fermentative glycerol metabolism, these regulatory and kinetic complexities involving FBPases and interconnected enzymes and intermediates may have resounding consequences. Several key enzymes involved in fermentative glycerol metabolism, including the previously discussed glycerol kinase (Section 4.2.1) as well as pyruvate kinase (Section 4.2.3), are directly regulated by F1,6BP underlying the direct importance of the levels of this metabolite. In addition, a key physiological regulator of FBPases, PEP, is a metabolic intermediate of great importance during fermentative glycerol metabolism and is directly involved in several essential pathways. The fermentative glycerol dissimilation pathway requires PEP for the phosphorylation of DHA and the importance of pathways involving the carboxylation of PEP are also detailed in subsequent sections (Section 4.2.3). The central role of PEP as metabolic intermediate in these pathways during fermentative glycerol metabolism likely makes the PEP availability of extreme importance and thus the direct regulation of FBPases through intracellular PEP levels may be vastly different compared to respiratory glycerol metabolism. This interconnection between FBPase function and regulation, glycerol dissimilation pathways, intracellular PEP pools, and other important factors could explain the involvement of multiple FBPases. Under the conditions present during fermentative glycerol metabolism, the varying kinetic, catalytic, and regulatory properties between the type I FBPase encoded by *fbp* and the two type II FBPases encoded by *glpX* and *yggF* may dictate sub-optimal activity

for each individual FBPase, however the combined expression and activity of multiple FBPases could result in appropriate overall FBPase activity required for optimal metabolite and regulation levels. Overall, this metabolic and regulatory interconnection between key enzymes, metabolic intermediates, and other factors is a key theme during fermentative glycerol metabolism, and illustrates how the switch from respiratory to fermentative conditions can have a widespread impact on numerous areas of metabolism.

4.2.3. PEP/Pyruvate nodes and carboxylating enzymes

With the conversion of DHAP into PEP through well established glycolytic enzymes (Romeo and Snoep, 2005), the next major area for investigation involves the PEP/pyruvate nodes and the carboxylating enzymes responsible for the formation of required 4 carbon intermediates (Figure 4.9). In the lack of external electron acceptors, this area of metabolism is critical, as the multiplicity of possible routes and end products with differing oxidation states and acidity enables *E. coli* to grow fermentatively on a broader range of substrates than would otherwise be possible. The high degree of reduction of glycerol, as well as the inherent coupling between glycerol dissimilation and the conversion of PEP to pyruvate resulting from the involvement of the PEP-dependent DHAK, makes this area of metabolism of great interest, as the vast differences between glycerol and more traditional carbon sources likely result in varying pathway involvement and activity.

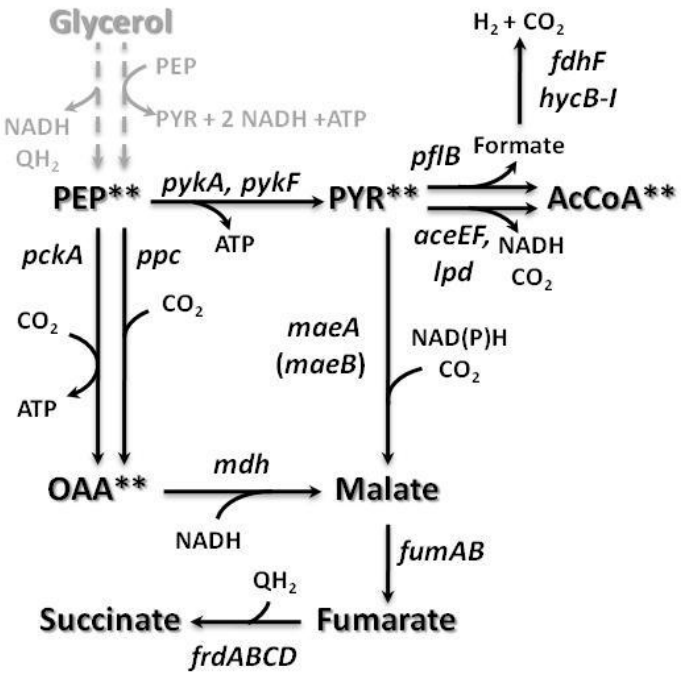

Figure 4.9 Pathways involved at the PEP/pyruvate nodes during fermentative metabolism. Broken lines illustrate multiple steps. Relevant reactions are represented by the gene(s) coding for the enzymes. See text for description. Abbreviations: AcCoA, acetyl-CoA; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate. ** denotes precursor metabolite required for cell biosynthesis.

From the PEP node, the most established routes during fermentative metabolism involve the conversion of PEP into pyruvate through either pyruvate kinase I (encoded by *pykF*) or II (encoded by *pykA*), or the carboxylation of PEP to oxaloacetate (OAA) mediated by PEP carboxylase (encoded by *ppc*) (Sauer and Elkmanns, 2005). OAA, a precursor metabolite needed for biosynthesis, can ultimately be reduced to succinate, while under fermentative conditions pyruvate conversion to acetyl-CoA (which can be subsequently converted to acetate or ethanol) is thought to take place through pyruvate formate lyase (PFL, encoded by *pflB*) with the generation of formate in the process (Sawers and Clark, 2004). Pyruvate dehydrogenase (PDH, subunits encoded by *aceEF* and *lpd*), which converts

pyruvate to acetyl-CoA with the generation of CO_2 and NADH, is typically active under respiratory conditions and the absence of activity under anaerobic conditions has been explained by the sensitivity towards NADH inhibition (de Graef et al., 1999; Sawers and Clark, 2004). While PDH activity during fermentative glucose (Murarka et al., 2010a) and glucuronate (Murarka et al., 2010b) metabolism has recently been reported, these studies demonstrated the important role of PDH to be more significant with a higher oxidized state of the carbon source, likely making its role negligible with a highly reduced substrate such as glycerol. In addition to these established routes, PEP carboxykinase (PCK, encoded by *pckA*) and both NAD^+ and $NADP^+$ linked malate dehydrogenase (encoded by *maeA* and *maeB*, respectively) are involved in the PEP/pyruvate node, although under physiological conditions the decarboxylation reaction is thought to be favored (i.e. formation of PEP or pyruvate from OAA or malate, respectively) (Figure 4.9) (Sauer and Elkmanns, 2005).

As expected under fermentative conditions, the deletion of *pflB* (encoding PFL) completely abolished glycerol fermentation, while the disruption of PDH (through the deletion of *aceF*) had minimal effects (Figure 4.10), indicting the lack of *in vivo* activity of PDH during fermentative glycerol metabolism and the importance of PFL for the formation of acetyl-CoA (from which ethanol can be synthesized). Pyruvate kinase activity appeared to be important during fermentative glycerol metabolism, with the deletion of *pykA* (encoding pyruvate kinase II) significantly decreasing glycerol consumption levels, while the disruption of pyruvate kinase I (encoded by *pykF*) had a negligible impact (Figure 4.10). When combined with the fact that a strain containing gene deletions for both pyruvate kinase I and II (i.e.

Δ*pykA*Δ*pykF*) showed similar glycerol consumption and ethanol production levels to the Δ*pykA* strain, it appears that pyruvate kinase II is the important isozyme and pyruvate kinase I expression/activity alone cannot support fermentative glycerol utilization at wild-type levels.

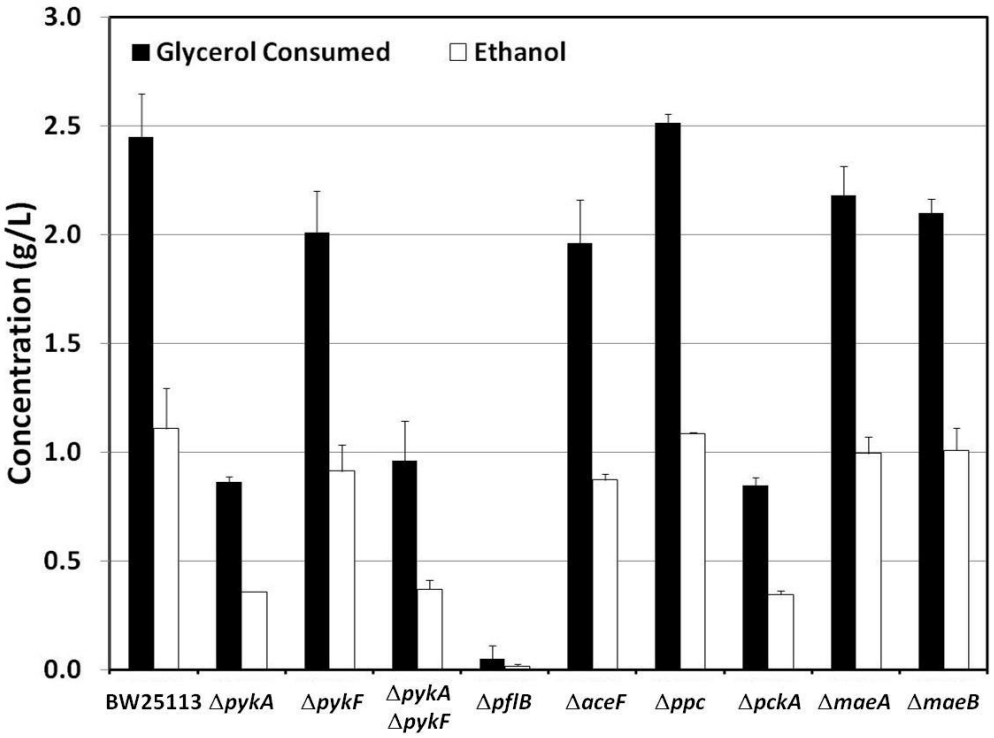

Figure 4.10 Effect of the disruption of enzymes at the PEP/pyruvate node on glycerol consumption and ethanol production. Fermentations conducted in 96 hr closed Hungate tube experiments. Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

Another surprising finding is the role of PEP carboxylating enzymes, as a mutant lacking PEP carboxykinase (Δ*pckA*) showed significantly reduced levels of glycerol consumption, while the deletion of PEP carboxylase (encoded by *ppc*), an enzyme believed to be essential during respiratory glycerol metabolism (Joyce et al., 2006), had no significant effect (Figure 4.10). To confirm the essential nature of *ppc*

during respiratory conditions, and the lack thereof for *pckA*, aerobic shake flasks were run with these strains in which glycerol utilization was abolished in the Δ*ppc* strain, while the Δ*pckA* strain exhibited wild-type glycerol consumption levels under respiratory conditions (data not shown).

The important role of pyruvate kinase during fermentative glycerol metabolism likely reflects the activity of the respiratory glycerol dissimilation pathway, in the fact that the fermentative glycerol dissimilation route directly converts PEP to pyruvate (through PEP-dependent DHAK) circumventing the need for pyruvate kinase. Glycerol dissimilation through glycerol kinase and G3P dehydrogenase however, does not involve PEP and therefore would require the activity of pyruvate kinase. This coupling between glycerol dissimilation and PEP synthesis/conversion to pyruvate and the decoupling of this mechanism through the activity of the respiratory glycerol dissimilation pathway and pyruvate kinase, has significant effects on fermentative glycerol metabolism, and is discussed in great detail in the following section. Another interesting facet in this area of fermentative glycerol metabolism is the fact that the deletion of pyruvate kinase II (Δ*pykA*) had a significant impact on glycerol consumption, while the deletion of pyruvate kinase I (Δ*pykF*) had minimal effects. While these isozymes in *E. coli* catalyze the same reaction, they differ in physical and chemical properties as well as in their kinetic behavior and are under independent genetic control (Bledig et al., 1996; Garrido-Pertierra and Cooper, 1983; Malcovati and Valentini, 1982). Of special note, is the fact that pyruvate kinase I is strongly activated by F1,6BP, while pyruvate kinase II

is activated by AMP and mono-phosphorylated sugars (Malcovati and Valentini, 1982). Therefore, the activation of pyruvate kinase I is directly linked to the complicated network of interactions involving F1,6BP, PEP, and FBPase activity, an area which has been shown to be of extreme importance during fermentative glycerol metabolism (Section 4.2.2). As a result, the observed importance of pyruvate kinase II and minimal effects of pyruvate kinase I deletion may be related to post-translational regulatory effects which pyruvate kinase I is subject to under fermentative glycerol metabolism conditions, which are circumvented by pyruvate kinase II. For example, while the K_M of pyruvate kinase I and II for PEP in the presence of activators (F1,6BP for type I and AMP for type II) are 0.15 mM and 0.1 mM respectively, these values were shifted to 8 mM in type I and 0.7 mM in type II without the presence of activators (Kotlarz et al., 1975), underlying just how critical F1,6BP levels may be for the ability of pyruvate kinase I to compete with other essential enzymes for PEP.

Interestingly, this same network of interactions involving F1,6BP, PEP, and FBPase activity may also play a role in the observed importance (or lack thereof) of PEP carboxylating enzymes. PEP carboxylase (encoded by *ppc*), whose deletion had negligible effects on glycerol consumption under fermentative conditions while required under respiratory conditions, is also strongly activated by F1,6BP, among other compounds (Yoshinaga, 1977). In the presence of F1,6BP (with no other activators), the K_M of PEP carboxylase for PEP was reported to be 1.3 mM, while in the absence of F1,6BP, this number shifted to 25 mM (NOTE: the K_M for PEP was reported to be 0.25 mM in presence of all activators) (Izui et al., 1983). Therefore,

as with pyruvate kinase I, the level of F1,6BP is of critical importance for the activity of PEP carboxylase and can directly affect the ability of this enzyme to compete with other enzymes for PEP. For example, PEP-dependent DHAK, an enzyme essential for fermentative glycerol metabolism, has a significantly higher affinity for PEP (K_M for PEP: 0.045 mM) (Gutknecht et al., 2001) than PEP carboxylase even with full activation, let alone possible low levels of F1,6BP. In contrast, PEP carboxykinase, while an enzyme believed to function in the decarboxylation direction, has the capability to compete with PEP-dependent DHAK from an enzyme kinetics standpoint (K_M for PEP: 0.07 mM) (Krebs and Bridger, 1980), and is not subject to regulation by any key intermediates in fermentative glycerol metabolism. These key enzymatic properties may underlie the rationale behind the involvement of PEP carboxykinase in place of PEP carboxylase during fermentative glycerol metabolism, which represents another significant divergence when compared to fermentative carbohydrate or respiratory glycerol metabolism.

4.2.4. Pathways and mechanisms ensuring the availability of 3-carbon intermediates

The influence of the respiratory glycerol dissimilation pathway observed through the decreases in glycerol consumption in glycerol kinase or glycerol-3-phosphate dehydrogenase (Section 4.2.1) deficient strains leaves little doubt to the importance of this pathway during fermentative glycerol metabolism. This however, is a surprising finding considering the essential nature of the fermentative glycerol dissimilation pathway consisting of glycerol dehydrogenase and PEP-

dependent dihydroxyacetone kinase. In order to fully understand fermentative glycerol metabolism, the underlying reasons behind the required activity of both pathways for maximum glycerol utilization and the influence this has on other areas of metabolism is critical. From a stoichiometric standpoint, the key difference between the fermentative and respiratory glycerol dissimilation pathways is the requirement for PEP as the phosphate group donor in the conversion of DHA to DHAP. This has the effect of creating a cycle in the metabolic network, as every mole of DHA converted to DHAP requires the availability of one mole of PEP, which in essence couples glycerol dissimilation with pyruvate synthesis (Figure 4.11). In all probability, this puts a significant strain on the intracellular PEP pool, as any carbon diverted to required precursor metabolites upstream of PEP (or PEP itself) has the effect of lowering the PEP levels. For example, any DHAP and GAL3P converted to 6-carbon intermediates reduces the amount of these molecules converted to PEP through the glycolytic pathways (Figure 4.11). Therefore, in order for glycerol dissimilation to proceed at maximum efficiency, the availability of precursor metabolites is severely limited without the regeneration of the PEP pool from a non-coupled pathway. Pyruvate can be converted back to PEP by the gluconeogenic enzyme phosphoenolpyruvate synthetase (encoded by *pps*) through an energy intensive reaction requiring an ATP be converted to AMP in the process (Figure 4.11). The respiratory dissimilation of glycerol also represents a means of regenerating the PEP pool without the coupled conversion of PEP to pyruvate, and with the observed activity of this pathway, could explain its involvement.

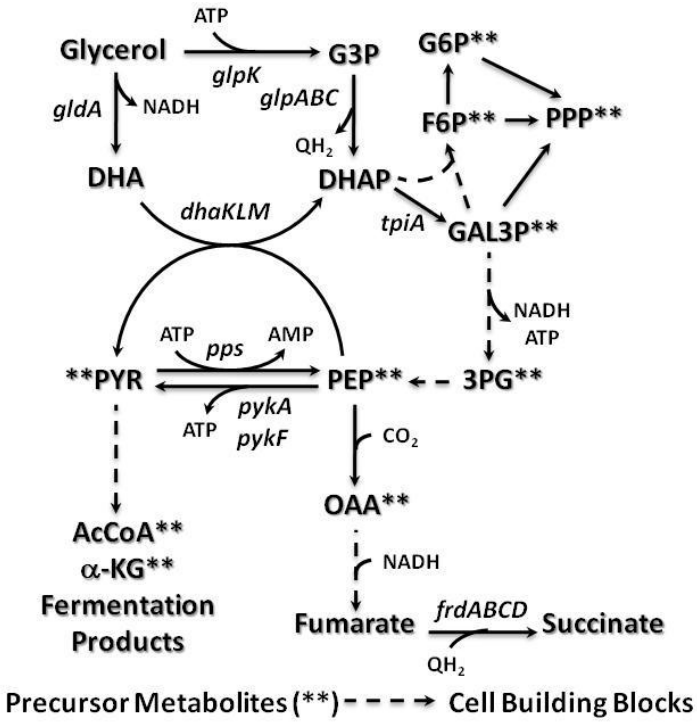

Figure 4.11 Metabolic cycle created by the involvement of PEP-dependent DHAK. Broken lines illustrate multiple steps. Relevant reactions are represented by the gene(s) coding for the enzymes. Abbreviations: 3PG, 3-phospoglycerate; α-KG, α-ketoglutarate; AcCoA, acetyl-CoA; DHA, dihydroxyacetone; DHAP, DHA phosphate; F1,6-BP, fructose-1,6-bisphosphate; F6-P, fructose-6-phosphate; G3P, glycerol-3-phosphate; G6-P, glucose-6-phosphate; GAL3P, glyceraldehyde-3-phosphate; OAA, oxaloacetate; PPP, pentose phosphate pathway; PEP, phosphoenolpyruvate; PYR, pyruvate; ** denotes precursor metabolite required for cell biosynthesis.

While the respiratory glycerol dissimilation pathway can partially decouple glycerol dissimilation and pyruvate synthesis, several other issues are created with its involvement during fermentative glycerol metabolism. In this pathway, after glycerol phosphorylation through glycerol kinase, G3P is oxidized by anaerobic G3P dehydrogenase to form DHAP (Figure 4.11). However, as opposed to the oxidation reaction in the fermentative glycerol dissimilation pathway in which the electron acceptor is NAD^+, the electron acceptor in the process is coupled the quinone pool

(Schryvers and Weiner, 1981). In the absence of externally provided electron acceptors (such as nitrate), these electrons must be passed through the electron transport chain with internally generated fumarate as the terminal acceptor (Schryvers and Weiner, 1981). Therefore, any activity of the respiratory glycerol dissimilation pathway would require the reduction of fumarate to succinate through fumarate reductase (encoded by *frdABCD*) (Figure 4.11). In addition, the involvement of the respiratory glycerol dissimilation pathway would also likely require the activity of pyruvate kinase for conversion of PEP to pyruvate, an enzyme shown to be important during fermentative glycerol metabolism (Section 4.2.3).

In order to test this hypothesis behind the importance of the respiratory glycerol dissimilation pathway, strains in which the proposed mechanisms were inhibited were tested for their effects on fermentative glycerol metabolism (Figure 4.12). As expected with the involvement of the respiratory glycerol dissimilation pathway, the deletion of *frdA* (encoding a catalytic domain subunit of fumarate reductase) lead to a decrease in glycerol consumption under fermentative conditions compared to wild-type BW25113, indicating the importance of the reduction of fumarate to succinate (Figure 4.12). In addition, further evidence to the requirement for a mechanism to decouple glycerol dissimilation and pyruvate synthesis was given by the fact that while a *pps* deletion alone had only a minor impact, when other decoupling mechanisms were impaired, the deletion of *pps* had a significant impact on glycerol consumption levels (i.e. Δ*glpK*Δ*pps* and Δ*frdA*Δ*pps*) (Figure 4.12).

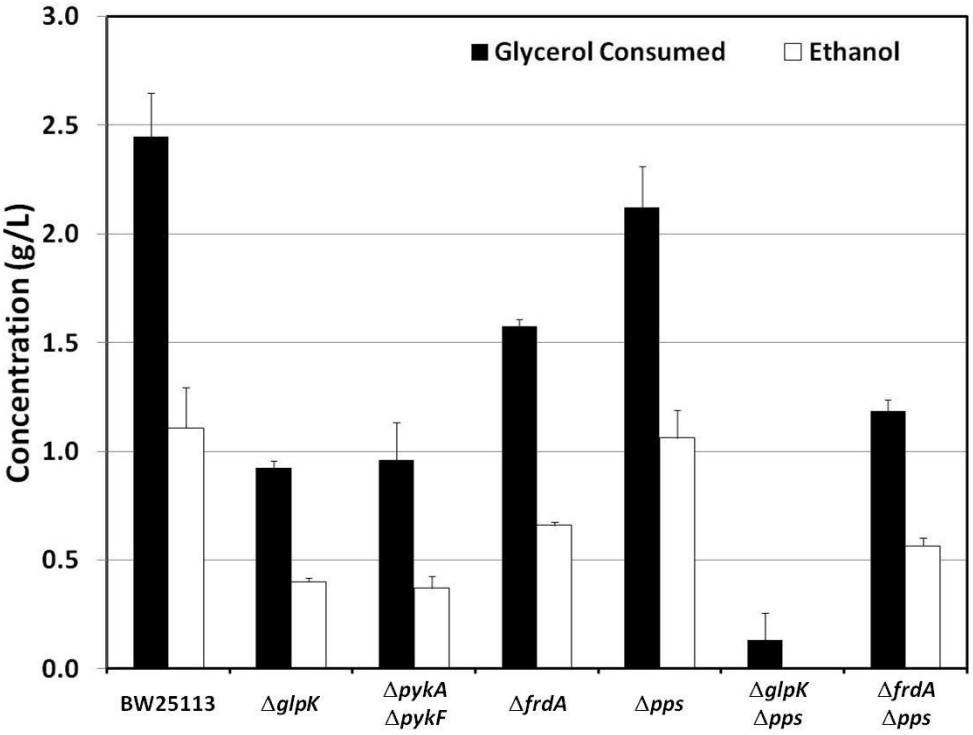

Figure 4.12 Effects of the deletion of proposed decoupling mechanisms on glycerol consumption and ethanol production. Fermentations conducted in 96 hr closed Hungate tube experiments. Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

In fact, the disruption of both the respiratory glycerol dissimilation pathway and phosphoenolpyruvate synthetase resulted in glycerol consumption levels similar to those seen in strains lacking the fermentative glycerol dissimilation pathway, indicating the essential nature of a mechanism for decoupling glycerol dissimilation and pyruvate synthesis during fermentative glycerol metabolism.

Further evidence to support the role of these mechanisms as means of decoupling glycerol dissimilation and pyruvate synthesis, as well as illustrate inefficient nature of these decoupling mechanisms is provided by the expression of an ATP-dependent DHAK from *Citrobacter freundii* (*dhaKL*), which utilizes ATP as

the phosphate group donor instead of PEP (Daniel et al., 1995). Expression of this enzyme in *E. coli* provides a glycerol dissimilation pathway decoupled from pyruvate synthesis, whose oxidation reaction is still liked to NAD^+, thus negating the need for reactions linked to the electron transport chain.

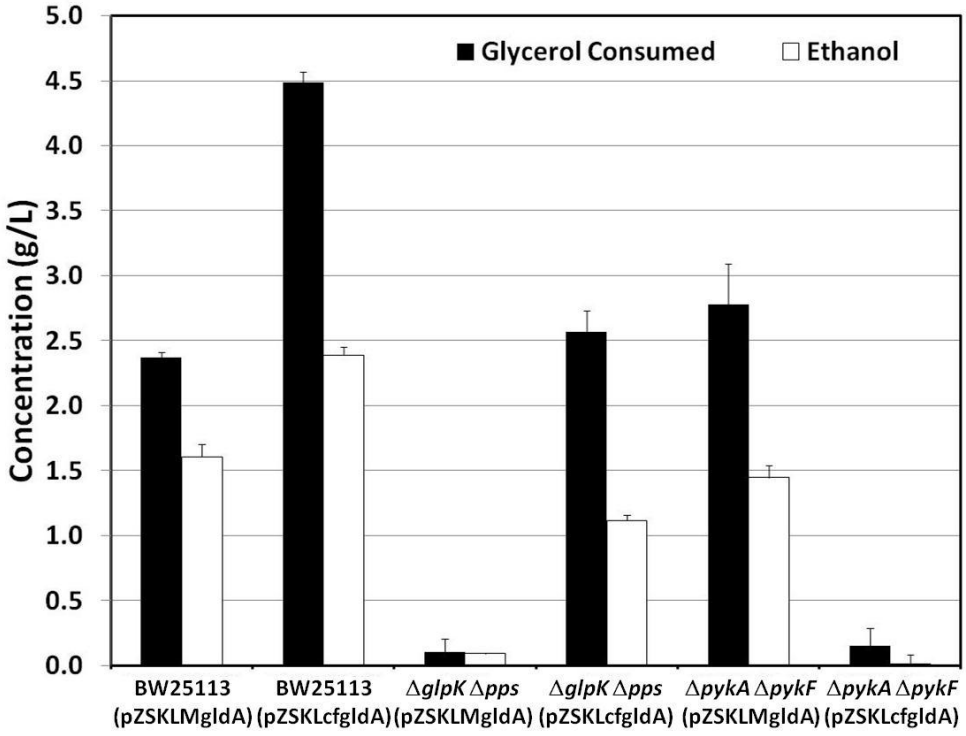

Figure 4.13 Glycerol consumption and ethanol production upon the overexpression of ATP- or PEP-linked fermentative glycerol dissimilation pathways. Fermentations conducted in 96 hr closed Hungate tube experiments. Δ"*gene*" represents gene deletion. Gene overexpressions are indicated by the plasmid name in parenthesis (i.e. pZSKLMgldA expressing *E. coli* PEP-dependent DHAK and *E. coli* glycerol dehydrogenase and pZSKLcfgldA expressing *C. freundii* ATP-dependent DHAK and *E. coli* glycerol dehydrogenase). Error bars represent standard deviations for a minimum of triplicate measurements.

The more efficient nature of this decoupled pathway is seen from the significant increase in glycerol consumption when the ATP-linked fermentative glycerol dissimilation pathway is expressed (i.e. BW25113 with pZSKLcfgldA) compared to

that with the PEP-linked fermentative glycerol dissimilation pathway is expressed (i.e. BW25113 with pZSKLMgldA) (Figure 4.13). In addition, the important role of the decoupling mechanisms is further emphasized by the fact that only the expression of the ATP-linked fermentative glycerol dissimilation pathway enables recovery of glycerol utilization ability in strains deficient in the respiratory glycerol dissimilation pathway and phosphoenolpyruvate synthetase (Δ*glpK*Δ*pps*), while pyruvate kinase activity is essential when expression ATP-linked fermentative glycerol dissimilation linearizes the pathway from glycerol to pyruvate (Figure 4.13).

Overall, the coupling between glycerol dissimilation and pyruvate synthesis created by the involvement of PEP-dependent DHAK is a critical component of fermentative glycerol metabolism and the need to regenerate the PEP pool for maximum glycerol utilization has resounding impacts on other areas of metabolism. From a fundamental standpoint, the elucidation of the decoupling mechanisms through the involvement of the respiratory glycerol dissimilation pathway and phosphoenolpyruvate synthetase greatly increases the knowledge of the challenges associated with fermentative glycerol metabolism and the variations in key areas of metabolism created by the use of glycerol as a carbon source. This is also an important paradigm from an application standpoint, as the conversion of an intermediate upstream of PEP to a product of interest may prove difficult due to the coupling mechanism involved in fermentative glycerol dissimilation.

4.2.5. Regulation of fermentative glycerol metabolism

In light of these new findings regarding the fermentative metabolism of glycerol in *E. coli*, very little is known about the regulation of glycerol metabolism as the current knowledge of regulation is centered on the respiratory model of glycerol metabolism. Glycerol kinase is considered the pacemaker in the current regulatory model of respiratory metabolism of glycerol and it has been reported that glucose-glycerol diauxie can be abolished by the constitutive production of a GK mutant resistant to inhibition by F1,6BP (Zwaig et al., 1970). However, while important, the fact that the glycerol kinase is not the central enzyme in glycerol dissimilation requires that the current regulatory models based on glycerol kinase be revisited. On the other hand, catabolite repression exerted by glycerol (on sugars such as maltose) is mediated by the control G3P exerts over the stimulation of adenylate cyclase by phosphorylated $EIIA^{Glc}$, the latter a component of the glucose PTS system (Eppler and Boos, 1999). Evidence from the previously discussed sections indicate that pathways mediating fermentative glycerol metabolism involve both G3P as an intermediate and interact with the PTS, a system involved in carbohydrate uptake in addition to the control of carbon metabolism. This interaction could be a central piece in new regulatory models of glycerol utilization, as the second enzyme of the fermentative glycerol dissimilation pathway, PEP-dependent DHAK, uses PTS as a source of high-energy phosphate and therefore becomes integrated in and put under the control of the PTS system (Bachler et al., 2005). Additionally, two subunits of PEP-dependent DHAK (DhaK and DhaL) exhibit feedback control over their own expression and could potentially exert a regulatory action on other

operons (Bachler et al., 2005). Overall, the limited knowledge regarding the regulation of fermentative metabolism of glycerol highlights the need for further studies to understand the key regulatory mechanisms.

In order to determine the key regulation aspects of fermentative glycerol metabolism, the effects of the deletion of genes encoding known global regulatory proteins on glycerol consumption were tested (Figure 4.14). These deletions included *crp* (encoding the CRP dual transcription regulator), *cyaA* (encoding adenylate cyclase), *arcA* (encoding the ArcA transcriptional dual regulator), *arcB* (encoding the ArcB sensory histidine kinase), *creB* (encoding the CreB transcriptional regulator), *creC* (encoding the CreC sensory histidine kinase), *dgsA* (encoding the DgsA DNA-binding transcriptional regulator, as known as Mlc), *fruR* (encoding the FruR DNA-binding transcriptional dual regulator, also known as Cra, Catabolite repressor activator), *fnr* (encoding the FNR DNA-binding transcriptional dual regulator), *frsA* (encoding the FrsA fermentation/respiration switch protein), *hns* (encoding the H-NS DNA-binding transcriptional dual regulator), *uspA* (encoding the UspA universal stress global stress response regulator), and *ompR* (encoding the OmpR response regulator). In order to ascertain if any observed effects were key only under fermentative conditions or if the deletions were influential under all conditions (i.e. respiratory and fermentative), aerobic shake flasks were also conducted with these gene deletion strains (Figure 4.14).

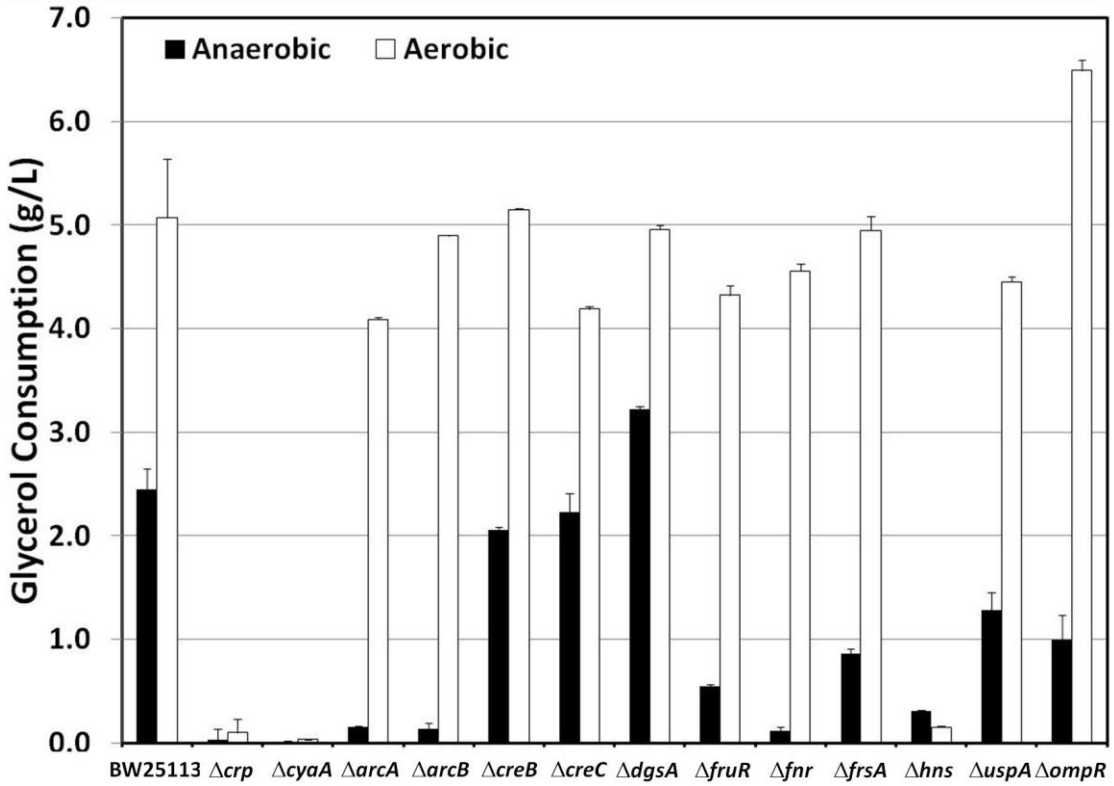

Figure 4.14 Effects of the deletion of genes encoding global regulatory proteins on glycerol consumption under fermentative and respiratory conditions. Anaerobic fermentations conducted in 96 hr closed Hungate tube experiments while aerobic experiments run in 24 hr shake flasks. Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

While the determination of the specific underlying causes behind the effects of the deletion of regulators is difficult due to the large number of genes many of these proteins regulate, several interesting aspects are apparent from the effects their deletion has on glycerol consumption. The deletion of *crp*, *cyaA*, and *hns* were the only gene deletions resulting in a significant impairment of glycerol consumption under both fermentative and respiratory conditions (Figure 4.14). *crp* and *cyaA*, encoding the CRP dual transcription regulator and adenylate cyclase, respectively, are essential components of the CRP-cAMP DNA-binding

transcriptional dual regulator, as the binding of cyclic AMP (cAMP) synthesized by adenylate cyclase to the CRP receptor protein is required for the activation of the transcriptional regulator (Deutscher et al., 2006). While the effects of CRP-cAMP disruption on respiratory glycerol consumption can be attributed to involvement of this complex in carbon catabolism and specifically in the regulation of genes involved in the respiratory glycerol dissimilation pathway (Deutscher et al., 2006), the negative effects of the deletions under fermentative conditions could indicate the involvement and regulation of the fermentative glycerol dissimilation pathway due to its link to CRP-cAMP and the PTS through the PEP-dependent DHAK (See above). The H-NS ("Histone-like nucleoid structuring protein") DNA-binding transcriptional dual regulator (encoded by *hns*) functions almost exclusively as a transcriptional repressor which plays an important role in the regulation of many genes in response to environmental changes and adaptation to stress (Dorman, 2004), making its importance under both fermentative and respiratory conditions expected.

The severe impairment of fermentative glycerol consumption with the deletion of *arcA*, *arcB*, and *fnr* (Figure 4.14) can be explained by their roles in the activation of operons involved in fermentative metabolism. The ArcA transcriptional dual regulator, which requires the membrane-associated histidine kinase ArcB and FNR DNA-binding transcriptional dual regulator for full expression and activation (Compan and Touati, 1994; Iuchi and Lin, 1992), is a regulator for anoxic redox control which acts primarily as a negative transcriptional regulator under anaerobic conditions repressing operons involved in respiratory metabolism

(Gunsalus and Park, 1994; Iuchi and Lin, 1991). However, this system has also been shown to activate a few operons encoding proteins involved in fermentative metabolism (Brondsted and Atlung, 1994; Lynch and Lin, 1996), including pyruvate formate-lyase (PFL, encoded by *pflB*) (Sawers and Suppmann, 1992), which as previously shown is essential during fermentative glycerol metabolism (Section 4.2.3) linking the effects on glycerol consumption of *arcA*, *arcB*, and *fnr* deletion to *pflB* expression. The impact of the deletion of the FruR DNA-binding transcriptional dual regulator on fermentative glycerol utilization (Figure 4.14) can also be explained by its regulation of key fermentative operons. FruR plays a pleiotropic role to modulate the direction of carbon flow through different metabolic pathways of energy metabolism independently of the CRP regulator (Ramseier et al., 1995), and has been implicated in the expression of a large number of operons that encode enzymes comprising central pathways of carbon metabolism (Bledig et al., 1996; Ramseier et al., 1993; Ramseier et al., 1995). Specifically for fermentative glycerol metabolism, FruR has been shown to activate a number of important gluconeogenic enzymes including *pckA* (Ramseier et al., 1995) and *pps* (Ramseier et al., 1993), as well as playing a role in the regulation of the *ptsHI-crr* operon (Ramseier et al., 1993; Shimada et al., 2005) required for the phosphotranfer to DHA in the fermentative glycerol dissimilation pathway. Also of note is the fact that FruR forms a complex with DNA in the absence of fructose 1-phosphate and fructose 1,6-bisphosphate (F1,6BP) and is inactivated upon interaction with these metabolites (Bledig et al., 1996; Ramseier et al., 1993; Ramseier et al., 1995), linking another key mechanism during fermentative glycerol metabolism to the intracellular F1,6BP

concentration. In addition to the impact of these gene deletions, the deletion of *frsA*, *uspA*, and *ompR* also had a negative effect on fermentative glycerol utilization (Figure 4.14), although the exact mechanisms behind their involvement is unclear due to the relative lack of knowledge of these global regulators.

While the impact of the majority of global regulator deletions can be attributed to their regulation of key aspects of fermentative metabolism, the importance of the CRP-cAMP complex indicated by the severe impairment of glycerol utilization upon deletion of *cyaA* or *crp*, could directly relate to the regulation of key aspects of glycerol metabolism. The transcriptional dual regulator CRP-cAMP regulates the expressions of over 180 genes in *E. coli* (Grainger et al., 2005; Zheng et al., 2004), and its impact on fermentative glycerol metabolism could be related to the regulation on any number of genes. However, one key aspect of CRP-cAMP regulation is the fact that many of the regulated genes are involved in the catabolism of secondary carbon sources (Deutscher, 2008) and as such CRP-cAMP plays a direct role in catabolite repression and inducer exclusion. These systems are inherently linked to the phosphotransferase system through the interplay between CRP, adenylate cyclase, cAMP, and the glucose-specific-enzyme IIA ($EIIA^{Glc}$) PEP:sugar PTS (Deutscher et al., 2006). With the essential involvement of PTS enzymes in the fermentative glycerol dissimilation pathway for the phosphorylation of DHA through PEP-dependent DHAK (See above and Section 4.2.1), it stands to reason that the impacts of *crp* and *cyaA* deletion could be related to the transcriptional regulation of components of this key enzyme. In addition, the transcriptional regulation of the *ptsA-fsaB-gldA* operon, with PtsA showing

similarity to sugar PTS components and glycerol dehydrogenase an essential enzyme for glycerol fermentation in *E. coli*, is poorly understood and the severe impairment of glycerol utilization with a *crp* or *cyaA* deletion could also be explained by the lack of expression of this operon.

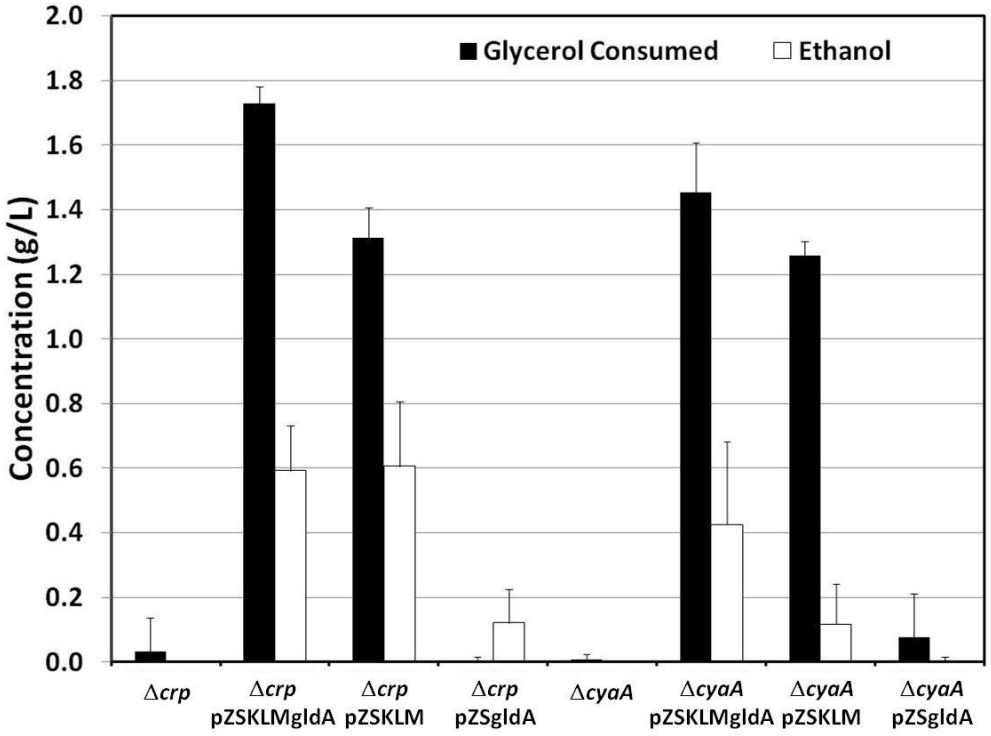

Figure 4.15 Effect of overexpressing *dhaKLM* and *gldA* in CRP-cAMP deficient genetic bacgrounds on fermentative glycerol utilization. Fermentations conducted in 96 hr closed Hungate tube experiments. Δ"*gene*" represents gene deletion. Gene overexpressions are indicated by the plasmid name (i.e. pZSKLMgldA expressing *E. coli* PEP-dependent DHAK and glycerol dehydrogenase, pZSKLM expressing *E. coli* PEP-dependent DHAK, and pZSgldA expressing *E. coli* glycerol dehydrogenase). Error bars represent standard deviations for a minimum of triplicate measurements.

In order to assess the possible CRP-cAMP dual transcription factor activation of the transcription of the *dhaKLM* and/or *ptsA-fsaB-gldA* operons, *crp* and *cyaA* deletion strains containing plasmids allowing controlled expression of *gldA*

(pZSgldA), *dhaKLM* (pZSKLM) or both (pZSKLMgldA) were tested for their ability to utilize glycerol under fermentative conditions (Figure 4.15). While the expression of *gldA* in these deletion mutants did not result in the ability to utilize glycerol, expression of *dhaKLM* and co-expression of *dhaKLM* and *gldA* in either the Δ*crp* or Δ*cyaA* background enabled the fermentative utilization of glycerol (Figure 4.15). These results clearly demonstrate that the independent expression of *dhaKLM* in strains deficient in the CRP-cAMP dual transcriptional regulator confers the ability for the fermentative utilization of glycerol, which implies the CRP-cAMP activation of the *dhaKLM* operon. This is a key finding in regards to regulatory aspects of fermentative glycerol utilization from both a fundamental knowledge standpoint, as well as an applied perspective, as any catabolite repression exerted on fermentative glycerol utilization by glucose (or other carbon sources) through a CRP-cAMP mechanism can be abolished through the independent expression of *dhaKLM*. This could have resounding importance for the conversion of thin stillage, a complex mixture of chemicals including low concentration of various sugars (namely glucose and maltose) and up to 2% glycerol which is generated in large amounts during the current industrial ethanol production process, to fuels and chemicals (Rausch and Belyea, 2006).

4.3. Kinetics of fermentative glycerol utilization

While previous sections detailed the elucidation of key pathways, mechanisms, and regulatory aspects that provide a detailed knowledge base on the

fermentative metabolism of glycerol, another key aspect, especially from an application standpoint, relates to the rate by which glycerol is fermented. Compared to glucose fermentation, the utilization of glycerol by *E. coli* under fermentative conditions is significantly slower (Dharmadi et al., 2006), leading to lower productivities of product formation. In order to be viable on an industrial scale, the most efficient process for the conversion of glycerol to fuels and chemicals need to be identified. Therefore, a detailed quantitative analysis of the kinetics and control structure of fermentative glycerol utilization, complementing the previous findings, can be used gain a better understanding of glycerol fermentation and identify key targets for genetic manipulation that could enhance the rate of fuel and chemical from glycerol.

Previous kinetic modeling studies of glycerol fermentation with wild-type *E. coli* have recently been reported (Cintolesi, 2010), and serve as the basis for the experimental analysis of the kinetics of glycerol fermentation. In this study, the kinetic model focused on the glycerol to ethanol pathway, utilizing the fermentative glycerol dissimilation route (i.e. glycerol dehydrogenase and PEP-dependent dihydroxyacetone kinase) with kinetic expressions and parameters for all enzymatic reactions taken from literature and experimental data (Cintolesi, 2010). The dynamic model, consisting of mass balance equations for extracellular glycerol, ethanol, biomass and 11 intracellular metabolites, was able to adequately represent the experimental concentration profiles of glycerol, ethanol, and biomass (Cintolesi, 2010). The kinetic model then enabled the use metabolic control analysis (MCA) (Stephanopoulos et al., 1998) to elucidate the control architecture of the pathways

involved in the fermentative metabolism of glycerol in *E. coli*. Calculation of the flux control coefficients (FCCs) (Stephanopoulos et al., 1998) revealed a clear picture of how the flux through the pathway mediating glycerol utilization and its conversion to ethanol is controlled, as two steps in the pathway, namely the glycerol dissimilation reactions catalyzed by glycerol dehydrogenase and dihydroxyacetone kinase, exhibited flux control coefficients of 0.44 and 0.56, respectively, while all other had values at or close to zero (Table 4.2). These results indicate that glycerol dehydrogenase and dihydroxyacetone kinase have nearly total control of the overall pathway flux.

Table 4.2 Calculated flux control coefficients for the glycerol to ethanol fermentative pathway (Cintolesi, 2010)

Enzyme/Reaction	Flux control coefficient (FCC)
GlpF (glycerol transporter, encoded by *glpF*)	0.003
glyDH (glycerol dehydrogenase, encoded by *gldA*)	0.44
DHAK (PEP-dependent dihydroxyacetone kinase, encoded by *dhaKLM*)	0.56
TPI (triose phosphate isomerase, encoded by *tpiA*)	0
GAPDH (glyceraldehyde 3-phosphate dehydrogenase, encoded by *gapA*)	0
PGK (phosphoglycerate kinase, encoded by *pgk*)	0
PGM (phosphoglycerate mutase, encoded by *gpmM*, *gpmA*)	0
ENO (enolase, encoded by *eno*)	0
PFL (pyruvate formate-lyase, encoded by *pflB*)	0
ALDH (aldehyde dehydrogenase, encoded by *adhE*)	0
ADH (alcohol dehydrogenase, encoded by *adhE*)	0

Based on these findings, the overexpression of glycerol dehydrogenase and dihydroxyacetone kinase should significantly increase the metabolic fluxes associated with glycerol utilization and ethanol synthesis. In order to validate these findings, glycerol dehydrogenase and dihydroxyacetone kinase were overexpressed

in wild-type *E. coli* MG1655, singularly and in combination. The kinetics of glycerol fermentation of these strains in a controlled environment (SixFors multi-fermentation system, see Materials and Methods) were observed to provide the most detailed assessment of the impact of these overexpressions. Confirmation of the amplification of these enzymes was conducted via a functional characterization of the enzyme activities in these strains (Table 4.3).

Table 4.3 Enzyme activities and glycerol utilization and ethanol synthesis fluxes for wild-type MG1655 and strains overexpressing glycerol utilization enzymes

Strain	Enzyme Activity [a]		Metabolic Fluxes [b]	
	Glycerol dehydrogenase	Dihydroxyacetone kinase	Glycerol utilization	Ethanol synthesis
MG1655 (pZSblank)	0.114 ± 0.002	0.016 ± 0.001	4.6±0.7	4.1±0.5
MG1655 (pZSgldA)	0.62 ± 0.03	NM	4.3±0.9	3.9±0.8
MG1655 (pZSKLM)	NM	0.03 ± 0.03	6.4±0.9	6±2
MG1655 (pZSKLMgldA)	0.41 ± 0.01	0.037 ± 0.001	10.7±0.8	10±1

[a] Activities (μmol/mg protein/min) were measured during active growth as described in Materials and Methods. NM: not measured

[b] Fluxes (mmol/g CDW/hr) represent average values for fluxes calculated during the active growth phase

While individual overexpression of glycerol dehydrogenase or dihydroxyacetone kinase alone had only a minor impact on glycerol metabolism, simultaneous overexpression of both enzymes led to a 2.4-fold increase in the glycerol-utilization and ethanol-synthesis fluxes (Table 4.3). The magnitude of these changes can be better appreciated when the kinetics of glycerol utilization of strains MG1655 (pZSblank) and MG1655 (pZSKLMgldA) are compared (Figure 4.16). For example, after 24 hours of cultivation, strain MG1655 (pZSKLMgldA) consumed about 80% of the total glycerol initially present in the medium (Figure 4.16) and synthesized close

to 4 g/L of ethanol (data not shown). This is approximately 6 times the amount of glycerol consumed and ethanol synthesized by strain MG1655 (pZSblank) over the same period of time (Figure 4.16).

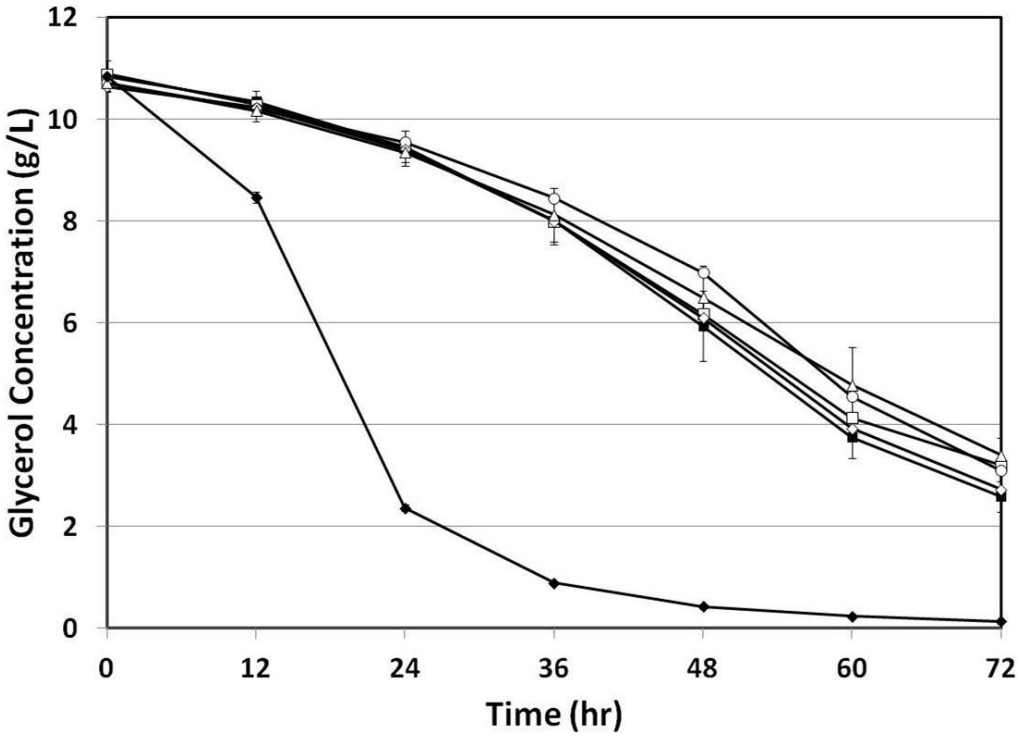

Figure 4.16 Effect of the overexpression of enzymes/proteins involved in glycerol metabolism on the kinetics of glycerol utilization. Experiments conducted in fully controlled fermentor at pH 6.3. Glycerol concentration shown for MG1655 expressing pZSblank (■), pZSglpF (○), pZStpiA (□), pZSpflB (◊), pZSadhE (Δ), and pZSKLMgldA (♦). Gene overexpression indicated by plasmids name. See text for details.

In order to further support the results predicted by the kinetic and metabolic control analyses, the effect of increasing the level of other enzymes/proteins that were predicted to have no control over the flux of the pathway was also tested. For this purpose the overexpression of *glpF* (glycerol facilitator protein), *tpiA* (triose phosphate isomerase), *pflB* (pyruvate formate lyase) and *adhE* (aldehyde/alcohol

dehydrogenase) were selected given their important role in the metabolism of glycerol (See previous sections). In agreement with the model predictions and MCA, overexpression of *glpF*, *tpiA*, *pflB* or *adhE* did not affect glycerol utilization or ethanol synthesis (Figure 4.16).

These findings complement the elucidated pathways, mechanisms, and regulatory aspects of fermentative glycerol metabolism and help to provide a clear picture of the kinetics of glycerol fermentation and the enzymes influential in dictating these kinetics. While the pathways modeled do not include the entirety of important pathways during fermentative glycerol metabolism, the experimental results indicate that the predicted control structure can accurately represent the entire metabolic network. In the context of glycerol dissimilation, these findings verify the essential importance of the fermentative glycerol dissimilation pathway on not only the ability for *E. coli* to utilize glycerol under fermentative conditions, but also the rate at which it does so. While this not only significantly increases the breadth of the knowledge base on fermentative glycerol metabolism in *E. coli*, from a application standpoint this is a critical observation as the overexpression of these two key enzymes could likely complement any downstream metabolic engineering strategies aimed at fuel and chemical production from glycerol to provide a means of increasing the rate at which the target product is produced.

4.4. Improved model for fermentative glycerol metabolism in *Escherichia coli*

The comprehensive study into the fermentative metabolism of glycerol in *E. coli* has elucidated several key elements that help provide more overall clarity into important pathways and mechanisms mediating this metabolic process. At the heart of these new findings are the challenges associated with the use of glycerol as a carbon source which results in several stark contrasts between fermentative glycerol metabolism and the fermentative metabolism of less reduced carbon sources, such as common sugars. A summary of the improved understanding of the metabolic network during fermentative glycerol metabolism in *E. coli* is provided in Figure 4.17. This improved model for fermentative glycerol metabolism includes fundamental knowledge on both essential and important pathways as well as regulatory and kinetic aspects to provide the detailed understanding needed for the rational design of metabolic engineering strategies for fuel and chemical production of glycerol.

Under fermentative conditions, the dissimilation of glycerol into the glycolytic intermediate DHAP was found to require the activity of both the recently discovered fermentative dissimilation pathway, as well as the established respiratory pathway for maximal glycerol utilization. This phenomenon was found to be inherently linked to the coupling of the fermentative glycerol dissimilation pathway to pyruvate synthesis through the essential nature of PEP-dependent DHAK for DHA phosphorylation.

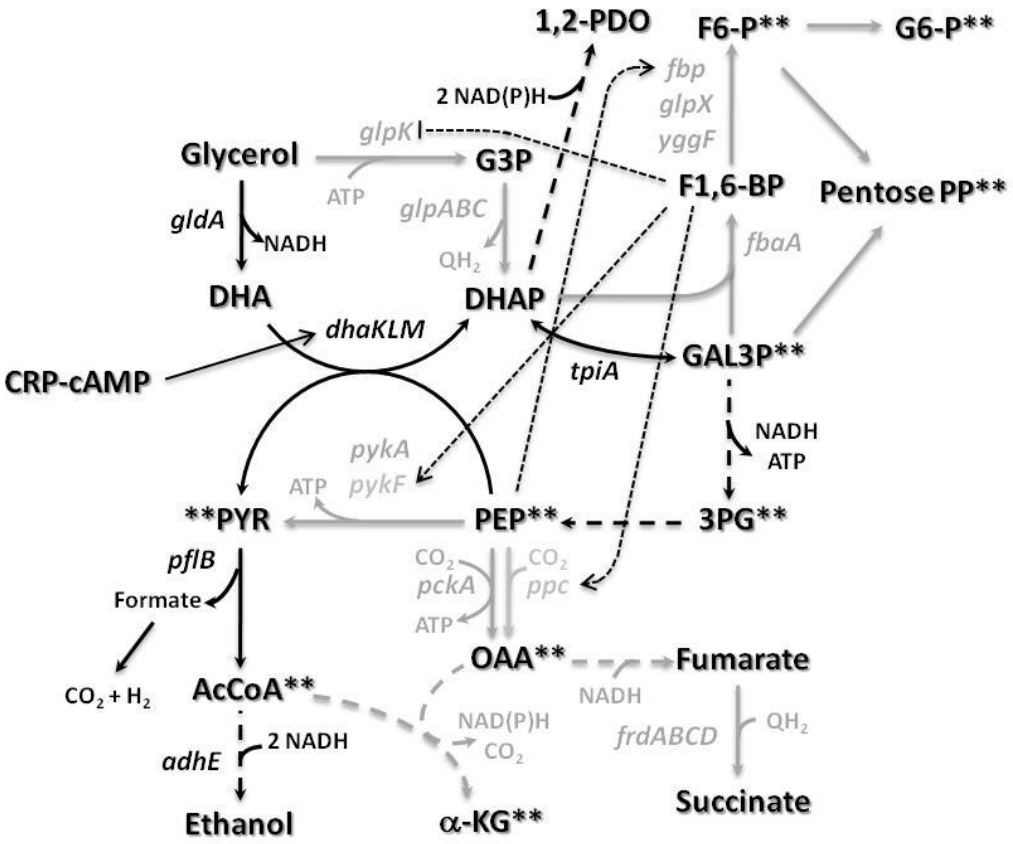

Figure 4.17 Improved model of fermentative glycerol metabolism in *Escherichia coli*. Relevant reactions are represented by the gene(s) coding for the enzymes. Thick broken lines illustrate multiple reaction steps. Black thick lines and genes represent reactions/genes established to be essential for fermentative glycerol metabolism in this study. Gray lines and genes represent reactions/genes important but not essential during fermentative glycerol metabolism. Regulatory aspects represented by thin solid (transcritpional regulation) or thin dashed (post-translational regulation) with ⊥ arrowheads for negative repression/inhibition and ↓ arrowheads for postitive regulation/activation. Abbreviations: 1,2-PDO, 1,2-propanediol; 3PG, 3-phospoglycerate; α-KG, α-ketoglutarate; AcCoA, acetyl-CoA; DHA, dihydroxyacetone; DHAP, DHA phosphate; F1,6-BP, fructose-1,6-bisphosphate; F6-P, fructose-6-phosphate; G3P, glycerol-3-phosphate; G6-P, glucose-6-phosphate; GAL3P, glyceraldehyde-3-phosphate; OAA, oxaloacetate; Pentose PP, pentose phosphate pathway; PEP, phosphoenolpyruvate; PYR, pyruvate.. ** denotes precursor metabolite required for cell biosynthesis.

The coupling between these two key areas of fermentative glycerol metabolism resulted in limited availability of 3-carbon intermediates upstream of pyruvate

without the activity of a decoupling mechanism. Several of these intermediates are not only required for biosynthetic purposes, but are also key components of important pathways and mechanisms, including 1,2-PDO synthesis (DHAP) and the fermentative glycerol dissimilation pathway (PEP). While phosphoenolpyruvate synthetase could regenerate the intracellular PEP pool, enabling continued glycerol dissimilation through the fermentative pathway, the ATP intensive nature of this process resulted in the activity of the respiratory glycerol dissimilation pathway providing a more efficient decoupling mechanism. Glycerol dissimilation through this pathway is not constrained by requirement for PEP generation, and as such provides an effective decoupling mechanism to enable increased metabolite availability upstream of PEP. However, the activity of this pathway has significant impacts in other areas of metabolism, as the linear pathway to pyruvate created by the dissimilation pathway necessitates the activity of pyruvate kinase, and the electron acceptor in the oxidation of glycerol-3-phosphate adds additional complexity. The oxidation mediated by anaerobic-G3P dehydrogenase is coupled to the quinone pool, and in the absence of externally provided electron acceptors, these electrons must be passed through the electron transport chain with internally generated fumarate as the terminal acceptor. Thus the reduction of fumarate to succinate through fumarate reductase was also found be an important element during fermentative glycerol metabolism, enabling the flux through the respiratory glycerol dissimilation pathway.

The formation of 6-carbon intermediates from 3-carbon intermediates is another unique area of fermentative glycerol metabolism and the importance of

fructose-1,6-bisphosphotases (FBPases) were discovered from investigation of this area of metabolism. The 3-carbon nature of glycerol dictates the involvement of gluconeogenic pathways for 6-carbon intermediate formation, and while the formation of fructose-1,6-bisphosphate (F1,6BP) was inferred to take place through traditional routes, the conversion of F1,6BP to fructose-6-phosphate proved to be an intriguing area. Under respiratory conditions, the type I FBPase encoded by *fbp* has been shown to be the only FBPase essential for growth on glycerol, however under fermentative conditions it was determined that in addition to *fbp*, the type II FBPases encoded by *glpX* and *yggF* also play a role in glycerol metabolism. This demonstrates a possible physiological role for these two type II FBPases (*glpX* and *yggF*), as their functions in *E. coli* have yet to be determined. The varying kinetic properties, substrate affinities and catalytic efficiencies of the three FBPases, and the network of interactions involving F1,6BP, PEP, and FBPase activity could explain the involvement of the three distinct FBPases. Of critical importance are the concentrations of F1,6BP and F6P, two regulatory hexoses that exhibit regulatory affects on several key enzymes, including glycerol kinase (encoded by *glpK*), pyruvate kinase I (encoded by *pykF*), and PEP carboxylase (encoded by *ppc*). This fact, combined with the regulatory affects of PEP on FBPase activity creates a complicated network of regulatory interactions involving key metabolic intermediates of fermentative glycerol metabolism. Specifically, the low intracellular levels of PEP likely seen during fermentative glycerol metabolism (due to the coupling between fermentative glycerol dissimilation and PEP to pyruvate conversion) can explain the observed effects of FBPase deletions. While the type I

FBPase encoded by *fbp* is the only required FBPase during respiratory conditions, the fact that this enzyme is strongly activated by PEP would result in less than optimal activity during fermentative glycerol metabolism with low intracellular PEP levels. In the absence of additional FBPases, the accumulation of F1,6BP would lead to a decrease in the activity of the respiratory glycerol dissimilation pathway, as F1,6BP serves as an allosteric inhibitor of glycerol kinase. Therefore, the observed importance of the type II FBPases encoded by *glpX* and *yggF* is likely a result of the ability for these enzymes to complement the decreased type I FBPase activity, as these enzymes have not be definitively shown to be activated by PEP. While these enzymes can complement the decreased type I FBPase activity, their comparative lower affinity for F1,6BP (K_M for F1,6BP: *fbp*, 15.4 μM; *glpX*, 35 μM; *yggF*, 100 μM) and lower catalytic efficiency underlie the requirement for all three enzymes for maximal glycerol utilization under fermentative conditions.

The key role of intracellular F1,6BP levels also underlies the finding of minimal pyruvate kinase I and PEP carboxylase involvement during fermentative glycerol metabolism. While pyruvate kinase activity was found to be important, due to the involvement of the respiratory glycerol dissimilation pathway, only the deletion of pyruvate kinase II (encoded by *pykA*) had a significant impact on the ability to utilize glycerol under fermentative conditions. This could be a result of the fact that while pyruvate kinase II is independent of F,1,6BP activation, the levels of F1,6BP are critical for pyruvate kinase I activity, and without proper activation the affinity of pyruvate kinase I for PEP make it difficult to compete with other essential enzymes for PEP, such as PEP-dependent DHAK and PEP carboxykinase. This same

phenomenon can also explain the requirement for PEP carboxykinase, an enzyme thought to function in the decarboxylation direction, in place of PEP carboxylase for the carboxylation of PEP during fermentative glycerol metabolism. PEP carboxylase, an enzyme required for respiratory glycerol metabolism, is strongly activated by F1,6BP, and as with pyruvate kinase I, lack of activation by F1,6BP significantly impacts its ability to compete with other enzymes for PEP. On the other hand, PEP carboxykinase is not subject to regulation by any key metabolic intermediates of fermentative glycerol metabolism and as a result can compete with other enzymes for PEP during fermentative growth on glycerol.

The theme of complex interactions of key enzymes with certain metabolic intermediates is also evident in the importance of PEP-dependent DHAK from both a pathway and regulatory standpoint. While an essential component of the required fermentative glycerol dissimilation pathway, the link between this enzyme and the phosphotransferase system of *E. coli* was also found to be the key regulatory mechanism of fermentative glycerol metabolism. As with the majority of PTS enzymes, PEP-dependent DHAK was determined to be under transcriptional control of the CRP-cAMP complex, and without CRP-cAMP transcriptional activation of the *dhaKLM* operon fermentative glycerol utilization is severely impaired. In the context of global control of fermentative glycerol metabolism, this key regulatory finding nicely ties the specific control of glycerol dissimilation to the global regulation of fermentative metabolism in general, as many regulators known to control key aspects of fermentative metabolism were also important under fermentative glycerol metabolism. The fermentative glycerol dissimilation pathway

was also found to be the critical component in the kinetics of glycerol fermentation, as experiments verified the metabolic control glycerol dehydrogenase and PEP-dependent DHAK have over glycerol fermentation, and identified the key genetic manipulations needed to increase the rate of glycerol utilization during fermentative glycerol metabolism.

Overall, this study into fermentative glycerol metabolism resulted in the elucidation of several important aspects that not only significantly increase the knowledge base on fermentative glycerol metabolism in *Escherichia coli*, but also provide fundamental knowledge needed to design effective metabolic engineering strategies for the efficient production of fuels and chemicals from glycerol. The influence of the coupling between glycerol dissimilation and pyruvate synthesis can have resounding complications for the availability of intermediates upstream of PEP, and any strategy aimed at the conversion of these intermediates to a desired product must take this into account. In addition, the key involvement of the fermentative glycerol dissimilation pathway from a kinetic and regulatory standpoint provides another important engineering target, as the manipulation of this pathway can help to increase productivities or break regulatory interactions that may aid in the efficient production of fuels and chemicals from a number of industrial relevant glycerol waste streams. In this context, the results obtained from this study can prove invaluable for the ability to efficiently produce value added products from glycerol. To demonstrate this potential, the next chapter focuses on the application of this fundamental knowledge for the design and implementation of metabolic engineering strategies to produce target fuels and reduced chemicals.

Chapter 5

Metabolic Engineering of *Escherichia coli* for the Efficient Production of Reduced Chemicals and Fuels from Glycerol

Advancements in the efficient microbial production of fuels and chemicals have been shaped by the development of tools for the understanding and manipulation of cellular metabolism, including the ability to transfer genes, modulate gene expression, and engineer proteins (Stephanopoulos 2007). Increased production of useful compounds through strategies designed to engineer cells and their metabolic pathways have been used extensively over the past few decades (Bailey 1991; Keasling, 2010; Stephanopoulos 2002). While the availability of genetic tools and the large genomic, metabolic, and physiological knowledge base for *E. coli* has facilitated the ease at which this organism can be engineered as an efficient biocatalyst, the lack of fundamental knowledge of fermentative glycerol

metabolism has limited the ability to utilize *E. coli* for efficient fuel and chemical production from glycerol.

Until recently, it was thought that *E. coli* was only able to metabolize glycerol under respiratory conditions, minimizing its potential for the production of biofuels and biochemicals from glycerol. The use of glycerol as a carbon source under fermentative conditions was limited to studies in which genes encoding the glycerol dehydratase and 1,3-PDO dehydrogenase enzymes from organisms such as *Klebsiella pneumoniae* (Skraly et al. 1998) and *Citrobacter freundii* (Daniel and Gottschalk 1992) were expressed to enable glycerol utilization. However, these studies focused on the production of 1,3-propanediol (1,3-PDO), a product naturally produced in these organisms but not by *E. coli*. 1,3-PDO production from glycerol was accomplished through the expression of a synthetic 1,3-PDO pathway which directly converts glycerol into 1,3-PDO without entering the central carbon metabolism of *E. coli*. Therefore, additional carbon sources other than glycerol were usually required to help sustain cell growth and the production of other products from glycerol was limited.

While the recent discovery that *E. coli* is able to utilize glycerol in a fermentative manner (Dharmadi et al., 2006; Gonzalez et al., 2008; Murarka et al., 2008) revealed the potential for the use of glycerol as a carbon source, the increased understanding of fermentative glycerol metabolism detailed previously (Chapter 4) provides the essential platform to design metabolic engineering strategies for the use of *E. coli* as a biocatalyst for the conversion of glycerol into fuels and reduced

chemicals. The detailed knowledge of essential pathways and mechanisms, as well as kinetic and regulatory aspects of fermentative glycerol metabolism can be utilized to provide the most effective engineering design to maximize the titers and yields of target fuels and chemicals from glycerol. In order to demonstrate the potential for the conversion of glycerol waste streams into value added products, as well as how the fundamental understanding of fermentative glycerol metabolism can be applied for this purpose, *E. coli* was engineered for the production of both reduced chemicals (1,2-propanediol) and fuels (ethanol and hydrogen) utilizing key elements uncovered during the study of fermentative glycerol metabolism. While the metabolic engineering strategies implemented for these products are vastly different due to the nature of the target product(s), the key elements of fermentative glycerol metabolism are an underlying theme, illustrating both the advantages of the use of glycerol as a carbon source and the limiting constraints that must be overcome to efficiently produce fuels and reduced chemicals at maximum titers and yields.

5.1. Metabolic engineering of *Escherichia coli* for the production of 1,2-propanediol from glycerol

1,2-propanediol (1,2-PDO: $C_3H_8O_2$), a commodity chemical with global demand estimated around 3 billion lb/yr, has a major role in applications such as antifreeze and heat-transfer fluids, plasticizers and thermoset plastics, and cosmetics (Shelley, 2007). Recent processes for the microbial production of 1,2-

PDO from sugars, predominantly glucose, have used microorganisms such as *Thermoanaerobacterium thermosaccharolyticum* (Altaras et al., 2001; Sanchez-Riera et al., 1987), *Clostridium sphenoides* (Trandin and Gottschalk, 1985), *Saccharomyces cerevisiae* (Jung et al., 2008; Lee and DaSilva, 2006), and *E. coli* (Altaras and Cameron, 1999; Altaras and Cameron, 2000; Huang et al., 1999). Utilizing batch cultures, 1,2-PDO titers from glucose ranged from 0.49 g/L with *E. coli* (Altaras and Cameron, 1999) to 1.11 g/L with *S. cerevisiae* (Jung et al., 2008) and 9.0 g/L with *T. thermosaccharolyticum* (Sanchez-Riera et al., 1987). An optimized fed-batch culture was used to achieve the highest titer of 1,2-PDO produced by *E. coli* from glucose (4.5 g/L) at a yield of 0.19 g/g (Altaras and Cameron, 2000).

Despite the modest success of these efforts, the higher degree of reduction of glycerol (compared to glucose) offers the opportunity for increased 1,2-PDO yields from an inexpensive and readily available carbon source (Section 2.1). As previously discussed in Section 2.1, under the assumption that no external source of energy is available other than the given carbon source, the maximum theoretical yield of 1,2-PDO from glycerol (0.72 g/g) is clearly higher than that from glucose (0.63 g/g). Capitalizing on this fact, the work reported here utilizes rational metabolic engineering strategies based on the detailed understanding of glycerol fermentation, to design and engineer *E. coli* for the efficient production of 1,2-PDO from glycerol.

5.1.1. Proposed metabolic engineering strategies for the conversion of glycerol into 1,2-PDO

Metabolic engineering strategies must be designed to enable 1,2-PDO synthesis while satisfying two key constraints governing fermentative metabolism, namely redox balance and ATP generation by substrate-level phosphorylation. The production of 1,2-PDO from glycerol results in the net consumption of reducing equivalents (Figure 5.1). Consequently, the production of a more oxidized co-product, such as lactate ($C_3H_6O_3$) or acetate ($C_2H_4O_2$), would maintain an overall redox balance as seen from the following balances.

1,2-PDO/Acetate: $3C_3H_8O_3 \rightarrow 2C_3H_8O_2 + C_2H_4O_2 + CH_2O_2 + H_2O$

1,2-PDO/Lactate: $2C_3H_8O_3 \rightarrow C_3H_8O_2 + C_3H_6O_3 + H_2O$

The synthesis of acetate could support higher 1,2-PDO yields as the molar ratio of 1,2-PDO to acetate required to achieve redox balance is 2:1 while that of 1,2-PDO to lactate is 1:1. It is important to note however, that the molar ratio of either acetate or lactate to 1,2-PDO required to enable redox poise would result in a net ATP balance of zero, placing a significant metabolic constraint on the system due to the ATP required for cell growth and maintenance. Therefore, the production of ethanol, whose synthesis along with formate (or CO_2 and H_2) from glycerol represents a redox balanced pathway that generates ATP (Figure 5.1, Murarka et al., 2008), may also be vital for 1,2-PDO production by providing the ability to generate ATP without disturbing redox poise.

The proposed pathways mediating the conversion of glycerol into 1,2-PDO are shown in Figure 5.1.

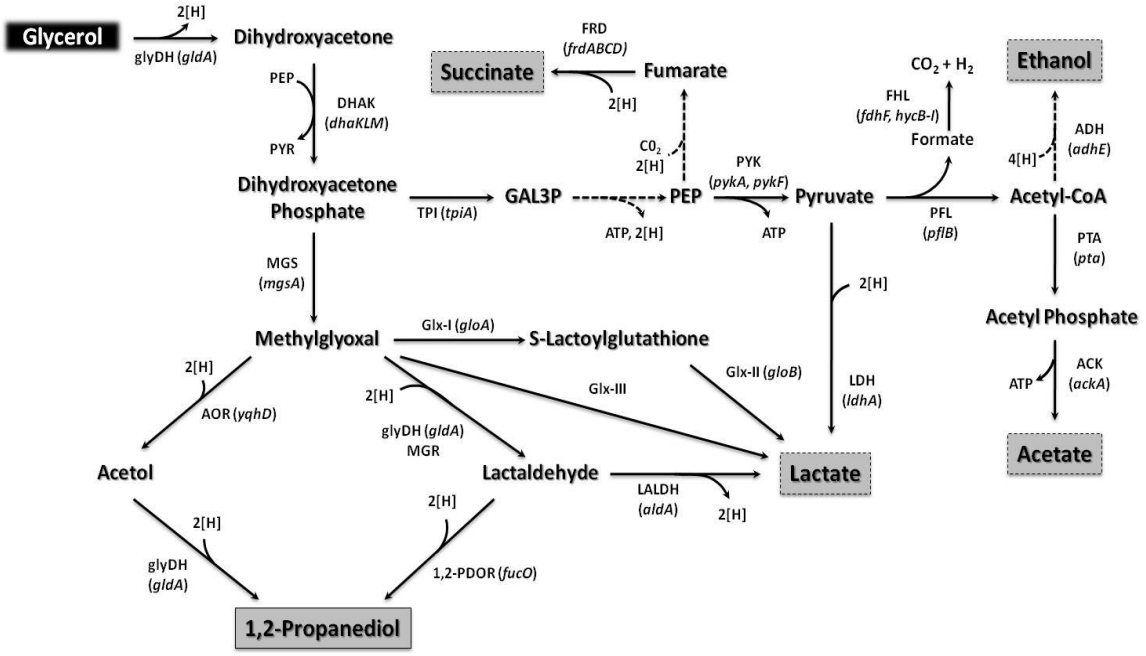

Figure 5.1 *E. coli* pathways involved in the synthesis of 1,2-PDO and other fermentation products during the fermentative metabolism of glycerol. Broken lines illustrate multiple steps. Relevant reactions are represented by the enzyme names with the gene(s) coding for the enzymes in parenthesis: ACK, acetate kinase; ADH, acetaldehyde/alcohol dehydrogenase; AOR, aldehyde oxidoreductase; DHAK, dihydroxyacetone kinase; FHL, fomate hydrogen lyase complex; FRD, fumarate reductase; Glx-1, Glyoxylase type I; Glx-II, Glyoxylase type II; Glx-III, Glyoxylase type III; glyDH, glycerol dehydrogenase; LALDH, lactaldehyde dehydrogenase; LDH, lactate dehydrogenase; MGR, methylglyoxal reductase; MGS, methylglyoxal synthase; PFL, pyruvate formate-lyase; PTA, phosphate acetyltransferase; PYK, pyruvate kinase; TPI, triose phosphate isomerase; 1,2-PDOR, 1,2-propanediol reductase. Abbreviations: GAL3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; 2[H], NADH/NADPH/$FADH_2$.

Glycerol dehydrogenase (glyDH) and dihydroxyacetone kinase (DHAK) catalyze the conversion of glycerol into the glycolytic intermediate dihydroxyacetone phosphate (DHAP) (Gonzalez et al., 2008). DHAP is converted to methylglyoxal (MG) by the action of methylglyoxal synthase (MGS, *mgsA*) (Hopper and Cooper, 1972). The

conversion of MG into 1,2-PDO takes place through two alternate pathways, one with acetol as an intermediate through the action of aldehyde oxidoreductase (AOR, *yqhD*) (Lee et al., 2010; Soucaille et al., 2008) and glycerol dehydrogenase (glyDH, *gldA*) (Subedi et al., 2008; Tang et al., 1979), and the other with lactaldehyde as an intermediate, mediated by glyDH (Altaras and Cameron, 1999; Subedi et al., 2008) and 1,2-PDO reductase (1,2-PDOR, *fucO*) (Zhu and Lin, 1989) (Figure 5.1). Based on these pathways, the overexpression of *mgsA* in combination with either alternative 1,2-PDO pathway will be investigated as a means of increasing 1,2-PDO production. In addition, the coupling between glycerol dissimilation and pyruvate synthesis through the fermentative glycerol dissimilation pathway could make the availability of DHAP for conversion into methylglyoxal (MG) a concern. This coupling is a critical aspect of fermentative glycerol metabolism and despite the fact that a product downstream of pyruvate must be co-produced with 1,2-PDO (i.e. acetate, lactate, or ethanol) the conversion of an upstream intermediate (i.e. DHAP) to 1,2-PDO may be limited in the presence of this coupling. Therefore the availability of DHAP will also be investigated, as it represents a critical node where partitioning of carbon flux between 1,2-PDO synthesis and glycolysis occurs.

Methylglyoxal, a key intermediate in the 1,2-PDO pathway, is a very toxic metabolite whose accumulation at sub-millimolar concentrations can lead to significant growth inhibition and even cell death (Booth et al., 2003; MacLean et al., 1998). Several detoxification pathways exist, the most important of which is glutathione (GSH)-dependent GlxI/GlxII (Glyoxylase type I/II, encoded by *gloA*/*gloB*) pathway, whose net result is the conversion of MG into lactate (MacLean

et al., 1998) (Figure 5.1). In addition, the glyoxylase III pathway (Misra et al., 1995) and the conversion of lactaldehyde into lactate through the action of lactaldehyde dehydrogenase (encoded by *aldA*) (Caballero et al., 1983) are other non-1,2-PDO forming MG detoxification pathways found in *E. coli* (Figure 5.1). Since these pathways shuttle carbon away from the production of 1,2-PDO, their disruption could provide higher 1,2-PDO production.

Due to the fact that the 1,2-PDO synthesis pathway is both redox and ATP consuming, it is likely that in addition to 1,2-PDO production, other fermentation products will also be produced. Therefore, the effect of the disruption and amplification of the fermentative pathways responsible for the production of lactate (*ldhA*), succinate (*frdABCD*), ethanol (*adhE*) and acetate (*ackA-pta*), in combination with the engineered 1,2-PDO pathway, will be investigated.

5.1.2. Engineering of 1,2-propanediol synthesis pathways

Two plasmids were constructed to overexpress the genes involved in the synthesis of 1,2-PDO from the intermediate DHAP (Figure 5.1): pTHgldAmgsAyqhD, expressing *gldA*, *mgsA*, and *yqhD* and pTHgldAmgsAfucO expressing *gldA*, *mgsA*, and *fucO*. While the expression of either pathway in wild-type MG1655 resulted in the production of similar amounts of 1,2-PDO, the product titers and yields were still low (less than 0.1 g/L) (Figure 5.2).

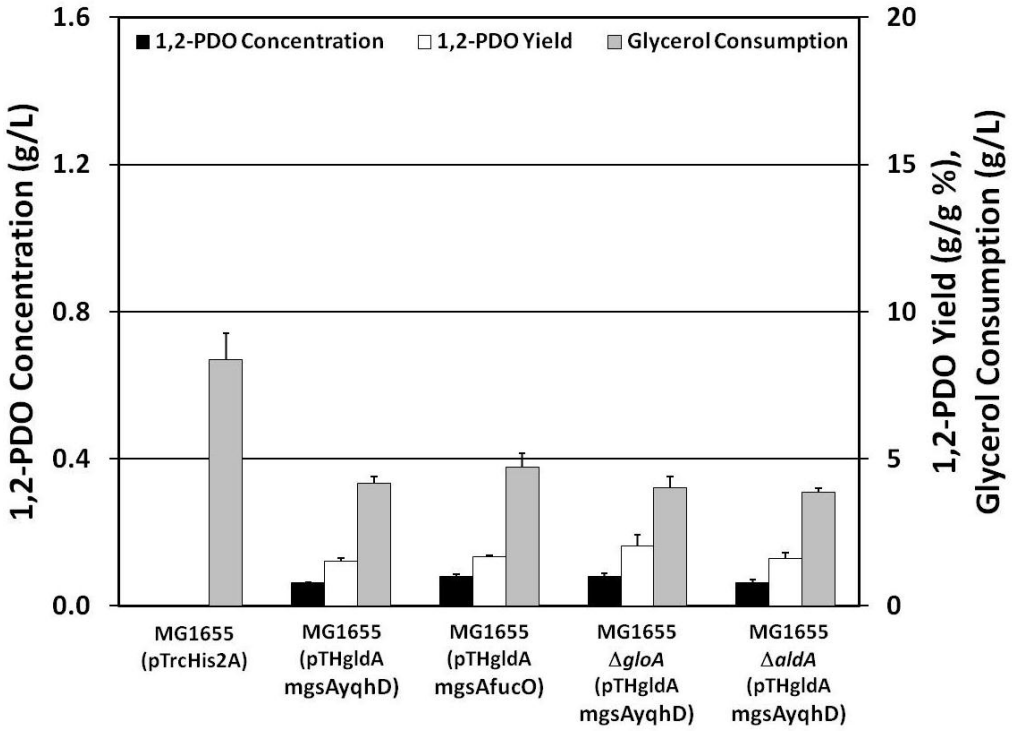

Figure 5.2 Effect of overexpression of 1,2-PDO-synthesis pathways on 1,2-PDO production and glycerol consumption. Fermentations conducted in 96-hour sparged-tube experiments. Gene overexpressions are indicated by the plasmid name in parenthesis (i.e. pTrcHis2A: control vector; pTHgldAmgsAyqhD expressing glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase; pTHgldAmgsAfucO expressing glycerol dehydrogenase, methylglyoxal synthase and 1,2-PDO reductase). Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

In addition to the overexpression of a 1,2-PDO pathway, the disruption of genes involved in MG detoxification pathways could potentially increase the titers and yields of 1,2-PDO by minimizing the conversion of MG into other products (see previous section). This was investigated through the deletion of the *gloA* or *aldA* genes in combination with the expression of the 1,2-PDO pathway. However, the disruption of either gene did not result in significant increases in 1,2-PDO production or changes to the distribution of fermentation products (Figure 5.2),

which suggests that the carbon flux through the glyoxylase systems might be negligible in this strain.

In order to ensure that inappropriate expression of the genes contained in each vector was not the cause of the low 1,2-PDO production, a functional characterization of the plasmids through enzyme assays was conducted (Table 5.1).

Table 5.1 Functional characterization of constructs used in the overexpression of 1,2-PDO synthesis and glycerol utilization pathways. Reported values are from 96 hr sparged tube cultures.

Enzyme Assayed	Activity (μmol/mg protein/min)[a,b]			
	pTrcHis2A	pTHgldAmgsA yqhD	pTHgldAmgsA fucO	pTHKLcfgldA mgsAyqhD
1,2-PDO Synthesis Pathways				
Methylglyoxal synthase	0.116±0.009	2.96±0.09	3.18±0.08	2.61±0.07
Methylglyoxal reducing (NADH)	0.020±0.001	1.43±0.04	1.8±0.1	0.60±0.02
Methylglyoxal reducing (NADPH)	0.51±0.03	5.62±0.04	4.69±0.08	1.90±0.09
Glycerol dehydrogenase (Acetol reducing)	0.010±0.001	0.56±0.02	NM	0.173±0.003
1,2-PDO reductase	0.170±0.009	4.0±0.2	4.5±0.2	NM
Glycerol Utilization Pathways				
Glycerol dehydrogenase (Glycerol oxidation)	0.075±0.003	NM	NM	1.47±0.03
ATP-dependent dihydroxyacetone kinase	0.178±0.004	NM	NM	0.586±0.007

[a]Activities (μmol/mg protein/min) were measured during active growth as described in Materials and Methods and values reported as average ± standard deviation for triplicate measurements. NM: not measured

[b]Activities measured in wild-type MG1655 containing the listed plasmids overexpressing the specific enzyme(s) (native *E. coli* genes unless otherwise specified): i.e., pTrcHis2A: control vector; pTHgldAmgsAyqhD expressing glycerol dehydrogenase, methylglyoxal synthase, and aldehyde oxidoreductase; pTHgldAmgsAfucO expressing glycerol dehydrogenase, methylglyoxal synthase, and 1,2-PDO reductase; pTHKLcfgldAmgsAyqhD expressing C. freundii ATP-dependent dihydroxyacetone kinase, glycerol dehydrogenase, methylglyoxal synthase, and aldehyde oxidoreductase.

MGS activity (conversion of MG from DHAP), MG reducing activities (both NADPH and NADH dependent for the reduction of MG into either acetol or lactaldehyde),

acetol reduction activity (for 1,2-PDO production from acetol), and 1,2-PDOR activity (for 1,2-PDO production from lactaldehyde) were all assayed (Table 5.1). The MGS, MG reducing, and acetol reducing activities (encoded by the pTHgldAmgsAyqhD plasmid) are all more than 10-fold higher than those of the control vector (pTrcHis2A) indicating the amplification of this 1,2-PDO pathway. In the case of MG reducing activities, an increase in both NADH- and NADPH-dependent reduction of MG was observed. While the AOR encoded by *yqhD* is believed utilize NADPH as a co-factor (Lee et al., 2010), the fact that the MG reducing assay cannot distinguish between the reduction of MG to acetol and that to lactaldehyde opens the possibility that the high activity of the NADH-dependent MG reducing assay is a result of the overexpression of glyDH, which can reduce MG to lactaldehyde. In addition, *E. coli* has a number of native aldo-keto reductases (both NADH and NADPH dependent) (Misra et al., 1996) that have been shown to reduce MG, which may be highly active in the presence of the larger MG pool likely seen with the overexpression of MGS. Similar results were obtained for pTHgldAmgsAfucO, as the MGS, MG reducing, and 1,2-PDO reductase activities are all more than 9-fold higher than those of pTrsHis2A. Taken together, the results from these enzyme assays indicate that the low 1,2-PDO production was not the result of poor gene expression or pathway functionality and further analysis is required to ascertain the reasons behind the low titers.

5.1.3. Manipulation of glycerol utilization pathways

As previously discussed (Section 4.2.4), the fact that the *E. coli* DHAK utilizes phosphoenolpyruvate (PEP) as the phosphate group donor in the phosphorylation of dihydroxyacetone (DHA) has the effect of coupling the native fermentative pathway for glycerol dissimilation with the requirement for the synthesis of PEP. This has significant consequences during fermentative glycerol metabolism and may limit the amount of DHAP (an intermediate upstream of PEP synthesis) available for the conversion into MG and subsequently 1,2-PDO as a result. The low levels of 1,2-PDO seen as a result of the engineered 1,2-PDO pathway (Figure 5.2) show that this may be a major issue.

In order to increase the availability of DHAP for 1,2-PDO synthesis, glycerol utilization was decoupled from the requirement for PEP synthesis by expressing an ATP-dependent DHAK from *Citrobacter freundii* (*dhaKL*), which utilizes ATP as the phosphate group donor instead of PEP (Daniel et al., 1995). In essence, this creates an ATP-linked fermentative glycerol dissimilation pathway which still utilizes NAD^+ for the oxidation reaction, providing the most efficient decoupling mechanism for glycerol utilization when the target product comes from intermediates upstream of pyruvate, as is the case with 1,2-PDO. A vector was constructed containing the previously engineered 1,2-PDO pathway (i.e. *gldA*, *mgsA*, and *yqhD* from *E. coli*) in addition to *C. freundii dhaKL* (pTHKLcfgldAmgsAyqhD). Once transformed with this vector, MG1655 produced nearly 12-fold higher levels of 1,2-PDO (over 8-fold

increase in 1,2-PDO yield) and consumed approximately 33% more glycerol than that seen from the expression of only the engineered 1,2-PDO pathway (Figure 5.3).

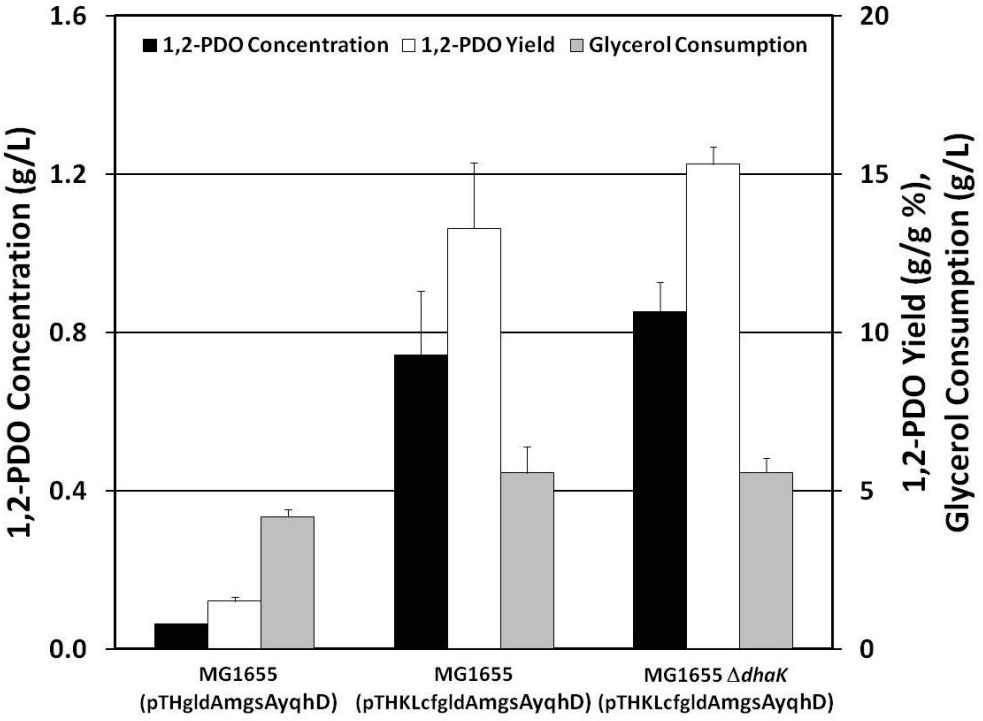

Figure 5.3 Manipulation of glycerol utilization pathways for increased 1,2-PDO production. Fermentations conducted in 96-hour sparged-tube experiments. Gene overexpressions are indicated by the plasmid name in parenthesis (i.e. pTHgldAmgsAyqhD expressing *E. coli* glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase; pTHKLcfgldAmgsAyqhD expressing *C. freundii* ATP-dependent DHA kinase, and *E. coli* glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase). Δ"*gene*" represents gene deletion. Error bars represent standard deviations for a minimum of triplicate measurements.

This dramatic increase in 1,2-PDO production and rise in the amount of glycerol consumption shows the combined advantages of increasing both DHAP availability for 1,2-PDO production and the rate of glycerol utilization. The latter results from the simultaneous overexpression of glyDH and DHAK, which as determined from the

kinetic studies on glycerol fermentation represent the two key enzymes shown to have control over of the rate of glycerol utilization (Section 4.3).

Proper gene expression was investigated by the functional characterization of pTHKLcfgldAmgsAyqhD through the use of enzyme assays. The activity of the steps within the 1,2-PDO pathway were significantly higher with the expression of pTHKLcfgldAmgsAyqhD when compared to the control vector (Table 5.1). In addition, the increased ATP-dependent DHAK activity shows proper expression and when combined with the increase in glycerol oxidation activity, indicates the functionality of both activities required for increased glycerol utilization rates. The ATP-dependent DHAK activity found in the control strain is due to the action of glycerol kinase (GK, *glpK*), an enzyme involved in the respiratory glycerol utilization pathway, and which acts on both glycerol and DHA (Garcia-Alles et al., 2004; Jin et al., 1982).

While the overexpression of the ATP-dependent DHAK from *C. freundii* helps alleviate the requirement of PEP synthesis for glycerol dissimilation and significantly increases 1,2-PDO production, the additional disruption of the native *E. coli* DHAK (through the deletion of *dhaK*) will completely decouple the two pathways. An *E. coli* mutant lacking a functional PEP-dependent DHAK (Δ*dhaK*) was constructed and transformed with the pTHKLcfgldAmgsAyqhD plasmid. This strain showed a slight increase (~15%) in both 1,2-PDO concentration and yield when compared to wild-type *E. coli* MG1655 expressing the same plasmid (Figure 5.3). Based on these results, it is apparent that the complete decoupling of glycerol

dissimilation and PEP formation through the replacement of *E. coli* PEP-dependent DHAK with the *C. freundii* ATP-dependent DHAK provides the most efficient route to 1,2-PDO synthesis. While this represents a promising result, it is important to note that significant amounts of ethanol, succinate, acetate, and lactate were also synthesized (Table 5.2).

5.1.4. Engineering of major fermentation pathways

Based on the significant amounts of ethanol, lactate, acetate, and succinate produced along with 1,2-PDO upon overexpression of the ATP-dependent DHAK and 1,2-PDO pathway (Table 5.2), the minimization of these by-products may provide the opportunity for increased 1,2-PDO titer and yield. For this purpose, *E. coli* mutants with deletions of key genes in the synthesis of fermentation products lactate (Δ*ldhA*), succinate (Δ*frdA*), ethanol (Δ*adhE*), and acetate (Δ*ackA-pta*) were transformed with the pTHKLcfgldAmgsAyqhD plasmid (Figure 5.4). Disruption of fumarate reductase (Δ*frdA*) eliminated almost all succinate production but had a negative impact on 1,2-PDO synthesis, the latter apparently due to the reduction in hydrogen recycling (see next section for details). In addition, the deletion of the *adhE* gene, resulting in the inability to produce ethanol, nearly eliminated glycerol consumption and 1,2-PDO synthesis (Figure 5.4), in agreement with the requirement of ethanol synthesis for glycerol fermentation (Gonzalez et al., 2008; Murarka et al., 2008).

Table 5.2 Product yields and carbon recovery in *E. coli* strains engineered for the production of 1,2-PDO

Strain[a]	Glycerol consumed (g/L)	Product mass yields (% g/g)							Carbon Recovery[b]
		1,2-PDO	Ethanol	Acetate	Lactate	Succinate	Formate	Pyruvate	
Engineering of 1,2-PDO synthesis pathways									
MG1655 (pTrcHis2A)	8.4 (0.9)	0.0 (0.0)	45.5 (1.3)	7.5 (1.2)	0.0 (0.0)	11.7 (3.5)	2.0 (1.1)	0 (0)	1.12
MG1655 (pTHgldAmgsAyqhD)	4.2 (0.2)	1.5 (0.1)	36.7 (1.1)	17.6 (0.4)	0.0 (0.0)	12.0 (0.1)	9.2 (0.5)	0 (0)	1.11
MG1655 (pTHgldAmgsAfucO)	4.7 (0.5)	1.7 (0.0)	38.0 (0.7)	14.0 (0.4)	0.0 (0.0)	11.5 (0.4)	7.3 (1.0)	0 (0)	1.08
MG1655 Δ*gloA* (pTHgldAmgsAyqhD)	4.0 (0.4)	2.0 (0.4	44.3 (1.3)	14.2 (1.4)	0.0 (0.0)	10.3 (1.0)	11.0 (1.7)	0 (0)	1.21
MG1655 Δ*aldA* (pTHgldAmgsAyqhD)	3.9 (0.1)	1.6 (0.2)	45.5 (1.0)	14.1 (1.3)	0.0 (0.0)	10.8 (0.8)	11.5 (0.7)	0 (0)	1.23
Manpulation of glycerol utilization pathways									
MG1655 (pTHKLcfgldAmgsAyqhD)	5.6 (0.8)	13.3 (2.1)	25.6 (3.3)	8.6 (2.3)	19.7 (4.9)	5.8 (1.9)	0.3 (0.8)	0 (0)	1.05
MG1655 Δ*dhaK* (pTHKLcfgldAmgsAyqhD)	5.6 (0.4)	15.3 (0.6)	25.9 (1.0)	8.3 (1.2)	21.1 (2.0)	7.4 (0.4)	0.0 (0.0)	0 (0)	1.10
Engineering of major fermentation pathways									
MG1655 Δ*frdA* (pTHKLcfgldAmgsAyqhD)	3.9 (0.5)	11.5 (0.6)	24.7 (1.8)	15.3 (2.2)	21.0 (1.6)	1.0 (2.4)	0.0 (0.0)	0 (0)	1.08
MG1655 Δ*ldhA* (pTHKLcfgldAmgsAyqhD)	7.5 (1.7)	11.5 (4.6)	35.1 (4.0)	9.1 (3.0)	3.1 (1.1)	6.1 (1.4)	0.0 (0.0)	0 (0)	1.06
MG1655 Δ*ackA-pta* (pTHKLcfgldAmgsAyqhD)	8.3 (1.2)	14.6 (0.8)	29.7 (2.8)	1.2 (0.5)	20.9 (2.9)	3.2 (0.8)	0.2 (0.4)	0 (0)	1.03
MG1655 Δ*adhE* (pTHKLcfgldAmgsAyqhD)	1.1 (0.0)	2.1 (0.3)	0.0 (0.0)	61.0 (0.6)	0.0 (0.0)	14.4 (2.1)	0.0 (0.0)	0 (0)	1.07
Kinetics of glycerol consumption and product formation in *E. coli* strain engineered for 1,2-PDO production									
MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD)	26.2 (0.1)	21.3 (0.1)	33.0 (0.1)	0.3 (0.1)	0.0 (0.0)	4.5 (0.1)	15.1 (0.2)	5.2 (0.1)	1.02
MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK*Δ*aceF* (pTHKLcfgldAmgsAyqhD)	19.9 (0.2)	21.2 (0.1)	32.9 (0.3)	0.3 (0.1)	0.0 (0.0)	5.2 (0.2)	17.3 (0.2)	7.3 (0.2)	1.05
MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK*Δ*fdhF* (pTHKLcfgldAmgsAyqhD)	17.9 (0.2)	21.6 (0.5)	30.0 (0.2)	0.3 (0.1)	0.0 (0.0)	4.7 (0.1)	29.1 (0.4)	12.6 (0.3)	1.04
MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK*Δ*aceF*Δ*fdhF* (pTHKLcfgldAmgsAyqhD)	13.7 (0.1)	19.8 (0.2)	31.6 (0.1)	0.4 (0.1)	0.0 (0.0)	5.4 (0.2)	29.7 (0.3)	11.5 (0.2)	1.04
MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD) Crude Glycerol	18.9 (0.5)	23.9 (1.7)	32.7 (1.5)	0.4 (0.1)	0.0 (0.0)	4.6 (1.2)	6.1 (0.3)	6.2 (1.3)	1.05

[a] Data represent the average of a minimum of three independent samples (standard deviations shown in parenthesis) taken from 96-hour sparged tube cultures, except those in "*Kinetics of glycerol consumption and product formation in E. coli strain engineered for 1,2-PDO production*" section which represent experiments in controlled fermentors with samples taken at 72 hours.

[b] Carbon recovery is expressed as the ratio of the mol of carbon in products per mol of carbon in glycerol consumed, assuming that moles of acetate *plus* moles of ethanol *equals* moles of 1-C compounds (formate *plus* CO_2) generated by the dissimilation of pyruvate (Dharmadi et al., 2006; Murarka et al., 2008).

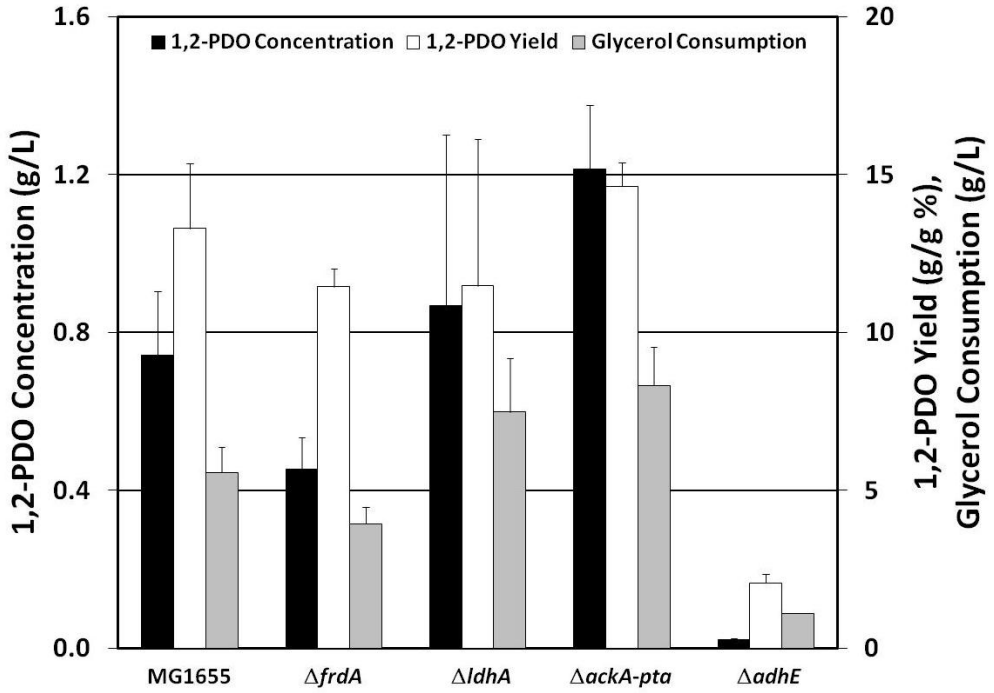

Figure 5.4 Effect of the disruption of major fermentative pathways on 1,2-PDO production and glycerol consumption. Fermentations conducted in 96-hour sparged-tube experiments. Δ"*gene*" represents gene deletion. All strains containing pTHKLcfgldAmgsAyqhD expressing *C. freundii* ATP-dependent DHA kinase and *E. coli* glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase. Error bars represent standard deviations for a minimum of triplicate measurements.

Deletion of the *ldhA* gene encoding lactate dehydrogenase, the enzyme responsible for the conversion of pyruvate into lactate, resulted in a 1.17-fold increase in 1,2-PDO concentration, although the 1,2-PDO yield slightly decreased (Figure 5.4). It is important to note that this deletion would only eliminate the synthesis of lactate from pyruvate (fermentative route) and the activity of several methylglyoxal detoxification pathways could also result in lactate production (Figure 5.1). While minimal amounts of lactate were seen with the overexpression of the 1,2-PDO pathway, the increases in lactate production when DHAP availability

is increased may be a result of these methylglyoxal detoxification pathways (Table 5.2). Specifically, the overexpression of both AOR and glyDH may result in competition for MG between these enzymes, which could result in significant lactate production through lactaldehyde (Figure 5.1). However, the fact only minimal amounts of lactate were produced upon deletion of *ldhA* indicates that the majority of lactate production was a result of the fermentative route through pyruvate (Table 5.2). Therefore, it is likely that the lactate production accompanying the increased 1,2-PDO production is a result of the need to achieve redox poise as the production of lactate through pyruvate provides a means of generating reducing equivalents through an ATP generating pathway (Figure 5.1, note that glycerol conversion to lactate through this route also generates ATP). Interestingly, the deletion of *ldhA* and the subsequent decrease in lactate production was not accompanied by an increase in the yield of acetate produced as one would expect in order to achieve redox poise (Table 5.2). In fact, the disruption of the fermentative lactate pathway actually led to an increase in the yield of ethanol produced (Table 5.2).

Another unexpected finding is the fact that blocking the route for fermentative acetate synthesis from acetyl-CoA (*ackA-pta* operon) resulted in the highest 1,2-PDO titers achieved by any deletion, (1.2 g/L at a yield of 14.6% w/w) (Figure 5.4). The deletion of the acetate synthesis pathway also resulted in nearly 50% increase in glycerol consumption and an increase in ethanol concentration and yield (2.5 g/L at a yield of 29.7 % w/w) (Table 5.2). Overall, these findings indicate that, as found in previous studies with wild-type *E. coli* (Murarka et al., 2008), ethanol synthesis is required for fermentative glycerol utilization despite the

expression of a functional 1,2-PDO pathway. This dependence is further shown by the increases in ethanol production that result from the disruption of fermentative acetate and lactate production pathways. Both the requirement for and shift toward ethanol synthesis are likely the result of the aforementioned redox neutral and ATP producing nature of the glycerol to ethanol (and formate/CO_2 and H_2) pathway, which unlike the fermentative acetate and lactate pathways, enables ATP generation without any stoichiometric constraints required for redox poise.

Given the redox- and ATP-consuming nature of 1,2-PDO synthesis (Figure 5.1), overexpression of fermentative pathways facilitating increased rates of co-product production could aid in the generation of ATP or reducing equivalents which may favor 1,2-PDO production. A series of vectors were constructed to overexpress *E. coli ackA-pta* (pZSackApta), *ldhA* (pZSldhA), and *adhE* (pZSadhE) genes. However, the co-expression of either of these vectors with the 1,2-PDO pathway (i.e. pTHKLcfgldAmgsAyqhD) in MG1655 did not result in improved 1,2-PDO production over MG1655 expressing the 1,2-PDO pathway vector and the control vector pZSblank (data not shown). To ensure that the results from the overexpression of the acetate synthesis pathway were not affected by any kinetic limitation of phosphate acetyltransferase (*pta*) in competition with acetaldehyde/alcohol dehydrogenase (*adhE*) for acetyl-CoA, the *adhE* deletion strain expressing the 1,2-PDO pathway (MG1655 Δ*adhE* (pTHKLcfgldAmgsAyqhD)) was transformed with pZSackApta. However, this strain consumed only small amounts of glycerol with no improvement to 1,2-PDO yield or titer (data not shown), further showing the importance of ethanol synthesis.

5.1.5. Kinetics of glycerol consumptions and product formation in E. coli strain engineered for 1,2-PDO production

Based on the results presented in previous sections, there are several genetic manipulations that warrant combination in an attempt to engineer *E. coli* for efficient 1,2-PDO production from glycerol. These manipulations include the expression of the engineered 1,2-PDO pathway (*E. coli gldA*, *mgsA*, and *yqhD* genes), the expression of ATP-dependent DHAK (*C. freundii dhaKL*) in place of the native *E. coli* PEP-dependent DHAK (Δ*dhaK*), and blocking the fermentative pathways for the production of acetate (Δ*ackA-pta*) and lactate (Δ*ldhA*). While the disruption of the fermentative pathways for both acetate and lactate production is a deviation from the theoretical design of co-production of 1,2-PDO and an oxidized co-product, the dependence of 1,2-PDO synthesis on ethanol production as well as the increased ethanol production when either fermentative lactate or acetate production is blocked point to the fact that co-production of 1,2-PDO and ethanol may be the best strategy to maximize 1,2-PDO production. This appears to result from the additional ATP needed for cell growth and maintenance, as the amounts of acetate (or lactate) and 1,2-PDO co-produced to achieve redox balance would result in no net ATP generation. On the other hand, the production of ethanol would enable ATP generation without any constraints on its production due to the stoichiometric proportions needed for redox poise (Figure 5.1).

An *E. coli* strain containing gene deletions blocking the synthesis of acetate and lactate, and including a deletion for the disruption of the native *E. coli* DHAK

(MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK*) was transformed with pTHKLcfgldAmgsAyqhD expressing the engineered 1,2-PDO pathway and ATP-dependent DHAK. The performance of this strain was characterized in controlled fermentors (SixFors multi-fermentation system, see Materials and Methods) and using a higher initial glycerol concentration (~30 g/L).

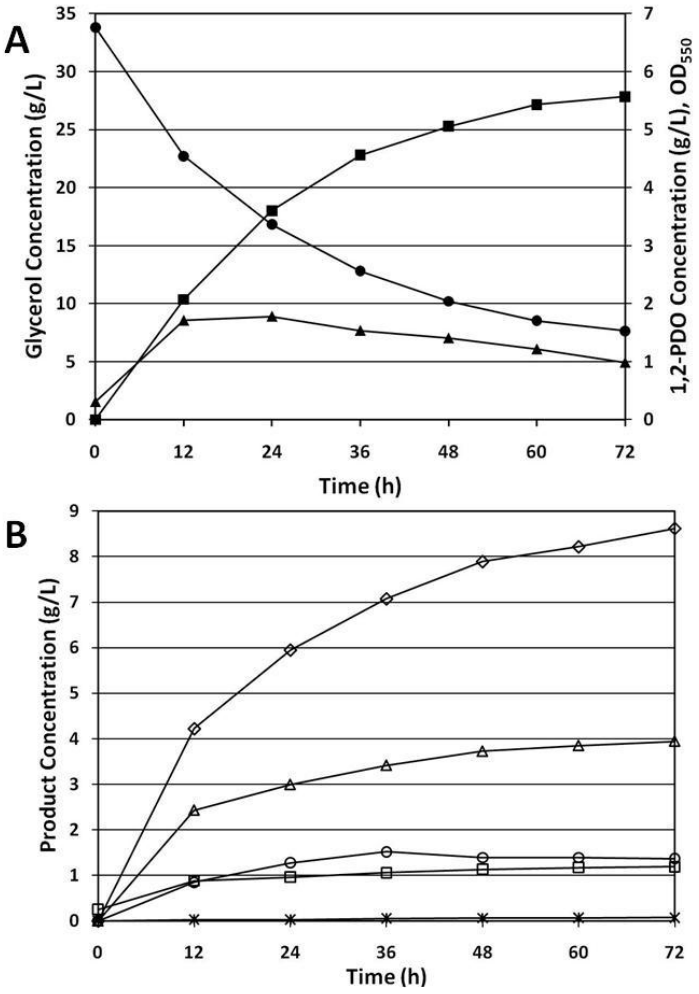

Figure 5.5 Kinetics of 1,2-PDO production by strain MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD). Experiments conducted in fully controlled fermentor at pH 7.5. A: Fermentation profile with concentration of cells (▲), glycerol (●), and 1,2-PDO (■). B: Concentration of by-products ethanol (◇), formate (△), succinate (□), acetate (*), and pyruvate (○). The coefficient of variation (standard deviation/mean x 100) was below 7% in all cases.

Glycerol fermentation with MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD) resulted in excellent 1,2-PDO production throughout the time-course of the fermentation, with a final 1,2-PDO concentration of 5.6 g/L after 72 hours (yield of 21.3% g/g) (Figure 5.5A). As expected, the production of 1,2-PDO was accompanied with the co-production of ethanol (Figure 5.5B). In addition, formate, pyruvate, succinate, and minor amounts of acetate (which could be synthesized through pyruvate oxidase, encoded by *poxB*) were also produced (Figure 5.5B, Table 5.2).

With the disruption of fermentative pathways for the production of acetate and lactate in strain MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD), there is no obvious oxidized product that could be synthesized to enable redox balance. Other than the increased 1,2-PDO titers and yields, the most obvious difference in the product profile with the engineered strain is the synthesis of pyruvate, which was not produced by any other strain tested in this study (Table 5.2). While pyruvate does represent an oxidized product whose synthesis from glycerol generates reducing equivalents, the amount of pyruvate synthesized does not enable redox balance closure as the overall balance remains on the reduced side (i.e. negative value) (Table 5.3). The generation of the additional reducing equivalents required for redox poise can be accounted for by two possible scenarios, namely the activity of the pyruvate dehydrogenase complex (PDH) and/or the recycling of hydrogen evolved from formate hydrogen lyase (FHL). PDH, which converts pyruvate to acetyl-CoA with the generation of CO_2 and NADH, is typically active under respiratory conditions and the absence of activity under anaerobic

conditions has been explained by the sensitivity towards NADH inhibition (de Graef et al., 1999; Sawers and Clark, 2004). However, it has recently been shown that PDH plays a crucial role in fermentative glucuronate metabolism indicating that it can function under anaerobic conditions (Murarka et al., 2010b). In addition, the highly reduced nature of the product distribution in the engineered *E. coli* strain (Table 5.3) may result in a lower NADH/NAD^+ ratio which could relieve some of the NADH inhibition on PDH. The latter scenario involves the recycling of hydrogen evolved by FHL through the hydrogenase isoenzyme 1 or 2, a process which has been shown to play a key role in the fermentative metabolism of *E. coli* (Alam and Clark, 1989; Murarka et al., 2008). Either scenario, which is not accounted for in the redox balances shown in Table 5.3, would result in the generation of additional reducing equivalents possibly enabling redox poise.

In order to test the importance of these processes during 1,2-PDO production by the engineered strain, additional mutations to disrupt PDH activity (Δ*aceF*) and the evolution of hydrogen by FHL (Δ*fdhF*) were added both separately and in combination to the engineered strain. Fermentations with these strains were then conducted in identical conditions to the engineered strain. Based on the results seen in Table 5.2, both PDH and hydrogen recycling appear to play a role in 1,2-PDO production as 1,2-PDO titers were significantly lower with PDH (4.2 g/L) or FHL (3.9 g/L) disruption when compared to the engineered strain.

Table 5.3 Analysis of redox balance during 1,2-PDO production

Substrate consumed and products formed[a]	Oxidation State[b]	Strain							
		MG1655 Δ*ackA-pta* Δ*ldhA*Δ*dhaK* (pTHKLcfgldAmgsAyqhD)		MG1655 Δ*ackA-pta* Δ*ldhA*Δ*dhaK*Δ*aceF* (pTHKLcfgldAmgsAyqhD)		MG1655 Δ*ackA-pta* Δ*ldhA*Δ*dhaK*Δ*fdhF* (pTHKLcfgldAmgsAyqhD)		MG1655 Δ*ackA-pta* Δ*ldhA*Δ*dhaK*Δ*aceF*Δ*fdhF* (pTHKLcfgldAmgsAyqhD)	
		Redox Balance[c]	Carbon Recovery[d]	Redox Balance	Carbon Recovery	Redox Balance	Carbon Recovery	Redox Balance	Carbon Recovery
Substrate									
Glycerol	-2	-2.0	3.0	-2.0	3.0	-2.0	3.0	-2.0	3.0
Products									
1,2-PDO	-4	-1.030	0.773	-1.028	0.771	-1.048	0.786	-0.960	0.720
Ethanol	-4	-2.636	1.318	-2.629	1.314	-2.396	1.198	-2.526	1.263
Acetic Acid	0	0	0.008	0	0.008	0	0.010	0	0.012
Pyruvic Acid	2	0.109	0.164	0.152	0.228	0.264	0.396	0.241	0.361
Succinic Acid	2	0.071	0.141	0.081	0.162	0.073	0.146	0.084	0.168
Formic Acid	2	0.603	0.302	0.693	0.346	1.162	0.581	1.189	0.594
Carbon Dioxide[e]	4	1.446	0.361	1.259	0.315	0	0	0	0
Hydrogen[f]	-2	-0.723	0	-0.630	0	0	0	0	0
Subtotal-Products	-	-2.160	3.067	-2.102	3.144	-1.944	3.116	-1.974	3.118
Total	-	-0.160	0.067	-0.102	0.144	0.056	0.116	0.026	0.118

[a] Substrate consumption and product formation data taken from 72 hour samples shown in the "*Kinetics of glycerol consumption and product formation in E. coli strain engineered for 1,2-PDO production*" section of Table 5.3.

[b] The oxidation state of carbon atoms was calculated assuming oxidation states of -2 and +1 for oxygen and hydrogen, respectively (Sawers and Clark, 2004).

[c] Redox balance values obtained by multiplying the number of moles of product per mole of glycerol by the oxidation state of carbon atoms.

[d] Carbon recovery calculated by multiplying the number of moles of product per mole of glycerol by the number of carbon atoms in the molecule.

[e] Carbon dioxide was calculated assuming that moles of acetate *plus* moles of ethanol *equals* moles of 1-C compounds (formate *plus* CO_2) generated by the dissimilation of pyruvate (Dharmadi et al., 2006; Murarka et al., 2008).

[f] Hydrogen values were obtained assuming that all pyruvate dissimilation to acetyl-CoA takes place through pyruvate formate lyase (i.e. no PDH activity) (de Graef et al., 1999; Sawers and Clark, 2004) and hence moles of hydrogen equals moles of carbon dioxide evolved through formate hydrogen lyase (Sawers et al., 2004).

In addition, the disruption of both PDH and FHL in the engineered strain (i.e. MG1655 Δ*ackA-pta*Δ*ldhA*Δ*dhaK*Δ*aceF*Δ*fdhF* (pTHKLcfgldAmgsAyqhD)) resulted in the production of only 2.7 g/L 1,2-PDO, appreciably lower than the 1,2-PDO titers seen with only one of PDH or FHL disrupted (Table 5.2). The redox balances with these fermentations were significantly improved, with the strains containing the *fdhF* deletion shifting to a redox balance on the oxidative side (i.e. positive value) (Table 5.3). The closure of the redox balance in the case of PDH and FHL disruption is a direct consequence of forcing the assumptions used for the calculation of the redox balance (no PDH activity and hydrogen levels equal to the amount evolved by FHL) to hold true. The drastic difference in the redox balance of the engineered strain reflects the fact that these assumptions do not hold true with this strain, i.e. significant PDH activity and overestimation of hydrogen levels (due to hydrogen recycling) result in the calculation of a highly reduced redox balance. The measured PDH activity was 0.0125 ± 0.007 μmol/mg protein/min in the engineered strain and undetectable in the derivatives containing the *aceF* deletion. Since fumarate reductase (*frdABCD*) has been implicated in hydrogen recycling during glycerol fermentation (Murarka et al., 2008), the above results also support our observation that an *frdA* deletion had a negative impact on 1,2-PDO production (Figure 5.4 and previous section). Overall, these results show that a combination of both PDH activity and hydrogen recycling is the most likely scenario enabling redox poise to be achieved during 1,2-PDO production with the engineered strain, as both processes appear to be beneficial for 1,2-PDO production.

Additional fermentations with the engineered strain were carried out using crude glycerol generated from the production of biodiesel. The performance of the engineered strain was comparable to the fermentation with refined glycerol (Table 5.2), illustrating the potential for the industrial scale conversion of glycerol waste streams into 1,2-PDO. Overall, the engineered *E. coli* strains produced 1,2-PDO at yields and titers higher than those reported with the use of this organism for the production of 1,2-PDO from other carbon sources, demonstrating the advantageous nature of glycerol for the production of reduced chemicals when compared to more traditional carbon sources.

This study demonstrates both the potential for the industrial scale conversion of glycerol waste streams to value added products and the advantageous nature of glycerol as a carbon source for the production of reduced chemicals. Furthermore, this study also demonstrates how fundamental knowledge of cellular metabolism can facilitate the ease of which metabolic engineering principles can be applied in the design of strategies for fuel and chemical production. The central influence of the coupling between glycerol dissimilation and PEP to pyruvate conversion was elucidated during the fundamental studies of glycerol metabolism, and the understanding of this key principle enabled the design of effective strategies to circumvent the initial constraints which lead to significantly increased yields and titers of 1,2-PDO. The understanding of the kinetics of glycerol fermentation was also central to the efficient production of 1,2-PDO, as the elucidation of the genetic manipulations needed to increase glycerol utilization was applied as a means of increasing the productivity of 1,2-PDO production. Thus, despite the challenges

associated with the use of glycerol as a carbon source, the improved fundamental knowledge of fermentative glycerol metabolism in *E. coli* enabled strategies that could fully capitalize on the advantageous nature of glycerol for the production of 1,2-PDO.

5.2. Efficient co-production of ethanol and hydrogen from glycerol waste streams

Ethanol (C_2H_6O) has emerged as a important renewable and sustainable fuel, and despite the promise of emerging technologies for the sustainable production of gasoline like replacements, currently represents the most widely produced biofuel in the United States (Fortman et al., 2008). Industrial production of ethanol is dominated by the conversion of sugars from starch or sucrose to ethanol by the yeast *Saccharomyces cerevisiae* (Gray et al., 2006) and significant research efforts have focused on the engineering of other microorganisms to produce maximal ethanol yields and titers from lignocellulosic derived sugars (Ingram et al., 1999; Kim et al. 2007; Trinh et al. 2008; Yomano et al. 2008; Zhang et al., 1995). Despite the success of these engineering efforts, the oxidation state of sugars such as glucose dictates that a significant portion of carbon is lost as CO_2 during the fermentation process, significantly reducing the overall product yield. On the other hand, the reduced nature of glycerol permits the co-production of ethanol and formic acid (or ethanol and hydrogen), enabling increased overall product yields compared to sugars. While the ability to co-produce formic acid with ethanol represents a

significant economical advantage, from a fuel perspective, hydrogen co-production is an intriguing prospect, as biohydrogen gas has been proposed as an attractive energy carrier and microbial processes for biohydrogen production are currently being investigated (Hallenbeck and Ghosh, 2009). In order to capitalize on the high degree of reduction of glycerol for the co-production of ethanol and hydrogen, engineering efforts aimed at increasing the productivity of product formation must be undertaken to provide the most efficient process for the conversion of glycerol waste streams into these two fuels.

Another intrinsic advantage of the use of glycerol as a carbon source for the co-production of ethanol and hydrogen is the near homoethanologenic nature of glycerol fermentation in *E. coli* (Dharmadi et al., 2006). As previously discussed, the conversion of glycerol to ethanol and formate (or H_2/CO_2) represents the only redox balanced ATP generating pathway from glycerol in *E. coli*, underlying its key role and requirement during fermentative glycerol metabolism. For this reason, by-product formation during glycerol fermentation is minimal and from a metabolic engineering standpoint the most important issue is increasing the rate and productivity of ethanol production from glycerol. The co-production of hydrogen can be accomplished through an active formate hydrogen lyase (FHL) complex, ensuring the complete conversion of formate generated via the pyruvate formate lyase complex (PFL) to H_2 and CO_2. As previously discussed, pyruvate dissimilation to acetyl-CoA during fermentative glycerol utilization has been shown to take place exclusively through PFL, and with environmental conditions selected to ensure FHL

activity (i.e. acidic pH), this will result in hydrogen production in equimolar quantities to ethanol.

With ethanol and hydrogen co-production an inherent characteristic of the properties and requirements for glycerol fermentation in *E. coli*, the most important aspect to consider from a metabolic engineering standpoint is improving the productivity of ethanol production. This can be accomplished through application of the findings on the kinetics of glycerol fermentation, and the specific discovery that glycerol dehydrogenase and dihydroxyacetone kinase control the glycerol to ethanol pathway and their concurrent overexpression leads to increased glycerol utilization fluxes (Section 4.3). With these findings in mind, the performance of strain MG1655 (pZSKLMgldA) overexpressing these two enzymes was characterized in a controlled environment (SixFors multi-fermentation system, see Materials and Methods) and using a high initial concentration of crude glycerol (~50 g/L), a direct by-product of biodiesel production (Figure 5.6). While this strain exhibited the characteristic near homoethanologenic product profile with complete formate conversion to H_2 and CO_2, high rates of glycerol utilization were only observed for the first 24 hours, leaving approximately 30 g/L of glycerol remaining in the media after 84 hours (Figure 5.6). The incomplete glycerol utilization of this strain represents a significant concern, as this inefficiency would hinder this process from being viable for the industrial scale conversion of glycerol into value added products.

Several important characteristics from the fermentation profile of MG1655 (pZSKLMgldA) (Figure 5.6) can be seen which may provide clarity to the issues at hand and offer a means of achieving more complete and efficient glycerol utilization. One interesting facet of the product profile is the fact that while a small amount of acetate (~0.36 g/L) is produced during the initial 12 hours, after 24 hours of fermentation the acetate level has dropped to around 0.25 g/L, indicating acetate consumption during this time (Figure 5.6).

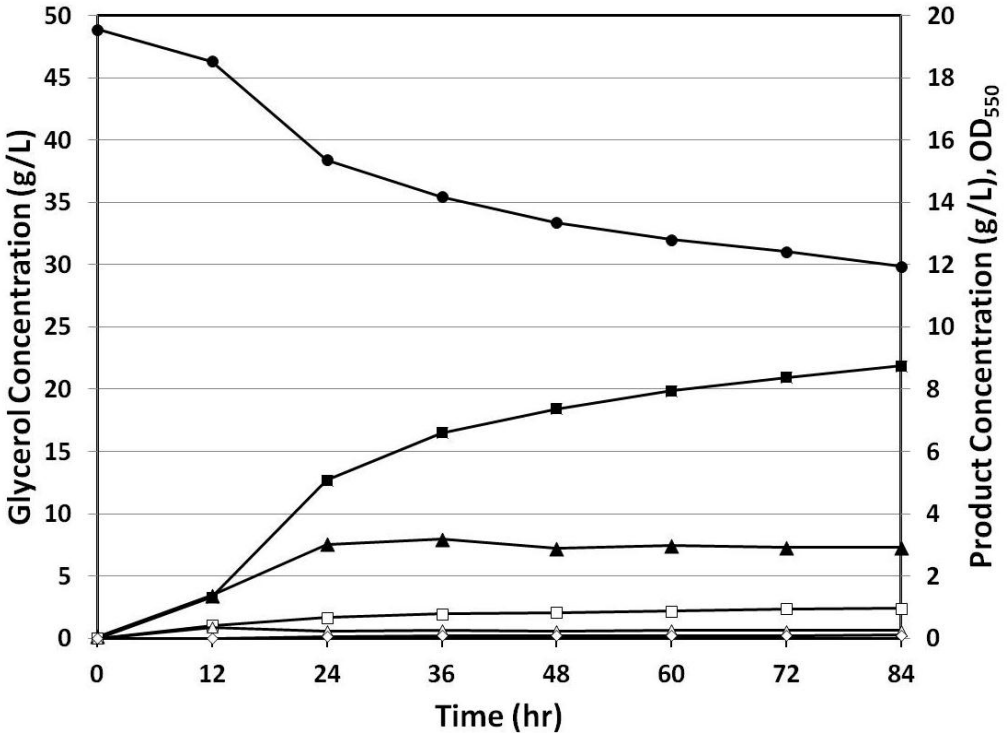

Figure 5.6 Crude glycerol utilization and product synthesis by MG1655 (pZSKLMgldA) in the presence of high initial glycerol concentrations. Experiments conducted in fully controlled fermentor at pH 6.3. Fermentation profile shown with concentration of cells (▲), glycerol (●), ethanol (■), 1,2-PDO (◇), acetate (△), and succinate (□).

This decrease in acetate concentration coincides with the highest observed glycerol utilization rates, which are seen between 12 and 24 hours (Figure 5.6),

demonstrating a possible link between the two observed dynamics. In addition, HPLC detectable levels of 1,2-PDO are also observed during the fermentation, a characteristic not seen previously with the use of lower starting glycerol concentrations in the media (Figure 5.6). These two key features of the fermentation could indicate several possible issues contributing to the incomplete and inefficient nature of glycerol utilization.

The production and subsequent consumption of acetate during the fermentation could be a result of carbon partitioning at the acetyl-CoA node, a key branch point where acetyl-CoA can be converted to acetate or reduced to ethanol. This node and the balancing of carbon flux to acetate and ethanol has been shown to be a critical factor during glucose fermentation (Gupta and Clark, 1989; Sawers and Clark, 2004), and while acetate production was minimal in wild-type strains, the increased glycerol utilization rates of this recombinant strain could have pronounced effects on importance of this node. Another critical factor with acetate production/consumption is the presence of rich nutrients (i.e. tryptone and yeast extract) in the fermentation media utilized for these experiments. While previous studies have shown that during glycerol fermentation the majority of products are synthesized from glycerol as opposed to the rich nutrients in a low supplementation media (Murarka et al., 2008), the presence of higher concentrations of tryptone and yeast extract could result in a slightly larger contribution to product synthesis. While the contribution to product synthesis would still only represent a small portion compared to that synthesized from glycerol, acetate production from the rich nutrients and its subsequent consumption could significantly contribute to the

overall redox balance. Under the assumption of that the production of acetate from rich nutrients results in minimal consumption or generation of reducing equivalents, the utilization of acetate and conversion to ethanol (through acetyl-CoA as an intermediate) would enable the consumption of two reducing equivalents in the reduction of acetyl-CoA to ethanol. With the ability for *E. coli* to utilize glycerol under fermentative conditions directly linked to the presence of pathway(s) for the consumption of excess reducing equivalents generated in the synthesis of cell mass from glycerol, the reduction of acetate to ethanol could help facilitate the increased glycerol utilization and cell growth rates by providing an additional sink for these excess reducing equivalents. This same issue of redox poise is also indicated by the detectable levels of 1,2-PDO observed during the fermentation (Figure 5.6). 1,2-PDO levels with the wild-type strain are only detectable through NMR analysis, and while the HPLC detectable levels could simply be a result of the increased amount of glycerol consumption, they could also reflect the need for an increased level of 1,2-PDO production as a means of consuming excess reducing equivalents when increased flux the through the glycerol to ethanol pathway is accompanied by an increased growth rate. The possible need for increased 1,2-PDO production also brings up the issue of DHAP availability and the coupling between glycerol dissimilation and pyruvate synthesis with the use of the PEP-dependent DHAK (Section 4.2.4). With one or any combination of these possible issues contributing to the incomplete and inefficient glycerol utilization, testing of each possibility is critical to determining a strategy for increased glycerol utilization enabling efficient ethanol and hydrogen co-production.

Prior to testing each possible scenario, an additional test with MG1655 (pZSKLMgldA) was run using refined glycerol to ensure that the issues at hand were not a result of any contaminants present in the crude glycerol obtained directly as a by-product of biodiesel production. However, the use of ~50 g/L refined glycerol resulted in no appreciable differences in fermentation profiles, as summarized by the glycerol consumption and ethanol production levels after 60 hours in Table 5.4.

Table 5.4 Glycerol consumption, ethanol synthesis, and 1,2-PDO production after 60 hours in fermentations aimed at improving overall glycerol utilization

Strain[a]	Glycerol Type	Acetate Addition (mM)	Glycerol Consumption (g/L)	Ethanol Produced (g/L)	1,2-PDO Produced (g/L)
MG1655 (pZSKLMgldA)	Crude	-	16.9	7.9	0.09
MG1655 (pZSKLMgldA)	Refined	-	16.1	7.3	0.04
MG1655 (pZSKLcfgldA)	Refined	-	13.2	5.9	0.29
MG1655 (pZSKLcfgldAmgsAyqhD)	Refined	-	19.0	8.5	0.48
MG1655 Δ*ackA-pta* (pZSKLMgldA)	Refined	-	11.2	5.2	0
MG1655 Δ*ackA-pta* (pZSKLcfgldAmgsAyqhD)	Refined	-	14.4	6.5	0.24
MG1655 (pZSKLMgldA)	Refined	10[b]	25.9	11.3	0.20
MG1655 (pZSKLMgldA)	Refined	30[c]	35.7	17.1	0.34

[a]All strains run in fully controlled fermentor at pH 6.3 with ~50 g/L glycerol. Δ"*gene*" represents gene deletion. Gene overexpressions are indicated by the plasmid name in parenthesis.
[b]10 mM sodium acetate added at 24 hours.
[c]10 mM sodium acetate added at 24, 36 , and 48 hours.

With the possibility of crude glycerol contaminants ruled out, additional experiments were conducted to directly test the impacts of manipulations affecting each of the aforementioned areas of metabolism (Table 5.4). Despite the improved efficiency of the ATP-linked fermentative glycerol dissimilation pathway seen

previously (Section 4.2.4), expression of the ATP-dependent DHAK in place of the PEP-dependent DHAK in combination with glycerol dehydrogenase (i.e. MG1655 (pZSKLcfgldA)) did not improve glycerol utilization. In fact, a slight decrease in glycerol consumption levels were seen with this strain after 60 hours when compared to MG1655 (pZSKLMgldA) (Table 5.4). It is also important to note that the expression of the decoupled fermentative glycerol dissimilation did result in an increased 1,2-PDO concentration, as over 6 times the amount of 1,2-PDO was produced compared to expression of the coupled pathway (Table 5.4). As a means of investigating the influence of increased 1,2-PDO production as a contributing factor for increased glycerol utilization, MG1655 carrying a low copy plasmid containing the 1,2-PDO production pathway and decoupled glycerol dissimilation pathway (Section 5.1.3) was tested (i.e. MG1655 (pZSKLcfgldAmgsAyqhD) expressing *C. freundii* ATP-dependent DHA kinase and *E. coli* glycerol dehydrogenase, methylglyoxal synthase and aldehyde oxidoreductase.). While the expression of a complete 1,2-PDO production pathway resulted in the production of over 10 times the 1,2-PDO produced by MG1655 (pZSKLMgldA), this increase was only accompanied by a minor improvement in glycerol utilization, as 19.0 g/L was consumed in 60 hours, compared to 16.1 g/L by MG1655 (pZSKLMgldA)(Table 5.4).

With increased 1,2-PDO production resulting in only modest improvements to glycerol utilization, manipulation of pathways at the acetyl-CoA node were investigated as a means for increasing glycerol utilization. In order to assess whether the synthesis of acetate results in a limitation at the acetyl-CoA node, minimizing ethanol production, a strain with a deletion of *ackA-pta* operon

(encoding acetate kinase and phosphate acetyltransferase) was transformed with pZSKLMgldA. However, the deletion in this strain (i.e. MG1655 Δ*ackA-pta* (pZSKLMgldA)) resulted in a significant decrease in glycerol utilization compared to MG1655 (pZKLMgldA), as only 11.2 g/L of glycerol were consumed in 60 hours (Table 5.4). Increased 1,2-PDO production in combination with the *ackA-pta* deletion was also investigated through the expression of the complete 1,2-PDO production pathway in this genetic background (i.e. MG1655 Δ*ackA-pta* (pZSKLcfgldAmgsAyqhD)). While a slight improvement in glycerol consumption was observed when compared to the expression of PEP-dependent DHAK and glycerol dehydrogenase only in this genetic background, the overall glycerol consumption was still lower than that by MG1655 (pZSKLMgldA) (Table 5.4). These results indicate that the *ackA-pta* operon, which is constitutively expressed (Brown et al., 1977), plays a role in enabling increased glycerol utilization under these conditions.

With the importance of the *ackA-pta* operon and the observed acetate consumption with MG1655 (pZSKLMgldA), the addition of small amounts of sodium acetate to the fermentation media was investigated as a means of increasing glycerol utilization. To this end, fermentations with MG1655 (pZSKLMgldA) were conducted in which 10 mM sodium acetate was added to the media after 24 hours of fermentation. This time corresponds to the point at which glycerol utilization rates decreased and acetate consumption creased in previous fermentations with this strain (Figure 5.6). The addition of this small amount of acetate resulted in significantly higher glycerol utilization, with 25.9 g/L glycerol consumed after 60

hours (Table 5.4). This increase in glycerol consumption was a result of significantly increased glycerol utilization rates in the 12 hours after acetate addition, with volumetric rates of utilization of 7.34 mmol/L/hr, 9.08 mmol/L/hr, and 3.11 mmol/L/hr for the 12 hour intervals between 12 and 48 hours respectively (data not shown). Despite these increases in glycerol utilization, a significant portion of the ~50 g/L glycerol initially present remained unfermented in the media after 60 hours. In an attempt to prolong these high glycerol utilization rates and improve the overall efficiency of the process, additional fermentations were run in which 10 mM sodium acetate additions were made at 24, 36 , and 48 hours. With these acetate additions, MG1655 (pZSKLMgldA) consumed 35.7 g/L of glycerol with the production of 17.1 g/L ethanol after 60 hours (Table 5.4), in large part to glycerol utilization rates remaining above 7 mmol/L/hr between 12 and 48 hours (data not shown).

Despite the obvious improvements to glycerol utilization with acetate addition, the underlying reasons behind this requirement are still unclear. The fact that exogenous acetate is utilized during the fermentation could indicate a significant contribution to the overall redox balance of the system. While the deletion of the *ackA-pta* operon resulted in decreased glycerol utilization, this is likely a result of the inability to produce acetate initially for consumption during the periods of high glycerol utilization. This is due to the fact that while the *ackA-pta* pathway can operate in both directions, the utilization of acetate through this pathway is thought to take place only under aerobic conditions or at high concentrations of acetate (El-Mansi et al., 2006; Wolfe 2005). On the other hand,

acetyl-CoA synthetase (encoded by *acs*), which converts acetate directly to acetyl-CoA with the conversion of ATP to AMP, is thought to function in a mainly anabolic role, scavenging acetate present in the extracellular medium even at low concentrations (Kumari et al., 2007; Wolfe, 2005). While this is a very ATP intensive process, the conversion of acetate to acetyl-CoA and the subsequent reduction to ethanol would result in the consumption of 2 reducing equivalents which could have significant effects during periods of high glycerol utilization and growth. This pathway could serve as an additional sink for the reducing equivalents generated during the synthesis of cell mass. While 1,2-PDO production accomplishes this in wild-type cells, the drastic increases in glycerol utilization rates and cell growth seen upon the overexpression of glycerol dehydrogenase and dihydroxyacetone kinase could result in the requirement for an increased rate of 1,2-PDO production that the native 1,2-PDO pathways are unable to achieve. This scenario is supported by the fact that overexpression of the entire 1,2-PDO production pathway resulted in increased glycerol consumption, albeit not nearly at levels seen with the addition of acetate (Table 5.4). In addition, the potential for acetate assimilation and conversion to ethanol to serve as a sink for reducing equivalents has been reported as a means of eliminating glycerol synthesis during ethanol production from glucose in *Saccharomyces cerevisiae*, as glycerol formation is essential for the reoxidation of NADH produced via biosynthetic processes during glucose fermentation with *S. cerevisiae* (Medina et al., 2010).

Despite this uncertainty, the improvements to glycerol utilization with the addition of acetate clearly demonstrate the viability of this strategy for improving

the efficiency of the co-production of ethanol and hydrogen from high glycerol concentrations. Utilizing this approach, additional fermentations were conducted with MG1655 (pZSKLMgldA) with crude glycerol to assess the potential for the conversion of glycerol waste streams produced as a by-product of biodiesel production to value added fuels ethanol and hydrogen. For these fermentations, 10 mM sodium acetate was added at 24, 36, and 48 hours, with an additional 5 mM added at each of 60 and 72 hours in an attempt to maximize glycerol utilization.

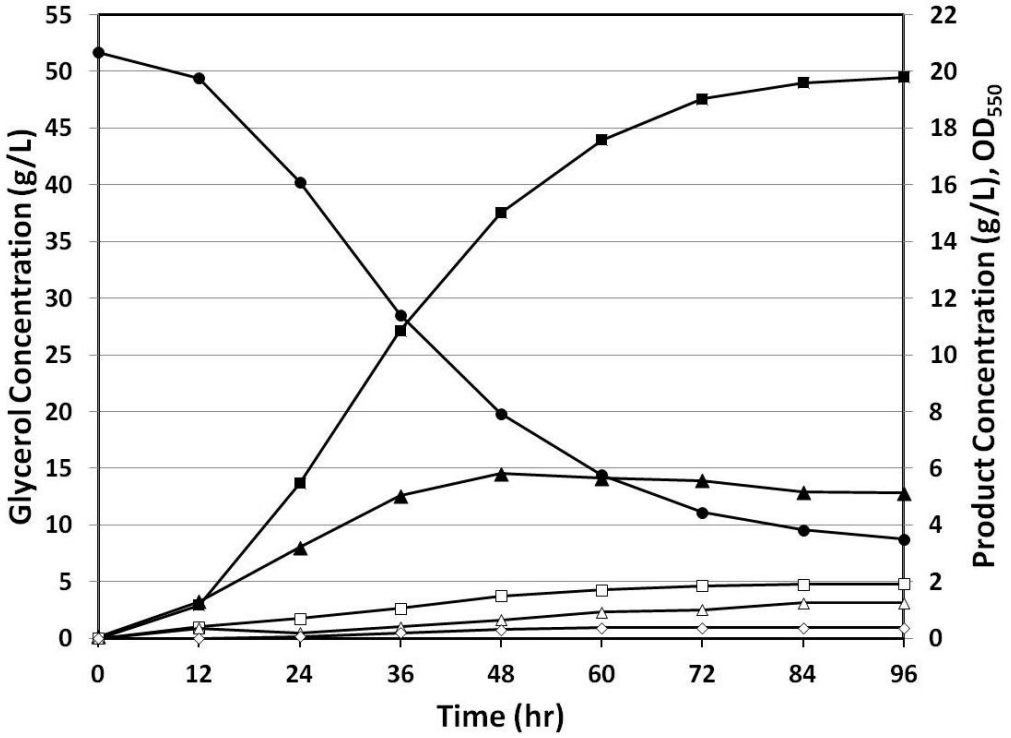

Figure 5.7 Efficient co-production of ethanol and hydrogen from crude glycerol by MG1655 (pZSKLMgldA). Experiments conducted in fully controlled fermentor at pH 6.3. Sodium acetate added at 10 mM at 24, 36, and 48 hours, with 5 mM additions at 60 and 72 hours. Fermentation profile shown with concentration of cells (▲), crude glycerol (●), ethanol (■), 1,2-PDO (◇), acetate (△), and succinate (□). The coefficient of variation (standard deviation/mean x 100) was below 10% in all cases.

This strategy enabled prolonged glycerol utilization, with nearly 43 g/L of crude glycerol consumed over the entirety of the fermentation (Figure 5.7). This resulted in the production of nearly 20 g/L ethanol with no formate accumulation, signifying the complete conversion of formate to hydrogen and carbon dioxide under these conditions (Figure 5.7). Under the assumption that all ethanol was synthesized from glycerol, and neglecting any glycerol converted to acetate, this would result in nearly 430 mM hydrogen gas co-produced along with ethanol, demonstrating the advantageous nature of glycerol from an overall product yield standpoint during ethanol production. However, a more conservative estimate under the assumption that all acetate consumed was converted to ethanol would result in approximately 400 mM hydrogen produced. In addition to enabling the conversion of high glycerol concentrations, the productivities of ethanol and hydrogen co-production (4.5 mmol ethanol/L/hr) obtained with this process were superior to others reported for the conversion of glycerol to ethanol-hydrogen (Hongbo and Wood, 2010; Ito et al., 2005; Sakai and Yagishita, 2007) demonstrating the advantages of a thorough knowledge of fermentative glycerol metabolism in the design of metabolic engineering strategies for its conversion to fuels and chemicals.

Overall, the combined application of fundamental knowledge of the fermentative metabolism of glycerol and kinetics of glycerol fermentation enabled an effective strategy for the co-production of two important biofuels from crude glycerol. The overexpression of the two key enzymes controlling the glycerol to ethanol pathway facilitated the increased rates of glycerol utilization and ethanol synthesis. However, the utilization of high concentrations of glycerol proved

difficult without further investigation into the underlying issues. The determination of acetate consumption as an enabling factor for increased glycerol utilization resulted in the ability to develop an efficient process for the conversion of high glycerol concentrations to value added fuels. Despite the challenges with the use of high glycerol concentrations and the ambiguity of the requirement for exogenous acetate addition, these results clearly demonstrate the potential for the industrial scale conversion of glycerol waste streams into the biofuels ethanol and hydrogen.

Chapter 6

Conclusions and Future Work

Due to its availability, low-price, and high degree of reduction, glycerol has become an attractive carbon source for the production of fuels and reduced chemicals. While the establishment of the ability for *Escherichia coli*, the workhorse of modern biotechnology, to utilize glycerol under fermentative conditions holds great promise for the conversion of glycerol into higher value fuels and chemicals, the relative lack of knowledge of fermentative glycerol metabolism in this bacterium limits the scope for which rational metabolic engineering strategies can be designed for the synthesis of high yields and titers of target products. In order to establish the knowledge platform required for the development of these efficient metabolic engineering strategies, this study sought to increase the fundamental understanding of fermentative glycerol metabolism in *E. coli*, whose principles were then applied as a means of increasing the yields and titers of fuels and reduced chemicals from glycerol.

The investigation into fermentative glycerol metabolism in *E. coli* revealed several key aspects, the involvement of which was linked to inherent system characteristics not seen with traditional carbon sources or respiratory glycerol metabolism. Central to many facets of fermentative glycerol metabolism was the involvement of a PEP-dependent dihydroxyacetone kinase (encoded by *dhaKLM*) required for the fermentative glycerol dissimilation pathway. The essential nature of this enzyme during fermentative glycerol utilization not only directly necessitates the activity of several additional pathways and mechanisms for maximal glycerol utilization, but the apparent consequences of the role of this enzyme on a system scale results in several secondary effects during fermentative glycerol metabolism. The coupling between glycerol dissimilation and the conversion of PEP to pyruvate created by this enzyme results in the involvement of the respiratory glycerol dissimilation pathway as a decoupling mechanism to increase the availability of precursor metabolites and enable maximum glycerol utilization. As a consequence of the oxidation reaction in the respiratory dissimilation pathway being tied to the quinone pool, the reduction of fumarate to succinate through fumarate reductase serves as an important mechanism in providing the terminal electron acceptor for these electrons generated during glycerol dissimilation.

From a system standpoint, the key role of PEP-dependent DHAK and the secondary impacts of its involvement also proved critical in the overall understanding of fermentative glycerol metabolism. The fermentative glycerol dissimilation pathway was found to be the pacemaker for glycerol utilization under fermentative conditions, with the transcription of the *dhaKLM* operon under control

of the CRP-cAMP complex providing the key link between the regulation of fermentative glycerol dissimilation and fermentative metabolism in general. In addition, the low intracellular PEP likely seen with the essential involvement of PEP-dependent DHAK may have resounding implications on several other areas of fermentative glycerol utilization. The importance of the type II fructose 1,6-bisphosphotases encoded by *glpX* and *yggF* in addition to the type I FBPase encoded by *fbp* is likely a result of the low intracellular PEP levels and the impact this has on the activity of the latter enzyme. Without sufficient PEP levels to provide maximal activity of this enzyme, the two type II enzymes become important for the conversion of F1,6BP to F6P, providing not only a means for the synthesis of required precursor metabolites, but also avoiding a detrimental accumulation of F1,6BP during fermentative glycerol metabolism. This compound also serves as a key metabolic intermediate from a regulatory perspective, as the activity or lack thereof of several enzymes during fermentative glycerol metabolism can be tied to intracellular F1,6BP levels, including glycerol kinase, PEP carboxylase, and pyruvate kinase I.

With the involvement and importance of several key enzymes directly tied to PEP and F1,6BP levels, investigation into the intracellular concentrations of these metabolites and the complex network of regulatory interactions they are involved in serves as the main area for future experimental work into fermentative glycerol metabolism in *E. coli*. Measurement of *in vivo* intracellular concentrations of these metabolites during fermentative glycerol metabolism and comparison to levels during respiratory metabolism as well as those during the metabolism of other

carbon sources can likely provide additional evidence to the key role of these metabolites and strengthen the current proposed regulatory role PEP and F1,6BP have on the involvement of certain enzymes. Furthermore, measurement of these metabolites in varying genetic backgrounds, including during the expression of the ATP-linked fermentative glycerol dissimilation pathway (i.e. expression of ATP-dependent DHAK) and in FBPase deletion strains, can help elucidate the proposed regulatory mechanisms and provide additional evidence for the role of the various FBPases involved during fermentative glycerol metabolism. Moreover, measurement of FBPase enzyme activity in varying FBPase deletion strains will provide further confirmation of the proposed roles of GlpX, Fbp, and YggF. In addition to this proposed work to strengthen the metabolic model for fermentative glycerol utilization by *E. coli*, the implementation of system wide experimental techniques can help to validate the involvement of the key pathways and mechanisms determined by this study and uncover additional important aspects not readily apparent from the current analysis. This includes the analysis of gene expression, protein levels, and metabolic flux analysis during fermentative glycerol metabolism which can provide additional information to both confirm the proposed model and develop new hypothesis about the role of specific genes, proteins, and pathways involved during fermentative glycerol metabolism.

Using the proposed metabolic model and improved understanding of fermentative glycerol metabolism in *E. coli* developed by this study as a platform, metabolic engineering strategies were developed for efficient conversion of glycerol into fuels and reduced chemicals. Specifically, 1,2-propanediol and the co-

production of ethanol and hydrogen were chosen as target compounds to demonstrate how the increased understanding of fermentative glycerol metabolism by *E. coli* can facilitate the ease of which the advantageous nature of glycerol can be exploited as a carbon source for production of high yields of fuels and reduced chemicals. Key to the production of 1,2-PDO is the affects of the coupling between fermentative glycerol dissimilation and pyruvate synthesis on the availability of DHAP, a critical node where partitioning of carbon flux between 1,2-PDO synthesis and glycolysis occurs. The overexpression of a functional pathway for 1,2-PDO synthesis from DHAP, the replacement of the native *E. coli* PEP-dependent DHAK with the ATP-dependent DHAK from *C. freundii* to ensure DHAP availability, and the disruption of the fermentative lactate and acetate pathways all contributed to the efficient production of 1,2-PDO. Since the synthesis of 1,2-PDO from glycerol is both redox- and ATP-consuming, the co-production of other fermentation products was required to ensure redox balance and ATP generation. Ethanol was determined to be the required co-product due to the ability of the glycerol to ethanol (and formate or H_2/CO_2) pathway to produce ATP without any stoichiometric limits of its production due to redox constraints. In addition, an anaerobically active PDH complex and hydrogen recycling through fumarate reductase were important during 1,2-PDO production with the engineered strain due to their ability to contribute to NADH generation. The final strain produced 5.6 g/L 1,2-PDO (at a yield of 21.3 % w/w), surpassing the performance of *E. coli* strains engineered to produce 1,2-PDO from glucose and was able to use crude glycerol, a by-product of biodiesel production.

Efficient ethanol and hydrogen co-production was accomplished through the implementation of metabolic engineering strategies based on the findings that glycerol dehydrogenase and dihydroxyacetone kinase control the glycerol to ethanol pathway and their concurrent overexpression leads to increased glycerol utilization fluxes. This fact combined with the homoethanologenic nature of glycerol fermentation and the utilization of environmental conditions ensure complete conversion of formate to CO_2 and H_2, was effective for the conversion of lower initial glycerol concentrations to ethanol and hydrogen, however the conversion of higher initial concentrations of glycerol proved more difficult. Testing of various hypotheses based on experimental observations resulted in the discovery that the addition and subsequent consumption of small amounts of acetate during glycerol fermentation enabled more efficient and complete glycerol utilization. This phenomenon is likely the result of the ability of acetate to ethanol conversion to provide an additional sink for reducing equivalents that may be needed during the high rates of glycerol utilization and cell growth seen with this engineered strain. Utilization of this strategy resulted in the production of nearly 20 g/L ethanol (with the co-production of approximately 400 mM hydrogen) from crude glycerol, a by-product of biodiesel production, indicating the potential for industrial scale conversion of waste glycerol to valuable fuels.

While the success of these metabolic engineering strategies for fuel and reduced chemical production from glycerol demonstrate the potential for the conversion of waste glycerol streams to value added products, additional future work could help to both improve these processes as well as elucidate underlying

mechanisms involved. In terms of 1,2-PDO production, the ATP consuming nature of the pathways native to *E. coli* involved in 1,2-PDO synthesis are a severe limiting factor during 1,2-PDO production. Improvements to the yield and titer of 1,2-PDO are envisioned through the exploitation of pathways that could circumvent the energy loss associated with the conversion of DHAP to methylglyoxal and inorganic phosphate. This could be accomplished through the identification of enzymes capable of reducing lactate to 1,2-PDO, which would enable 1,2-PDO production through a ATP generating pathway. While this may prove difficult with native *E. coli* enzymes, as no known enzyme in *E. coli* has been shown to catalyze the reduction of lactate to lactaldehyde, species of *Lactobacillus* have been shown to ferment lactic acid to 1,2-PDO and acetic acid (Oude Elferink *et al.*, 2001), indicating the involvement of enzymes capable of this conversion. Identification and expression of these or alternative enzymes in combination with alterative metabolic engineering strategies for lactate generation from glycerol based on the platform provided by the understanding of glycerol metabolism could provide increased titers and yields of 1,2-PDO.

Another intriguing area which may warrant future investigation is in the determination of the exact mechanisms involved in acetate consumption as an enabling factor for increased and complete glycerol utilization upon the overexpression of the rate limiting steps in the glycerol to ethanol pathway. While this study showed that the application of this strategy could improve glycerol utilization and provide increased ethanol and hydrogen co-production, the elucidation of underlying components of this phenomenon will enhance the

fundamental understanding of this process. Key to this investigation will be the determination of the enzyme(s) involved in acetate assimilation and whether this process takes place through acetyl-CoA synthetase (encoded by *acs*) or the enzymes encoded by the *ackA-pta* operon (i.e. acetate kinase and phosphate acetyltransferase). Analysis of the effects the deletion of these genes has on acetate consumption and glycerol utilization in strains overexpressing glycerol dehydrogenase and dihydroxyacetone kinase will provide evidence for the involvement of either pathway. This analysis is critical for a better understanding of the mechanism by which acetate assimilation enables increased glycerol utilization and how it is more beneficial that 1,2-PDO production if reducing equivalent consumption is indeed the enabling factor. While assimilation through acetate kinase and phosphate acetyltransferase would be more efficient from an ATP standpoint than assimilation through acetyl-CoA synthetase or 1,2-PDO production from glycerol (i.e. less ATP consumed per NADH consumed), this pathway has not been shown to be the active route for acetate assimilation outside of aerobic conditions with high acetate concentrations. On the other hand, acetyl-CoA synthetase, as with 1,2-PDO production from glycerol, would stoichiometrically consume an ATP equivalent with NADH consumption (i.e. 1 ATP equivalent consumed per NADH consumed), making the exact rationale for its advantageous nature unclear from a system standpoint. Investigation into this area will not only answer the fundamental questions regarding the mechanisms involved in this process, but also possibly provide a platform by which this process can be exploited

as a means of redox consumption during the production of other fuels or reduced chemicals enabling increased titers and yields.

Overall, this in-depth study into fermentative glycerol metabolism in *E. coli* has resulted in a significant improvement in the understanding of the essential and important pathways and mechanisms involved during the utilization of this abundant and inexpensive carbon source under fermentative conditions. The key principles elucidated during the study of cellular metabolism of such a highly reduced carbon source provided the required knowledge base enabling the development of efficient metabolic engineering strategies aimed at the production of 1,2-PDO and ethanol/hydrogen production exploiting the advantageous nature of glycerol for fuel and reduced chemical production. This not only demonstrates the potential for the industrial scale conversion of glycerol waste streams into value added products, but also provides a platform for future engineering efforts targeting a larger repertoire of fuels and chemicals, essential for the long term viability of the biofuel and biochemical industry.

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Appendix

Table A1 Verification primers for gene deletions used in this study

Verification Primers	Sequence[a]
v-dhaK	CGTGTCGTTGAACATCATCC GAGTGAGCCAGTTAACAATTTG
v-gldA	ATGGACCGCATTATTCAATCAC GCCTACAAAAGCACGCAAATTC
v-glpK	CGCTGTAATATGACTACGGGAC CGGCATAAACGCTTCATTCG
v-glpA	CGTTTCACTTTCGAATTATGAGCG TGACCACGAGTGACAATGG
v-glpB	ATCAGGGATTGTGTGGTCTG CACTTAATGCAGTTTTCGAAGC
v-glpC	TGCCGTCAGTGCTTTACATG GAATCGTGTACGCAAAAATGCC
v-glpD	GAAAGTGAATGAGGGCAGC TCTGACCTGATCACCTTACG
v-glpF	TGACTTTCACGCATACAACAAAC GTCATGTTTAATTGTCCCGTAGTC
v-ptsG	AAGCACCCATACTCAGGAG CCTGTAAAAAAGGCAGCCATC
v-ptsH	AGGCTAGACTTTAGTTCCACAAC ACCCTACCTTACTTGTGACTG
v-ptsI	GAAAGCGGTTGAACATCTGG TCCTTCTTGTCGTCGGAAAC
v-crr	AATCTGCTAATCCACGAGATGC CAGCATGAGAGCGATGAATTG
v-ptsA	GTGCGCTCTTATCCTGTAAAC TTAGCGGTGTCCAGATACAG
v-tpiA	GCGGCCATCTTCCTTTATTCG GATATGAAATCCGGCACCTG
v-fsaA	TGTGCGTCAACTGTTCAGAG ACTCCGCCACGTCATCAG
v-fsaB	GCTGCAACATGAACTGGAAC CGGTGATTGAATAATGCGGTC
v-fbaB	GACAATCCATAGCCGATATCC AACGCCTTATCCGTCCTAC
v-pgi	CTGTGACTGGCGCTACAATC CCTTATCCGGCCTACATATCG
v-zwf	AAGTATACCCTGGCTTAAGTACC CTGCGCAAGATCATGTTACC
v-fbp	GTTAAGATTGTTGCGGTCGC CAAGAGTCAACATTGCGCAG
v-glpX	TTCGCGCCATTCCTTACTGC AAGGACTGGAAGGCTCAATCG
v-yggF	AATATCTGCTGGCACAATTTGG AGCACATCATCTTCTGTCAGC
v-ybhA	GGTGAATGCTGAATTAAAACGCAG TGTACGCGCAAATTAAAAGTGTG
v-pykA	CCTGGTTGTTTCAGTCAACG GTTCAAGTTTGCTTCAGAATATTCG
v-pykF	GCAAGTTTCTCCCATCCTTCTC ATCAGGGCGCTTCGATATAC
v-pflB	AAATCCACTTAAGAAGGTAGGTG TCGTGGAGCCTTTATTGTAC
v-ppc	AGGATACAGGGCTATCAAACG AAAAGCACGAGGGTTTGC
v-pckA	GGTTAACACCCCCAAAAAGA GGCACGACAAAAGAAGGGTA
v-maeA	CCCAGGGATGGATATTCAAA GAAATAGCCCGGTAGCCTTC
v-maeB	GCTGTTGCCCACACACTTA TTGCAGCTAAACTGCTTACCC
v-pps	TGCAAAGATAAATGCGCAGAAATG ATCTTCGGGGATCACATAACC
v-crp	TATCTGGCTCTGGAGAAAGC GAAACAAAATGGCGCGCTAC
v-cyaA	TCATGACGGGTAGCAAATCAG AGATTGCATGCCGGATAAGC
v-gloA	GAATCTGTTAGCCATTTTGAGG CTTGTCGATGCAGTAAAGATG
v-fnr	TGCTTAGACTTACTTGCTCCC CAGAAGGATAGTGAGTTATGCGG
v-aldA	TGTCCTTTCACGATTCCGTC TATGGCTTCGTCACACAGC
v-frdA	TACCCTGAAGTACGTGGCTG AGGTAGTTGCGTCATAAGGC
v-ackApta	CATAAAACGGATCGCATAACG CATGATGATGCCAACG
v-adhE	CGAGCAGATGATTTACTAAAAAAG ATCGGCATTGCCCAGAAGG
v-ldhA	GCTTAAATGTGATTCAACATCACTGG AGAATAGAGGATGAAAGGTCATTG
v-aceF	CGTCTGGCGTAAGAGGTAAAAG CCGATTTGTCTATTCGCTAATAACC
v-fdhF	TGCGTGATTTGATTAACTGGAGC CGAAAGGAGGCTGTAGAAAGG

[a] "v" indicates the primer sequences (5' to 3') that were used for verification purposes during the creation of disruption mutants. The reverse sequence follows the forward sequence in each case. Genes or operons deleted are apparent from primer names.

Table A2 Primers used in the construction of plasmids

Construction Primers	Sequence[a]
pZSKLcfgldA	GACAGGATCCAGGAGCAATTATGGACCGCA
	CAAGCTACGCGTTTATTCCCACTCTTGCAGGA
pZSKLcfgldAmgsA	ACGTACGCGTAGGAGGGTACATTATGGAACTGACGACTC
	GATCACGCGTTTACTTCAGACGGTCCGCG
pZSackApta	CATTAAAGAGGAGAAAGGTACCATGTCGAGTAAGTTAG
	GATGCCTCTAGCACGCGTTTACTGCTGCTGTGC
pZSadhE	CATTAAAGAGGAGAAAGGTACCATGGCTGTTACTAATG
	GATGCCTCTAGCACGCGTTTAAGCGGATTTTTTCG
pZSglpF	TTAAAGAGGAGAAAGGTACCATGAGTCAAACATCAACCTTGAAA
	TGCCTCTAGCACGCGTTTACAGCGAAGCTTTTTGTTCTG
pZSldhA	CATTAAAGAGGAGAAAGGTACCATGAAACTCGCCG
	GATGCCTCTAGCACGCGTTTAAACCAGTTCGTT
pTHgldAmgsAyqhD	GCTATCGAGATCTATGGACCGCATTATTCAATC
	GGCGGAGATCTTTATTCCTCCTTATTTAATCG
pTHgldAmgsAfucO	GCTATCGAGATCTATGGACCGCATTATTCAATC
	GGCGGAGATCTTTATTCCTCCTTATTTAATCG
pTHKLcfgldAmgsAyqhD	CCATGGATCCGAGCTCGAGAATGTCTCAATTCTTTTTTAACC
	GCAGATTAAAGTTGTTCATCTCGAGCCTCCTTACTTCAGACGGTCCG
pTHKLcfgldAmgsAfucO	CCATGGATCCGAGCTCGAGAATGTCTCAATTCTTTTTTAACC
	CATTCTGTTAGCCATCATCTCGAGCCTCCTTACTTCAGACGGTCCG
pTHyqhD	CCGCTCGAGATGAACAACTTTAATCTGCAC
	GAGGTACCTTAGCGGGCGGC
pTHfucO	GATCCTCGAGATGATGGCTAACAGAATGATTC
	GATCGGTACCTTACCAGGCGGTATGGTAAAGC

[a.] Primers listed for the construction of plasmids were utilized according to the procedures in Materials and Methods. Genes cloned are apparent from primer names.

Table A3 FBA reaction network for fermentative glycerol metabolism in *E. coli*

Name	Reaction
Glycerol Uptake	Glycerol (external) -> Glycerol
Glycerol Dissimilation (Respiratory)	Glycerol + ATP -> T3P1 + NADH
Glycerol Dissimilation (Fermentative)	Glycerol -> DHA + NADH
Glycerol Dissimilation (Fermentative r2)	DHA + PEP -> T3P1 + PYR
Gluconeogenesis	2 T3P1 -> F6P
Glycolysis (r3)	G6P <-> F6P
Glycolysis (r4)	F6P + ATP -> 2 T3P1
Glycolysis (r5)	T3P1 <-> 1,3-P2DG + NADH
Glycolysis (r6)	1,3-P2DG <-> 3PDGL + ATP
Glycolysis (r7)	3PDGL <-> PEP
Glycolysis (r8)	PEP -> PYR + ATP
Pentose Phosphate Pathway (r9)	G6P -> RL5P + CO2 + 2 NADPH
Pentose Phosphate Pathway (r10)	RL5P <-> P5P
Pentose Phosphate Pathway (r11)	2 P5P <-> T3P1 + S7P
Pentose Phosphate Pathway (r12)	T3P1 + S7P <-> E4P + F6P
Pentose Phosphate Pathway (r13)	P5P + E4P <-> F6P + T3P1
Glycogen Metabolism	G6P + ATP -> Glycogen
1,2-Propanediol Synthesis	T3P1 + 2 NADH -> 1,2-PDO
Lactate Production	PYR + NADH -> Lactate + ACID
Acetyl CoA (PDH) (r16)	PYR -> AcCoA + CO2 + NADH
Acetyl CoA (PFL) (r17)	PYR -> AcCoA + Formate + ACID
Ethanol Production	AcCoA + 2 NADH -> Ethanol
Acetate Production	AcCoA -> Acetate + ATP + ACID
Formate Decomposition	Formate -> CO2
TCA Cycle (r21)	AcCoA + OAA -> AKG + NADPH + CO2
TCA Cycle (r22)	PEP + CO2 -> OAA
TCA Cycle (r23) (Succinate Production)	OAA + 2 NADH -> Succinate + ACID
Aspartate Synthesis	OAA + NADPH -> Asp
Asparagine Synthesis	OAA + 3 ATP + NADPH -> Asn
Glutamate Synthesis	AKG + NADPH -> Glu
Glutamine Synthesis	AKG + ATP + NADPH -> Gln
Alanine Synthesis	PYR + NADPH -> Ala
Arginine Synthesis	AKG + 7 ATP + 4 NADPH -> Arg + NADH
Proline Synthesis	AKG + ATP + 3 NADPH -> Pro
Leucine Synthesis	2 PYR + AcCoA + 2 NADPH -> Leu + NADH + 2 CO2
Valine Synthesis	2 PYR + 2 NADPH -> Val + CO2
Isoleucine Synthesis	OAA + PYR + 2 ATP + 5 NADPH -> Ile + CO2
Phenylalanine Synthesis	2 PEP + E4P + ATP + 2 NADPH -> Phe + CO2
Tyrosine Synthesis	2 PEP + E4P + ATP + 2 NADPH -> Tyr + NADH + CO2

Tryptophan Synthesis	PEP + E4P + 5 ATP + 3 NADPH + P5P -> Trp + 2 NADH + CO2
Histidine Synthesis	1C + P5P + 6 ATP + NADPH -> His + 3 NADH
Serine Synthesis	3PDGL + NADPH -> Ser + NADH
Glycine Synthesis	3PDGL + NADPH -> Gly + NADH + 1C
Cysteine	3PDGL + 4 ATP + 5 NADPH -> Cys + NADH
Threonine Synthesis	OAA + 2 ATP + 3 NADPH -> Thr
Lysine Synthesis	PYR + OAA + 3 ATP + 4 NADPH -> Lys + CO2
Methionine Synthesis	OAA + 7 ATP + 8 NADPH + 1C -> Met
AMP Synthesis	3PDGL + 1C + 9 ATP + NADPH + P5P -> AMP + 3 NADH
GMP Synthesis	3PDGL + 11 ATP + 1C + P5P -> GMP + 3 NADH
DAMP Synthesis	3PDGL + 9 ATP + 2 NADPH + 1C + P5P -> DAMP + 3 NADH
DGMP Synthesis	3PDGL + 11 ATP + NADPH + 1C + P5P -> DGMP + 3 NADH
UMP Synthesis	OAA + 5 ATP + NADPH + P5P -> UMP
CMP Synthesis	OAA + 7 ATP + NADPH + P5P -> CMP
DUMP Synthesis	OAA + 5 ATP + 3 NADPH + 1C + P5P -> DUMP
DCMP Synthesis	OAA + 7 ATP + 2 NADPH + P5P -> DCMP
1 Carbon Metabolism	Gly -> 1C + CO2 + NADH
Lipid Synthesis (r53)	T3P1 + NADPH -> GlyP
Lipid Synthesis (r54)	8.2 AcCoA + 7.2 ATP + 14 NADPH -> AVGFAT
UDPG Synthesis	G6P + ATP -> UDPG
CDPETN Synthesis	3PDGL + 3 ATP + NADPH -> CDPETN
OHMA Synthesis	7 AcCoA + 6 ATP + 11 NADPH -> OHMA
C14,0 Synthesis	7 AcCoA + 6 ATP + 12 NADPH -> C14,0
CMPKDO Synthesis	PEP + 2 ATP + P5P -> CMPKDO
NHEP Synthesis	1.5 G6P + ATP -> NHEP + 4 NADPH
TGSM Synthesis	F6P + 2 ATP -> TGSM
UDPNAG Production	F6P + AcCoA + 3 ATP -> UDPNAG
UDPNAM Synthesis	F6P + PEP + AcCoA + 4 ATP + NADPH -> UDPNAM
DAP Synthesis	OAA + PYR + 2 ATP + 3 NADPH -> DAP
ATP Maintainence	ATP -> ATPmaintainence
Transhydrogenase (r66)	NADH + 0.33333 ATP -> NADPH
Transhydrogenase (r67)	NADPH -> NADH
LPS Synthesis	0.105 UDPG + 0.1578 CDPETN + 0.1578 OHMA + 0.1578 C14,0 + 0.1578 CMPKDO + 0.1578 NHEP + 0.105 TGSM -> LPScell
Lipid Synthesis	0.25 GlyP + 0.25 Ser + 0.5 AVGFAT + 2 ATP -> LIPIDcell
Protein Synthesis	0.096044 Ala + 0.055304 Arg + 0.04507 Asn + 0.04507 Asp + 0.017123 Cys + 0.049203 Gln + 0.049203 Glu + 0.114544 Gly + 0.017713 His + 0.05432 Ile + 0.084235 Leu + 0.064161 Lys + 0.028735 Met + 0.034639 Phe + 0.04133 Pro + 0.040346 Ser + 0.047432 Thr + 0.010628 Trp

	+ 0.025782 Tyr + 0.079118 Val + 4.3 ATP -> PROTcell
DNA Synthesis	0.246507 DAMP + 0.253493 DCMP + 0.253493 DGMP + 0.246507 DUMP + 5.4 ATP -> DNA
RNA Synthesis	0.261905 AMP + 0.2 CMP + 0.322222 GMP + 0.215873 UMP + 4.4 ATP -> RNA
Peptidoglycan Synthesis	0.167 UDPNAG + 0.167 UDPNAM + 0.33 Ala + 0.167 DAP + 0.167 Glu + 0.833 ATP -> PEPTIDO
Cell Growth	0.014882 DNA + 0.02067 Glycogen + 0.076774 LIPIDcell + 0.025049 LPScell + 0.024639 PEPTIDO + 0.744113 PROTcell + 0.093673 RNA -> CELL

Table A4 Metabolite list for reaction network in Table A3

Abbreviation	Description
1C	1 carbon carrier with methyl group
12PDO	1,2 - Propanediol (external)
13P2DG	1,3-P-D glycerate
3PDGL	3-Phospho-D-Glycerate
Acetate	Acetate (external)
AcCoA	Acetyl Coenzyme A
ADP	Adenosine diphosphate
AKG	a-Ketoglutarate
Ala	Alanine
AMP	Adenosine monophosphate
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine triphosphate
ATPmaintainence	ATP required for cell maintenance
AVGFAT	Average fatty acid molecule
C14:0	Myristic acid
CDPETN	CDP-Ethanolamine
CELL	Average cell with composition corresponding to a molecular weight of 143
CMP	Cytidine monophosphate
CMPKDO	CMP-2-Keto-3-deoxyoctanoate
CO_2	Carbon dioxide (external)
Cys	Cysteine
DAMP	Deoxyadenosine monophosphate
DAP	Diaminopimelate
DCMP	Deoxycytidine monophosphate
DGMP	2-Deoxy-guanosine-5-phosphate
DHA	Dihydroxyacetone
DNA	Average DNA-bound nucleic acid (mol. wt. 309.38)
DUMP	Deoxyuridine monophosphate
E4P	Erythrose 4-phosphate
Ethanol	Ethanol (external)
F6P	Fructose 6-phosphate
Formate	Formate (external)
G6P	Glucose 6-phosphate
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
Glycerol (external)	Glycerol (external)

Glycerol	Glycerol
Glycogen	Glycogen
Glyp	Glyceraldehyde-3-phosphate
GMP	Guanosine monophosphate
His	Histidine
Ile	Isoleucine
Lactate	Lactate (external)
Leu	Leucine
LIPIDcell	Average LIPID building block (mol. wt. 176.36)
LPScell	Average Lipposaccharide building block (mol. wt. 202)
Lys	Lysine
Met	Methionine
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NHEP	Heptulose
OAA	Oxaloacetate
OHMA	Myristic acid
P5P	Pentose phosphate
PEP	Phosphoenolpyruvate
PEPTIDO	Average Peptidoglycan building block (mol. wt. 150.96)
Phe	Phenylalanine
Pro	Proline
PROTcell	Average proteinogenic amino acid (mol. wt. 110)
PYR	Pyruvate
RL5P	Ribulose 5-phosphate
RNA	Average RNA bound nucleic acid (mol. wt. 325.39)
S7P	Sedoheptulose-7-P
Ser	Serine
Succinate	Succinate (external)
T3P1	Glyceraldehyde-3-phosphate
TGSM	Glucosamine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UDPG	UDP-Glucose
UDPNAG	UDP N-acetyl glucosamine
UDPNAM	UDP-N-acetyl-D-muramate
UMP	Uridine monophosphate
Val	Valine

Table A5 FBA simulated fluxes during fermentative glycerol metabolism

Reaction Name	Fluxes (Normalized to Glycerol Uptake)		
	Wild-Type	No 1,2-PDO Production	No Respiratory Glycerol Dissimilation
Glycerol Uptake	1	1	1
Glycerol Dissimilation (Respiratory)	0.25189	0	-
Glycerol Dissimilation (Fermentative)	0.74811	1	1
Glycerol Dissimilation (Fermentative r2)	0.74811	1	1
Gluconeogenesis	0.0115	0	0
Glycolysis (r3)	-0.00229	0	0
Glycolysis (r4)	0	0	0
Glycolysis (r5)	0.80596	1	1
Glycolysis (r6)	0.80596	1	1
Glycolysis (r7)	0.78865	1	1
Glycolysis (r8)	0	0	0
Pentose Phosphate Pathway (r9)	0	0	0
Pentose Phosphate Pathway (r10)	0	0	0
Pentose Phosphate Pathway (r11)	-0.00211	0	0
Pentose Phosphate Pathway (r12)	-0.00211	0	0
Pentose Phosphate Pathway (r13)	-0.00625	0	0
Glycogen Metabolism	0.00162	0	0
1,2-Propanediol Synthesis	0.16329	-	0
Lactate Production	0	0	0
Acetyl CoA (lpdA) (r16)	0	0	0
Acetyl CoA (pflB) (r17)	0.7156	1	1
Ethanol Production	0.66936	1	1
Acetate Production	0	0	0
Formate Decomposition	0	0	0
TCA Cycle (r21)	0.01169	0	0
TCA Cycle (r22)	0.03225	0	0
TCA Cycle (r23) (Succinate Production)	0	0	0
Aspartate Synthesis	0.00263	0	0
Asparagine Synthesis	0.00263	0	0
Glutamate Synthesis	0.00319	0	0
Glutamine Synthesis	0.00287	0	0
Alanine Synthesis	0.00624	0	0
Arginine Synthesis	0.00322	0	0
Proline Synthesis	0.00241	0	0
Leucine Synthesis	0.00491	0	0
Valine Synthesis	0.00461	0	0
Isoleucine Synthesis	0.00317	0	0

Phenylalanine Synthesis	0.00202	0	0
Tyrosine Synthesis	0.0015	0	0
Tryptophan Synthesis	0.00062	0	0
Histidine Synthesis	0.00103	0	0
Serine Synthesis	0.00386	0	0
Glycine Synthesis	0.00727	0	0
Cysteine	0.001	0	0
Threonine Synthesis	0.00276	0	0
Lysine Synthesis	0.00374	0	0
Methionine Synthesis	0.00168	0	0
AMP Synthesis	0.00192	0	0
GMP Synthesis	0.00236	0	0
DAMP Synthesis	0.00029	0	0
DGMP Synthesis	0.0003	0	0
UMP Synthesis	0.00158	0	0
CMP Synthesis	0.00147	0	0
DUMP Synthesis	0.00029	0	0
DCMP Synthesis	0.0003	0	0
1 Carbon Metabolism	0.00059	0	0
Lipid Synthesis (r53)	0.0015	0	0
Lipid Synthesis (r54)	0.00301	0	0
UDPG Synthesis	0.00021	0	0
CDPETN Synthesis	0.00031	0	0
OHMA Synthesis	0.00031	0	0
C14,0 Synthesis	0.00031	0	0
CMPKDO Synthesis	0.00031	0	0
NHEP Synthesis	0.00031	0	0
TGSM Synthesis	0.00021	0	0
UDPNAG Production	0.00032	0	0
UDPNAM Synthesis	0.00032	0	0
DAP Synthesis	0.00032	0	0
ATP Maintainence	0	1	1
Transhydrogenase (r66)	0.18194	0	0
Transhydrogenase (r67)	0	0	0
LPS Synthesis	0.00196	0	0
Lipid Synthesis	0.00601	0	0
Protein Synthesis	0.05829	0	0
DNA Synthesis	0.00117	0	0
RNA Synthesis	0.00734	0	0
Peptidoglycan Synthesis	0.00193	0	0
Cell Growth (Optimization Function:MAX)	0.07834	0	0

Table A6 Optical density, pH, and extracellular metabolite fermentation data for strains tested in study of fermentative glycerol metabolism

Strain	OD	OD Std Dev	pH	pH Std Dev	Glycerol Consumsed (g/L)	GC Std Dev	Ethanol (g/L)	Eth Std Dev	Succinate (g/L)	Succinate Std Dev	Formate (g/L)	Formate Std Dev	Acetate (g/L)	Acetate St Dev
BW25113	0.53	0.03	6.60	0.09	2.4	0.2	1.1	0.2	0.14	0.01	0.31	0.02	0	0
BW25113 (pZSBlank)	0.33	0.03	6.75	0.06	1.4	0.2	0.7	0.1	0.09	0.01	0.35	0.04	0	0
BW25113 (pZSKLMgldA)	0.60	0.02	6.68	0.01	2.37	0.05	1.6	0.1	0.080	0.005	0.253	0.007	0	0
BW25113 (pZSKLcfgldA)	0.76	0.03	6.39	0.03	4.48	0.08	2.39	0.06	0.25	0.01	0.302	0.009	0	0
BW25113 Δ*gldA*	0.048	0.003	7.10	0.01	0.16	0.04	0	0	0	0	0	0	0	0
BW25113 Δ*dhaK*	0.033	0.006	7.08	0.04	0.09	0.03	0	0	0	0	0	0	0	0
BW25113 Δ*glpK*	0.30	0.01	6.83	0.03	0.92	0.03	0.40	0.02	0.067	0.004	0.439	0.005	0	0
BW25113 Δ*glpA*	0.10	0.01	7.01	0.05	0.26	0.04	0.07	0.02	0.05	0.01	0.16	0.02	0	0
BW25113 Δ*glpB*	0.153	0.006	7.06	0.01	0.33	0.03	0.119	0.008	0.041	0.001	0.206	0.003	0	0
BW25113 Δ*glpC*	0.16	0.02	7.05	0.01	0.34	0.06	0.13	0.01	0.04	0.00	0.21	0.03	0	0
BW25113 Δ*glpF*	0.22	0.02	6.72	0.05	2.0	0.1	0.91	0.09	0.138	0.007	0.30	0.05	0.06	0.04
BW25113 Δ*glpK*Δ*glpA*	0.30	0.02	6.75	0.04	0.91	0.04	0.38	0.02	0.06	0.00	0.422	0.009	0	0
BW25113 Δ*glpK*Δ*ppsA*	0.08	0.02	7.10	0.04	0.1	0.1	0	0	0.038	0.002	0.130	0.006	0	0
BW25113 Δ*ptsG*	0.48	0.03	6.62	0.06	2.29	0.07	0.9	0.2	0.13	0.01	0.32	0.02	0	0
BW25113 Δ*ptsH*	0.07	0.01	6.93	0.03	0.15	0.01	0	0	0.17	0.03	0	0	0	0
BW25113 Δ*ptsI*	0.048	0.003	7.00	0.01	0.11	0.18	0	0	0.039	0.003	0	0	0	0
BW25113 Δ*ptsA*	0.51	0.02	6.67	0.02	2.26	0.02	0.98	0.02	0.125	0.004	0.32	0.02	0	0
BW25113 Δ*crr*	0.43	0.01	6.32	0.03	1.9	0.0	0.9	0.2	0.119	0.001	0.282	0.005	0	0
BW25113 Δ*glpA* (pZSKLMgldA)	0.51	0.02	6.62	0.01	2.2	0.1	1.10	0.05	0.0393	0.0005	0.26	0.01	0	0
BW25113 Δ*glpK* (pZSKLMgldA)	0.48	0.01	6.61	0.01	2.01	0.04	0.97	0.09	0.0384	0.0007	0.28	0.02	0	0

Strain	OD	OD Std Dev	pH	pH Std Dev	Glycerol Consumsed (g/L)	GC Std Dev	Ethanol (g/L)	Eth Std Dev	Succinate (g/L)	Succinate Std Dev	Formate (g/L)	Formate Std Dev	Acetate (g/L)	Acetate St Dev
BW25113 Δ*glpK*Δ*ppsA* (pZSKLMgldA)	0.078	0.003	6.91	0.03	0.1	0.1	0.093	0.006	0.0443	0.0003	0.165	0.004	0	0
BW25113 Δ*glpK*Δ*ppsA* (pZSKLcfgldA)	0.59	0.01	5.8	0.1	2.6	0.2	1.11	0.04	0.0984	0.0003	1.19	0.01	0	0
BW25113 Δ*pykA*Δ*pykF* (pZSKLMgldA)	0.67	0.06	6.54	0.03	2.8	0.3	1.45	0.09	0.10	0.02	0.236	0.004	0	0
BW25113 Δ*pykA*Δ*pykF* (pZSKLcfgldA)	0.05	0.01	6.95	0.02	0.2	0.1	0.01	0.07	0.08	0.01	0.10	0.02	0	0
BW25113 Δ*ptsH* (pZSKLMgldA)	0.04	0.00	6.95	0.02	0.18	0.08	0.07	0.06	0.0383	0.0004	0	0	0	0
BW25113 Δ*tpiA*	0.03	0.01	6.87	0.02	0.20	0.02	0	0	0.02	0.01	0	0	0	0
BW25113 Δ*fsaA*	0.478	0.008	6.52	0.01	2.4	0.2	1.0	0.1	0.12	0.01	0.32	0.02	0	0
BW25113 Δ*fsaB*	0.51	0.02	6.47	0.01	2.4	0.2	0.98	0.04	0.117	0.004	0.318	0.005	0	0
BW25113 Δ*fsaA*Δ*fsaB*	0.47	0.01	6.51	0.01	2.01	0.06	0.84	0.01	0.101	0.003	0.33	0.02	0	0
BW25113 Δ*fbaB*	0.51	0.03	6.54	0.01	2.6	0.2	1.27	0.03	0.146	0.005	0.292	0.008	0	0
BW25113 Δ*zwf*	0.47	0.05	6.55	0.01	2.89	0.07	1.2	0.1	0.155	0.003	0.279	0.002	0.026	0.002
BW25113 Δ*pgi*	0.37	0.06	6.57	0.05	2.5	0.1	1.10	0.08	0.24	0.01	0.30	0.03	0.03	0.03
BW25113 Δ*glpX*	0.11	0.01	6.69	0.07	0.56	0.04	0.18	0.02	0.128	0.005	0.255	0.006	0	0
BW25113 Δ*fbp*	0.11	0.02	6.79	0.01	0.35	0.04	0.133	0.005	0.043	0.002	0.202	0.005	0	0
BW25113 Δ*yggF*	0.37	0.03	6.8	0.1	1.3	0.1	0.55	0.04	0.118	0.005	0.23	0.01	0	0
BW25113 Δ*ybhA*	0.49	0.01	6.52	0.02	2.34	0.02	1.00	0.01	0.161	0.007	0.271	0.009	0	0
BW25113 Δ*pykA*	0.27	0.07	6.67	0.07	0.9	0.3	0.4	0.2	0.15	0.02	0.26	0.02	0	0
BW25113 Δ*pykF*	0.47	0.03	6.1	0.2	2.0	0.2	0.91	0.06	0.120	0.002	0.95	0.08	0.05	0.02
BW25113 Δ*pykA*Δ*pykF*	0.28	0.06	6.6	0.2	1.0	0.2	0.37	0.05	0.16	0.04	0.31	0.01	0	0
BW25113 Δ*ppsA*	0.470	0.009	6.32	0.01	2.1	0.2	1.1	0.1	0.110	0.007	0.33	0.06	0.06	0.05
BW25113 Δ*pflB*	0.107	0.003	6.94	0.01	0.1	0.1	0.02	0.03	0.052	0.001	0.139	0.004	0	0
BW25113 Δ*aceF*	0.49	0.01	6.62	0.03	2.0	0.1	0.87	0.05	0.112	0.006	0.319	0.008	0	0
BW25113 Δ*ppc*	0.49	0.02	6.70	0.09	2.51	0.07	1.08	0.07	0.12	0.01	0.260	0.008	0	0
BW25113 Δ*pckA*	0.14	0.02	6.84	0.02	0.8	0.2	0.35	0.04	0.051	0.006	0.5	0.1	0.08	0.06

Strain	OD	OD Std Dev	pH	pH Std Dev	Glycerol Consumsed (g/L)	GC Std Dev	Ethanol (g/L)	Eth Std Dev	Succinate (g/L)	Succinate Std Dev	Formate (g/L)	Formate Std Dev	Acetate (g/L)	Acetate St Dev
BW25113 Δ*glpK*Δ*pckA*	0.40	0.04	6.88	0.01	1.22	0.07	0.57	0.03	0.042	0.002	0.43	0.02	0	0
BW25113 Δ*maeA*	0.51	0.02	6.65	0.03	2.2	0.1	0.99	0.06	0.125	0.007	0.284	0.006	0.025	0.003
BW25113 Δ*maeB*	0.50	0.05	6.64	0.03	2.1	0.3	1.0	0.2	0.12	0.02	0.290	0.007	0	0
BW25113 Δ*frdA*	0.29	0.01	6.72	0.02	1.58	0.03	0.66	0.01	0	0	0.193	0.002	0	0
BW25113 Δ*frdA*Δ*ppsA*	0.197	0.019	6.94	0.01	1.19	0.05	0.56	0.04	0	0	0.28	0.03	0.036	0.003
BW25113 Δ*adhE*	0.032	0.003	7.07	0.01	0.15	0.06	0	0	0.030	0.006	0	0	0	0
BW25113 Δ*cyaA*	0.028	0.003	7.03	0.01	0.01	0.02	0	0	0	0	0	0	0	0
BW25113 ΔcyaA (pZSKLMgldA)	0.48	0.01	6.83	0.01	1.5	0.2	0.4	0.3	0.0403	0.0002	0.18	0.02	0	0
BW25113 ΔcyaA (pZSKLM)	0.38	0.02	6.79	0.02	1.26	0.05	0.1	0.1	0.0460	0.0007	0.31	0.02	0	0
BW25113 ΔcyaA (pZSgldA)	0.038	0.006	7.01	0.02	0.1	0.1	-0.2	0.3	0	0	0	0	0	0
BW25113 Δ*crp*	0.03	0.01	7.02	0.02	0.0	0.1	0	0	0	0	0	0	0	0
BW25113 Δcrp (pZSKLMgldA)	0.512	0.003	6.71	0.01	1.73	0.05	0.6	0.1	0.0418	0.0004	0.176	0.002	0	0
BW25113 Δcrp (pZSKLM)	0.382	0.008	6.71	0.01	1.31	0.09	0.6	0.2	0.0493	0.0007	0.35	0.04	0	0
BW25113 Δcrp (pZSgldA)	0.04	0.01	6.99	0.02	0.00	0.02	0.1	0.1	0	0	0	0	0	0
BW25113 Δ*arcA*	0.053	0.008	7.02	0.03	0.15	0.01	0	0	0.038	0.002	0	0	0	0
BW25113 Δ*arcB*	0.055	0.005	7.04	0.01	0.14	0.05	0	0	0.027	0.007	0	0	0	0
BW25113 Δ*creB*	0.498	0.008	6.64	0.02	2.06	0.03	1.0	0.1	0.121	0.002	0.30	0.01	0	0
BW25113 Δ*creC*	0.513	0.008	6.60	0.02	2.2	0.2	1.02	0.07	0.14	0.01	0.30	0.01	0	0
BW25113 Δ*dgsA*(*mlc*)	0.447	0.006	6.47	0.01	3.22	0.03	1.45	0.01	0.22	0.01	0.25	0.01	0	0
BW25113 Δ*fruR*(*cra*)	0.15	0.005	6.85	0.04	0.54	0.03	0.20	0.02	0.105	0.008	0.30	0.02	0	0
BW25113 Δ*fnr*	0.045	0.000	7.02	0.02	0.12	0.04	0	0	0	0	0.05	0.05	0	0
BW25113 Δ*frsA*	0.29	0.01	6.83	0.03	0.86	0.05	0.37	0.03	0.074	0.003	0.325	0.004	0	0
BW25113 Δ*hns*	0.06	0.02	6.94	0.03	0.312	0.006	0.118	0.002	0.069	0.001	0.218	0.005	0	0
BW25113 Δ*uspA*	0.33	0.03	6.77	0.01	1.3	0.2	0.57	0.08	0.103	0.009	0.31	0.01	0	0
BW25113 Δ*ompR*	0.25	0.05	6.76	0.03	1.0	0.2	0.4	0.1	0.142	0.006	0.25	0.02	0	0