Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.
Abstract

Biological platforms for propionate production have been limited to anaerobic native microbial producers, such as *Propionibacterium* and *Clostridium*. In this work, we demonstrated high-level heterologous production of propionate under microaerobic conditions in engineered *Escherichia coli* (*E. coli*). Activation of the native Sleeping beauty mutase (Sbm) operon not only transformed *E. coli* to be propionogenic (i.e. propionate-producing) but also introduced an intracellular “flux competition” between the traditional C2-fermentative pathway (forming acetate and ethanol) and the novel C3-fermentative pathway (forming propionate and 1-propanol). The propionogenic *E. coli* was further engineered by inactivation or overexpression of various genes involved in the glycerol dissimilation pathways and their individual genetic effects on propionate production were investigated. Generally, knocking out genes involved in glycerol dissimilation (except *glpA*) can minimize levels of solventogenesis and shift more dissimilated carbon flux toward the C3-fermentative pathway. For effective propionate production, glycerol dissimilation should be channeled through the respiratory pathway and, upon suppressed solventogenesis with minimal production of highly reduced alcohols, the alternative NADH-consuming route associated with propionate synthesis can be critical for more flexible redox balancing. With the implementation of various biochemical and genetic strategies, high propionate titres of more than 11 g/L with high yields up to 0.4 g-propionate/g-glycerol (accounting for ~50% of dissimilated glycerol) were achieved, implying the potential for industrial application. To our knowledge, this represents the most effective non-native engineered microbial system for propionate production.
Acknowledgements

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Finally, I would not be where I am today without the support of my parents. Thank you for showing me how far drive, dedication, and perseverance can bring me. I give a special thank you to my sisters and best friends, Mona and Caroline, for your enthusiasm and creative encouragement.
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**Abbreviations:**

*ackA*, acetate kinase  
*adhE*, gene encoding acetaldehyde dehydrogenase/alcohol dehydrogenase  
AN, anaerobic  
Ap, ampicillin  
ATP, adenosine triphosphate  
cI857, gene encoding temperature-sensitive λ repressor  
Cm, chloramphenicol  
DHA, dihydroxyacetone  
*dhaKLM*, genes encoding dihydroxyacetone kinase  
DHAP, dihydroxyacetone phosphate  
*flp*, gene encoding *Saccharomyces cerevisiae* Flp recombinase  
FNR, fumarate and nitrate reductase  
*frdABCD*, fumarate reductase  
FRT, Flp recombination target  
FRT-Cm^R^-FRT, cassette carrying Cm^R^ marker  
FRT-Km^R^-FRT, cassette carrying Km^R^ marker  
G3P, glycerol-3-phosphate  
*gldA*, gene encoding glycerol dehydrogenase  
*glpABC*, genes encoding the anaerobic glycerol-3-phosphate dehydrogenase  
*glpD*, gene encoding the aerobic glycerol-3-phosphate dehydrogenase  
*glpK*, gene encoding glycerol kinase  
HPLC, high-performance liquid chromatography  
*hpr*, gene encoding histidine phosphoryl carrier protein  
IPTG, isopropyl β- D-thiogalactopyranoside  
Km, kanamycin  
LB, lysogeny broth  
*ldhA*, gene encoding lactate dehydrogenase  
MA, microaerobic  
NAD+/NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide
mgsA, gene encoding methylglyoxal synthase
OAA, oxaloacetate
PEP, phosphoenolpyruvate
ppc, phosphoenolpyruvate carboxylase
pflB, gene encoding pyruvate formate lyase I
pta, gene encoding phosphate acetyltransferase
ptsG, gene encoding glucose-specific phosphotransferase permease
ptsI, gene encoding phosphoenolpyruvate-protein phosphotransferase enzyme I
pyc, gene encoding pyruvate carboxylase
sbm, methylmalonyl-CoA mutase
TCA, tricarboxylic acid cycle
ts, temperature sensitive
WT, wild type
ygfD, gene encoding arginine kinase
ygfG, gene encoding methylmalonyl-CoA decarboxylase
ygfH, gene encoding propionyl-CoA:succinate-CoA transferase

Symbols:

Δ, deletion
[], denotes plasmid-carrier state
R, resistant/resistance
S, sensitive/sensitivity
Chapter 1- Overview

1.1 Research background

Propionate (CH₃CH₂COO⁻), the anion of the naturally occurring 3-carbon carboxylic acid propionic acid (Figure 1), is an important industrial chemical with a number of specialty applications. Mainly used as animal feed preservatives and antibiotics, food preservatives and herbicides, propionate salts also have minor applications in the perfume, pharmaceutical and plastic industries (Kirschner, 2009; Liu et al., 2012) (Table 1 and Figure 2). Given its connection to the food production market, propionate demand remains high, regardless of the state of the economy. The North American propionate market continues to grow at a forecasted annual rate of 2.3%, and as of 2009, the sale price reached $2.05-$2.13 per kilogram (Kirschner, 2009).

![Chemical structure of propionate](image)

**Figure 1**: Chemical structure of propionate

Currently, industrial production of propionate and propionic acid is by means of petrochemical processes including the Reppe process (from ethylene, CO and steam using nickel carbonyl as a catalysis) or the Larson process (from ethanol and CO using boron trifluoride as a catalyst) (Bertleff, 2000; Samel et al., 2000).
Figure 2: Propionate uses and applications
Adapted from Kirschner (2009).

Table 1: Propionate applications in industry

<table>
<thead>
<tr>
<th>Application</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed and grain preservative</td>
<td>Used as a mold inhibitor in animal feed, corn and grain. Inhibitory to <em>Aspergillus flavus</em>, aerobic <em>Bacillus</em>, <em>Salmonella</em> and yeast.</td>
</tr>
<tr>
<td>Food preservative</td>
<td>Used for mold prevention in breads and cakes</td>
</tr>
<tr>
<td>Herbicides</td>
<td>Used in synthesis of the herbicide sodium 2,2-dichloropropionate</td>
</tr>
<tr>
<td>Biopolymer production</td>
<td>Used as a precursor in the synthesis of the biopolymer cellulose acetate propionate for use in inks, varnishes and nail lacquers</td>
</tr>
<tr>
<td>Perfume intermediate</td>
<td>Used as a precursor of propionic ether and benzyl propionate, used as additives in food and cosmetics</td>
</tr>
<tr>
<td>Pharmaceutical intermediate</td>
<td>Used in synthesis of the pharmaceutical intermediates propionic anhydride and chloropropionic acid</td>
</tr>
<tr>
<td>Other applications</td>
<td>Used as an intermediate in the production of plastics, plasticizers, textile and rubber auxiliaries, and dye intermediates</td>
</tr>
</tbody>
</table>
Increased demands for green production platforms have brought considerable attention to biological production of propionate. Propionate fermentation using microbial platforms and renewable biomass resources is both sustainable and environmentally friendly in comparison to petrochemical processes. Recently, the U.S. Department of Energy identified propionate as one of the top 30 building block chemicals which can be produced from biomass resources, emphasizing its industrial significance and potential for large scale biological production (Werpy et al., 2004).

Current biological production of propionate is restricted to the use of native microbial producers, via the dicarboxylic acid pathway (also known as the methylmalonyl-CoA mutase pathway) in various species of Propionibacteria and the acrylate pathway in Clostridium propionicum. High-level production of propionate in these microorganisms, including P. ferudenreichii, P. acidipropionici, and C. propionicum etc., using glucose and glycerol as carbon sources has been demonstrated with high yields (Barbirato et al., 1997; Himmi et al., 2000; Kandasamy et al., 2013; Zhang and Yang, 2009; Zhu et al., 2010). Despite these successes, production platforms on the basis of these Gram-positive anaerobes are not ideal for various reasons, including their slow growth rate, the use of costly and complex media, and the lack of available genetic tools for strain engineering. Escherichia coli (E. coli), being the most common bacterial host for biomanufacturing but a non-native propionate producer, has recently been explored for potential propionate synthesis owing to its well-characterized physiology and genetics, simple and inexpensive cultivation methods, and the variety of existing technologies for genetic manipulation, synthetic biology, and metabolic engineering. Nevertheless, engineering propionate-producing E. coli strains has been unpopular to date, with few reports being published and low yields achieved.
While *E. coli* is a non-native propionate producer, it has a native pathway for extended dissimilation of succinate, i.e. the Sleeping beauty mutase (Sbm) pathway which is normally dormant but potentially relevant to the production of C3-fermentative products, 1-propanol and propionate (Haller et al., 2000). The Sleeping beauty mutase (Sbm) operon in the *E. coli* genome contains four genes whose encoding products (Sbm: methylmalonyl-CoA mutase, YgfD: an Sbm-interacting protein kinase, YgfG: methylmalonyl-CoA decarboxylase, and YgfH: propionyl-CoA/succinyl-CoA transferase) are similar to enzymes in the dicarboxylic (or methylmalonyl-CoA mutase) pathway of *Propionibacterium*. Although the structure, function, and relationship of these enzymes have been characterized, hardly any work has been performed to assess their practical application.
1.2 Research objectives

We hypothesize that high levels of propionate can be achieved by transcriptional activation of the Sbm operon in addition to manipulation of culture conditions and further strain engineering to favour propionate production over solvent production.

The overall objectives of this thesis include:

1. Establish a heterologous propionate production system in engineered *E. coli* by transcriptional activation of the Sbm operon.

2. Optimization of cultivation conditions, including use of carbon source and aeration regimes, to favour organic acid production (i.e. acetate and propionate) over solvent production (i.e. ethanol and 1-propanol).

3. Further engineering of the propionate-producing strain by the application of various biochemical, genetic and metabolic engineering strategies, including gene knockout and gene overexpression, targeting the cell’s glycerol dissimilation pathways to selectively produce C3 products and minimize C2 by-products, effectively enhancing propionate titres, yield and productivity.
1.3 Outline of thesis

Chapter 2 is a review of the recent trend towards biological production (or biomanufacturing) of chemical products. Specifically, the use of *Escherichia coli* as a production host and glycerol as a carbon source, including the current understanding of the fermentative and respiratory glycerol dissimilation pathways in *E. coli*, are described. Existing production schemes for biological synthesis of propionate in both native producers and *E. coli* are reviewed in addition to the structure and postulated function of the silent Sbm operon in *E. coli* as it relates to propionate synthesis. In Chapter 3, all materials and methods relevant to this study are described, including strain and plasmid construction, media and cultivation, analyses and calculation. In Chapter 4, the results of this study are stated including, (1) determination of culture conditions for propionate production where carbon sources and aeration regimes are compared; (2) the effect of inactivation of genes of the glycerol dissimilation pathways; and (3) the effect of overexpression of genes of the glycerol dissimilation pathways on biomass growth, the overall metabolite profile and specifically propionate production. Chapter 5 contains a discussion of the results presented in Chapter 4. Finally, Chapter 6 states the conclusions of this study and a proposal for future studies.
Chapter 2- Literature Review

2.1 Biomanufacturing for sustainable production of chemicals

The production of chemicals for industrial applications has a long history in Europe, Asia and North America. The chemical manufacturing sector is among the world’s top industries and continues to grow as demands for energy and food increase to support a growing population; it is a well-established industry and an important part of the global economy (Gavrilescu and Chisti, 2005). Currently, petroleum is the primary feedstock for production of chemicals; over 80 million tons of industrial chemicals valued at over $2 trillion are manufactured from petroleum feedstocks globally each year (Yang and Yu, 2013). However, the world’s dependence on petroleum cannot continue indefinitely given the rapid depletion of oil reserves. In addition, mounting environmental concerns regarding the use of unsustainable petroleum-based sources and environmentally damaging production processes has placed significant pressure on the chemical industry to develop more environmentally sustainable and socially responsible production methods. Among these alternatives is the use of biomanufacturing technologies (Gavrilescu and Chisti, 2005; Matlack, 2001; Poliakov et al., 2002).

Biomanufacturing is defined as the application of biological catalysts, including natural or modified whole cells and enzymes, to manufacture a chemical or protein product of interest. Developments in genetic and metabolic engineering have played a fundamental role in driving the transformation of the manufacturing industry toward bio-based production and away from traditional chemical synthesis for key products (Kim et al., 2000; Poppe and Novak, 1992). Genetic engineering and molecular biology techniques have been widely employed to create modified enzymes with superior properties compared to their natural counterparts. Metabolic
engineering (the manipulation of metabolic pathways at the molecular level), offers novel or enhanced capabilities for production of native and non-native chemicals in microorganisms.

2.1.1 Bio-based organic acid production

Organic acids (e.g. acetic acid, propionic acid, lactic acid, malic acid, succinic acid, etc.) are both valuable commodity chemicals and platform chemicals (building block chemicals) which may be further processed into higher value chemicals, solvents, or fuels (Sauer et al., 2008; Yu et al., 2011). Recently, the U.S. Department of Energy identified a list of the top 30 building block chemicals which can be produced from biomass resources. Among the top 30 are the organic acids lactic acid, propionic acid and succinic acid, emphasizing their industrial significance and emerging future in biomanufacturing. Selection of the top 30 chemicals was based on the following criteria (Werpy et al., 2004):

(1) relevance to current or future biorefinery operations
(2) current market volumes and prices
(3) cost of feedstock
(4) estimated processing costs
(5) the technical complexity of each part of the pathway transformation (sugars to building blocks and building blocks to derivatives)
(6) value of the building block and its derivatives as a replacement or novel chemical
(7) the building block’s potential to produce families or groups of similar derivatives

The use of microorganisms for the synthesis of organic acids from renewable carbon sources is a sustainable approach to production of these valuable chemicals (Yang and Yu, 2013). Bio-based organic acids are produced through microbial fermentation of carbohydrate sugars. Many different microorganisms are capable of producing a number of organic acids, either as part of their natural metabolism or through genetic modification of their central metabolic
pathways. Fermentation for organic acid production is complex, and the efficiency of production varies based on the production host, genetic factors and cultivation conditions (Gavrilescu and Chisti, 2005).

Some organic acids are currently or will soon be in commercial production. For example, succinic acid is manufactured by Myriant (United States), DSM (Netherlands) and BASF (Germany) (Yang and Yu, 2013). Table 2 lists select organic acids which are presently produced in high volumes, highlighting the potential for bio-based organic acid production. The development of biological systems through genetic and metabolic engineering for improved productivity and yields are on-going, both for organic acid bioprocesses already in commercial production as well as those which are not yet established (Sauer et al., 2008). Particularly in the last decade, the development of high-production strains has become increasingly popular. Table 3 lists recent studies to establish such strains for the production of a number of organic acid products using a variety of production hosts.

**Table 2: High volume production of organic acids**
Adapted from Sauer et al. (2008)

<table>
<thead>
<tr>
<th>Organic acid (Number of carbon atoms)</th>
<th>Total annual production (tons)</th>
<th>Annual production by microbial processes (tons)</th>
<th>Uses and applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid (C2)</td>
<td>7 000 000</td>
<td>190 000</td>
<td>Polymers and solvents</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Food, beverages,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cosmetics,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pharmaceuticals,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>biopolymers</td>
</tr>
<tr>
<td>Lactic acid (C3)</td>
<td>150 000</td>
<td>150 000</td>
<td>Food, feed, agriculture</td>
</tr>
<tr>
<td>Propionic acid (C3)</td>
<td>130 000</td>
<td>-</td>
<td>Food, feed, agriculture</td>
</tr>
<tr>
<td>Succinic acid (C4)</td>
<td>16 000</td>
<td>-</td>
<td>Bulk chemical, food, agriculture</td>
</tr>
<tr>
<td>Citric acid (C6)</td>
<td>1 600 000</td>
<td>1 600 000</td>
<td>Food additive</td>
</tr>
</tbody>
</table>
Table 3: Biological production platforms for organic acids
Adapted from Yang and Yu (2013)

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>Microorganism</th>
<th>Titre (g/L)</th>
<th>Productivity (g/L·h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td><em>Clostridium formicoaceticum</em></td>
<td>79</td>
<td>0.95</td>
<td>(Huang et al., 1998)</td>
</tr>
<tr>
<td>Lactic acid</td>
<td><em>Lactobacillus delbrueckii</em></td>
<td>135</td>
<td>3.40</td>
<td>(Kadam et al., 2006)</td>
</tr>
<tr>
<td>Lactic acid</td>
<td><em>Escherichia coli</em></td>
<td>138</td>
<td>3.54</td>
<td>(Zhu et al., 2007)</td>
</tr>
<tr>
<td>Propionic acid</td>
<td><em>Propionibacterium acidipropionici</em></td>
<td>97</td>
<td>0.05</td>
<td>(Zhang and Yang, 2009)</td>
</tr>
<tr>
<td>Succinic acid</td>
<td><em>Anaerobiosprillum succiniproducins</em></td>
<td>83</td>
<td>10.4</td>
<td>(Meynial-Salles et al., 2008)</td>
</tr>
<tr>
<td>Succinic acid</td>
<td><em>Cornebacterium gutamicum</em></td>
<td>146</td>
<td>3.2</td>
<td>(Okino et al., 2008)</td>
</tr>
<tr>
<td>Succinic acid</td>
<td><em>Escherichia coli</em></td>
<td>87</td>
<td>0.9</td>
<td>(Jantama et al., 2008)</td>
</tr>
</tbody>
</table>

2.1.2 *Escherichia coli as a production platform*

Depending on the target product, various microbial biocatalysts can be employed. These can be native producers, such as the use of *Lactobacillus* or *Propionibacterium* for production of lactic acid and propionic acid, respectively, whereby their existing biochemical and metabolic pathways are manipulated for enhanced production with reduced byproducts (Kadam et al., 2006; Zhang and Yang, 2009). Alternatively, a new biochemical pathway can be established in an engineered microorganism for the production of a non-native compound, though this approach requires a thorough understanding of the organism’s metabolism (Miller, 1992). Among the candidate microbial production platforms, *E. coli*, a Gram-negative rod shaped bacterium, is the most popular choice and has been used in numerous applications (Yim et al., 2011). *E. coli* is considered the user-friendly workhorse for biomanufacturing owing to its many advantages including its quick growth rate, inexpensive cultivation media, well understood physiology, well
characterized biological systems (e.g. genomic and proteomic), and extensive knowledge of its central carbon metabolism in addition to the many available genetic tools and technologies for strain manipulation and bioprocessing (Chen et al., 2013; Miller, 1992; Sauer and Eikmanns, 2005; Yim et al., 2011; Zaldivar et al., 2001). High-level production of a number of organic acids in *E. coli* has been demonstrated with the implementation of various biochemical and genetic strategies, such as overexpression of the key genes in the metabolic pathway, heterologous expression of genes from non-*E. coli* sources, or targeted gene knockout (Förster and Gescher, 2014). Table 4 summarizes some successful *E. coli* engineering approaches for production of acetic, succinic and lactic acids.
Table 4: Production of organic acids in metabolically engineered *E. coli*

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Titre (g/L)</th>
<th>Yield (g/g)</th>
<th>Engineering strategy</th>
<th>Fermentation process</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>51</td>
<td>0.5</td>
<td>Inactivation of <em>focA-pflB</em>, <em>frdBC, ldhA, atpFH, adhE, sucA</em></td>
<td>Aerobic fed-batch</td>
<td>(Causey et al., 2003)</td>
</tr>
<tr>
<td>Lactic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>32</td>
<td>0.87</td>
<td>Inactivation of <em>pta, adhE, frdA, dld</em>, overexpression of <em>E. coli glpK and glpD</em></td>
<td>Shake flask</td>
<td>(Mazumdar et al., 2010)</td>
</tr>
<tr>
<td>Glucose</td>
<td>138</td>
<td>0.99</td>
<td>Inactivation of <em>aceEF, pflB, poxB, pps and frdABCD</em></td>
<td>Two-phase aerobic-anaerobic fed-batch</td>
<td>(Zhu et al., 2007)</td>
</tr>
<tr>
<td>Succinic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>99.2</td>
<td>0.96</td>
<td>Inactivation of <em>ptsG, pflB and ldhA, overexpression of Rhizobium etli pyc</em></td>
<td>Two-phase aerobic-anaerobic fed-batch</td>
<td>(Vemuri et al., 2002)</td>
</tr>
<tr>
<td>Glucose</td>
<td>46.5</td>
<td>1.1</td>
<td>Inactivation of <em>adhE, ldhA, iclR, ack-pta, overexpression of Candida boidinii fdh</em></td>
<td>Anaerobic fed-batch</td>
<td>(Balzer et al., 2013)</td>
</tr>
</tbody>
</table>
2.1.3 Glycerol as a carbon source

The choice of carbon source is a fundamental aspect of biomanufacturing. It is generally perceived that uptake and dissimilation of the major carbon source during *E. coli* cultivation could critically affect biomass growth, metabolite profile and culture performance, such as titre/yield of recombinant proteins and target metabolites (Cheng et al., 2014; Chou et al., 1994; Martinez-Gomez et al., 2012; Sigüenza et al., 1999; Wong et al., 2008). Furthermore, the use of inexpensive carbon sources can contribute to reducing the cost of fermentation processes, facilitating the development of more economically competitive bio-based production methods (Wendisch et al., 2011). Glycerol (or glycerine), a highly reduced 3-carbon sugar-alcohol, can be taken up by facilitated diffusion and used as a carbon or energy source by many known microorganisms (Da Silva et al., 2009; Dobson et al., 2012; Pagliaro et al., 2007). It is inexpensive and presently in high abundance due to its generation as a by-product during biodiesel production. Consequently, it has the potential to be a valuable resource of biotechnological importance. The highly reduced nature of glycerol ($\gamma_{\text{glycerol}} = 4.7$ compared to $\gamma_{\text{glucose}} = 4$, where $\gamma$ is the degree of reduction per carbon) results in more reducing equivalents generated upon its dissimilation. In addition, since biomass synthesis from glycerol is associated with the generation of reducing equivalents, redox balance can be achieved by the production of reduced metabolite products as a means of NAD$^+$ regeneration (Murarka et al., 2008). To date, glycerol has successfully been used as the carbon source for production of a number of products, including succinic acid, lactic acid and ethanol in high yields (Blankschien et al., 2010; Mazumdar et al., 2010; Yazdani and Gonzalez, 2008).
2.1.3.1 *Glycerol dissimilation pathways of* Escherichia coli

*E. coli* has two pathways for glycerol dissimilation: the respiratory pathway, and the fermentative pathway converging at dihydroxyacetone phosphate (DHAP) (Durnin et al., 2009) (Figure 3). In the respiratory pathway, glycerol is first phosphorylated to glycerol-3-phosphate (G3P) by an ATP-dependent glycerol kinase, encoded by *glpK*. The subsequent reaction for conversion of G3P to DHAP is mainly catalyzed by the aerobic glycerol-3-phosphate dehydrogenase, encoded by *glpD* (Durnin et al., 2009). However, the anaerobic glycerol-3-phosphate dehydrogenase, encoded by *glpABC*, is active in the absence of oxygen, though using the anaerobic enzyme for this conversion is considered a minor pathway for glycerol dissimilation (Zhang et al., 2010). Alternatively, the fermentative pathway is functional particularly under anaerobic conditions, and it includes glycerol dehydrogenase (encoded by *gldA*) and a PTS-like phosphorelay system (with various enzymes encoded by *ptsI*, *hpr* and *dhaKLM*) for phosphorylation of dihydroxyacetone (DHA) using phosphoenolpyruvate (PEP) as a phosphate donor (Gutknecht et al., 2001; Jin and Lin, 1984).
The enzymes of the respiratory pathway are: GlpK, glycerol kinase, and the aerobic (GlpD) and anaerobic (GlpABC) glycerol-3-phosphate dehydrogenases. The fermentative pathway enzymes are: GldA, glycerol dehydrogenase, and the enzymes of the phosphorelay system, PtsI, phosphoenolpyruvate-protein phosphotransferase enzyme I, HPr, histidine phosphoryl carrier protein and DhaKLM, dihydroxyacetone kinase.

2.2 Biological production of propionate

2.2.1 In Gram-positive propionic acid bacteria

Current biological production of propionate is limited to Gram-positive anaerobic natural producers, known as propionic acid bacteria. This group of organisms includes various species of *Propionibacteria*, the most widely used genus of bacteria for propionate fermentation, which produce propionate via the dicarboxylic acid (or methylmalonyl-CoA mutase) pathway, and *Clostridium propionicum* via the acrylic acid pathway (Figure 4).
Figure 4: Natural microbial propionate-producing pathways

(A) The dicarboxylic acid (methylmalonyl-CoA mutase) pathway of Propionibacteria. Key enzymes in the pathway are: 1, pyruvate carboxylase; 2, malate dehydrogenase; 3, fumarase; 4, succinate dehydrogenase; 5, propionyl-CoA:succinyl-CoA transferase; 6, methylmalonyl-CoA mutase; 7, methylmalonyl-CoA epimerase; 8, methylmalonyl-CoA carboxytransferase (B) The acrylic acid pathway of Clostridium propionicum. Key enzymes in the pathway are: 9, lactate dehydrogenase; 10, propionyl CoA:lactyl-CoA transferase; 11, lactyl-CoA dehydratase; 12, acryl-CoA reductase). Adapted from Wang et al., 2013.

In Propionibacteria, the dicarboxylic acid pathway, is responsible for production of propionate from fermentation of carbohydrates such as glucose or glycerol (Wang et al., 2013). Often called the methylmalonyl-CoA mutase pathway, this cyclical pathway relies on the synthesis of oxaloacetate (OAA) by one of two enzymes: pyruvate carboxylase, or methylmalonyl-CoA carboxytransferase which catalyzes the transfer of a carboxyl-group from methylmalonyl-CoA to pyruvate, simultaneously generating oxaloacetate and propionyl-CoA.
Methylmalonyl-CoA is generated from succinyl-CoA by methylmalonyl-CoA mutase and methylmalonyl-CoA epimerase. Propionyl-CoA:succinyl-CoA transferase is responsible for the reversible transfer of the CoA moiety from propionyl-CoA to succinate, regenerating succinyl-CoA and producing the end product propionate (Figure 4A).

A number of investigations have demonstrated high-level propionate production in these organisms. Anaerobic batch cultivation (72h, 35°C and pH 6.5) of *P. ferudenreichii* spp. *shermanii* by Quesada-Chanto et al. (1998) reported a titre of 12.5 g/L (0.16 g/ g glucose) from a starting glucose concentration of 80 g/L. Similarly, when *P. ferudenreichii* spp. *shermanii* was used as a production host for anaerobic batch fermentation (30°C, pH 7.0) by Himmi et al. (2000), a titre of ~6.5 g/L (0.22 g/ g glucose), was achieved from an initial 20 g/L glucose. Under these same conditions, propionate titre and yield was higher (~8 g/L, 0.16 g/ g glucose) using *P. acidipropionici*. The use of glycerol as a carbon source improved propionate production during anaerobic cultivation of *P. ferudenreichii* spp. *shermanii* and *P. acidipropionici*, ~9 g/L (0.47 g/g glycerol) and ~12 g/L (0.63 g/g glycerol) respectively (Himmi et al., 2000). Another study by Barbirato et al. (1997) explored a comparison of glucose and glycerol as carbon sources for propionate batch fermentation of *P. acidipropionici*, and found that glycerol is a more suitable carbon source with a titre of ~13.6 g/L (0.68 g/g glycerol) in contract to glucose, with a titre of ~11.9 g/L (0.59 g/ g glucose). Furthermore, in a similar study using complex and undefined medium supplemented with 20 g/L glycerol as carbon source for *P. acidipropionici* fermentation, ~6.77 g/L of propionate was produced with a high yield of 0.72 g/g glycerol as there was no acetate produced as a byproduct (Coral et al., 2008).

Alternatively, the acrylic acid pathway of strict anaerobe *C. propionicum* produces propionate using lactate as a starting molecule (Figure 4B). First, pyruvate is reduced to lactate,
which is then converted to propionate through generation of lactyl-CoA, acrylyl-CoA and propionyl-CoA catalyzed by propionyl-CoA:lactyl-CoA transferase, lactyl-CoA dehydratase and acrylyl-CoA reductase. Specifically, the enzyme propionyl CoA:lactyl-CoA transferase facilitates the transfer of a Co-A moiety to from propionyl-CoA to lactate, producing lactyl-CoA and propionate (Wang et al., 2013). A propionate titre of ~12.8 g/L with a corresponding yield of 0.64 g/ g glycerol was achieved by anaerobic batch cultivation of *C. propionicum* on glycerol (Barbirato et al., 1997).

### 2.2.2 In Gram-negative *Escherichia coli*

Despite these successes, use of Gram-positive anaerobes such as *Propionibacteria* and *Clostridium* as production platforms is not ideal for various reasons, including their slow growth rate, the use of costly and complex media and the lack of available genetic tools for strain engineering which present a challenge to optimized and efficient production. *E. coli*, being the most common bacterial host for biomanufacturing but a non-native propionate producer, has recently been explored for potential propionate synthesis owing to its well-characterized physiology and genetics, simple and inexpensive cultivation methods, and the variety of existing technologies for genetic manipulation, synthetic biology, and metabolic engineering. Nevertheless, few reports have been published on engineering propionate-producing *E. coli* strains.

A recent study by Kandasamy et al. (2013) involved metabolically engineering *E. coli* with the acrylate pathway genes. These 3 genes, *pct*, *lcd* and *acr*, whose encoding products are propionyl CoA:lactyl-CoA transferase, lactyl-CoA dehydratase and acrylyl-CoA reductase, respectively, were PCR amplified. The *pct* and *acr* PCR products were cloned into two different
sites in a single vector, while \(lcd\) was cloned into a second vector. The two plasmids were co-transformed into \(E.\ coli\), creating a double plasmid expression system. This strain was cultivated in a bioreactor, by a two-phase aerobic-anaerobic batch fermentation method. Overall, expression of these genes in \(E.\ coli\) resulted in an impaired growth rate with low biomass accumulation and had a significant metabolic impact on the strain. With glucose as a carbon source, at 10 h post-induction, only 0.27 g/L propionate was detected by HPLC analysis with reduction in all other metabolites. Upon further investigation, the relative expression levels of each of the heterologously expressed genes were analyzed by relative mRNA abundance with qRT-PCR (quantitative real-time polymerase chain reaction). It was found that the \(acr\) gene product was produced at significantly lower levels in comparison to the \(pct\) and \(lcd\) products, and enzymatic assays showed that the activity of all three enzymes were lower in \(E.\ coli\) than in the native producer which may account for the low propionate titre in this engineered strain.

In addition, propionate production was reported as a byproduct during aerobic fed-batch cultivation of an engineered 1-propanol-producing \(E.\ coli\) strain which was developed based on an L-threonine-overproducing pathway (Choi et al., 2012). The genes of the threonine biosynthesis pathway were overexpressed in addition to three more native \(E.\ coli\) genes for efficient production of 1-propanol from the threonine precursor. This strain contained numerous mutations to remove the effect of feedback inhibition on threonine synthesis and to eliminate byproduct formation. While no propionate was detected during batch cultivation of this strain, up to 4 g/L propionate, an intermediate in this 1-propanol pathway, was produced after the first glycerol feeding during fed-batch cultivation. Though the exact reason for accumulation of propionate in this strain is unknown, it may be related to reduced activity of the downstream enzymes in later cultivation stages (Choi et al., 2012).
2.3 The methylmalonyl-CoA mutase pathway

The methylmalonyl-CoA mutase (MCM) pathway, is a vitamin B\textsubscript{12}-dependent pathway found in both eukaryotes and prokaryotes (Kannan, 2008). In animals, the MCM pathway is predominately active in the digestive system and functions in the reverse direction for oxidation of propionate to succinyl-CoA which can then enter the tricarboxylic acid (TCA) cycle (Kamoun, 1992; Ledley et al., 1990). While remnants of the MCM pathway can be found in most prokaryotes, this important metabolic pathway plays a central role in the metabolism of succinate to propionate in members of the *Streptomyces* and *Propionibacteriaceae* families. Specifically, in *Streptomyces cinnamonensis* and *P. ferudenreichii* spp. *shermanii*, the MCM pathway is involved in the production of propionate for maintenance of redox balance with propionate being one of the major fermentative products (Banerjee, 1997; Gruber and Kratky, 2001; Roy, 1996).

The MCM pathway is catalyzed by a group of enzymes of the crotonase superfamily. Generally they include a methylmalonyl-CoA mutase, methylmalonyl-CoA decarboxylase and propionyl-CoA:succinyl-CoA transferase, (Benning et al., 2000). In all cases, the first enzyme of the MCM pathway, methylmalonyl-CoA mutase, is an apoenzyme, and is dependent on vitamin B\textsubscript{12} as a coenzyme for formation of the holoenzyme and functional activity (Haller et al., 2000).

2.3.1 The Sleeping beauty mutase operon of Escherichia coli

The metabolic role of the MCM pathway is well understood in native propionate producers such as *Propionibacteria*. While *E. coli* is a non-native propionate producer, genomic analysis has identified that proteins of the MCM pathway are also encoded in the *E. coli* genome, the first of which shares high sequence similarity to methylmalonyl-CoA mutase found in
Propionibacteria (Haller et al., 2000). Located at 62.8 minutes on the E. coli chromosome, the genes are structured as a four-gene operon (sbm-ygfD-ygfG-ygfH) known as the Sleeping beauty mutase (Sbm) operon. The encoding products are Sbm, methylmalonyl-CoA mutase, YgfD, an Sbm-interacting protein kinase, YgfG, methylmalonyl-CoA decarboxylase, and YgfH, propionyl-CoA/succinyl-CoA transferase (Figure 5).

**Figure 5:** The four-gene E. coli Sleeping beauty mutase operon

Sbm encodes methylmalonyl-CoA mutase, ygfD encodes an Sbm-interacting protein kinase, ygfG encodes methylmalonyl-CoA decarboxylase and ygfH encodes propionyl-CoA/succinyl-CoA transferase.

The Sbm pathway is cyclical and composed of a series of biochemical conversions forming propionate as a fermentative product while regenerating the starting molecule, succinyl-CoA (Figure 6). First, Sbm catalyzes the isomerization of succinyl-CoA to methylmalonyl-CoA. The ygfD product is believed to interact with Sbm. Methylmalonyl-CoA is then decarboxylated to propionyl-CoA by YgfH. Finally, using propionyl-CoA and succinate as substrates, the CoA moiety is transferred to succinate by propionyl-CoA: succinyl-CoA transferase (YgfH) producing succinyl-CoA and propionate (Figure 6).

As its name suggests, this operon is dormant or silent in E. coli, but potentially relevant to extended dissimilation of succinate for production of C3 products. It is hypothesized that the operon genes are not expressed due to a weak or inactive promoter-operator system (Kannan, 2008). In addition, since E.coli neither produces the vitamer cyanocobalamin (which can be activated to vitamin B\textsubscript{12}) nor requires it for growth, exogenous supplementation of
cyanocobalamin would be required for an active Sbm pathway. It is hypothesized that the role of this pathway in *E. coli* is to facilitate the utilization of unusual carbon sources such as succinate or propionate (Kannan, 2008). *In vitro* studies by Haller et al. (2000) and Froese et al. (2009) provide evidence for this hypothesis, suggesting that Sbm, YgfG and YgfH are involved in the conversion of succinate to propionate with the YgfD kinase interacting with Sbm.

**Figure 6:** The *E. coli* Sleeping beauty mutase pathway

2.3.2 Significance of the Sbm pathway for production of propionate in engineered *Escherichia coli*

While the structure, relationship, and mechanism of the *E. coli* Sbm pathway enzymes have been characterized *in vitro*, hardly any work has been performed for their practical application. Recently, we explored heterologous production of 1-propanol by developing
propanogenic (i.e. 1-propanol-producing) *E. coli* strains engineered with an activated Sbm operon (Srirangan et al., 2013; Srirangan et al., 2014). Anaerobic cultivation of these strains favoured 1-propanol synthesis as a means of consuming excess reducing equivalents, producing low levels of propionate as a by-product. Similarly, we hypothesize that high levels of propionate can be achieved by transcriptional activation of the Sbm operon in addition to manipulation of culture conditions and further strain engineering to favour propionate production over solvent production (Figure 7).
**Figure 7:** The genetically engineered metabolic pathway for propionate production
Glucose and glycerol dissimilation pathways are shown under microaerobic conditions. The fermentative pathway for glycerol dissimilation is presented in a green box and the respiratory pathway for glycerol dissimilation is presented in a yellow box. The Sbm pathway is presented in a purple box. Red and blue arrows represent the route to the C2 and C3-fermentative products, respectively. The C2-fermentative pathway is presented in a red box, while the C3-fermentative pathway is presented in a blue box. Relevant enzymes for production of various fermentative products as well as the enzymes of the respiratory and fermentive glycerol pathways and the Sbm pathway and are in blue.
Chapter 3 - Materials and Methods

3.1 Bacterial strains and plasmids

All primers used and plasmids constructed are listed in Table 5. Genes of the respiratory glycerol dissimilation pathway (i.e. glpK, glpD and glpABC) as well as genes of the fermentative glycerol dissimilation pathway (i.e. gldA, dhaKLM and ptsI) were each amplified from E. coli BW25141 genomic DNA using the corresponding primers sets (i.e. c-glpK, c-glpD, c-glpABC, c-gldA, c-dhaKLM and c-ptsI). PCR amplifications were performed as conventional reactions using New England Biolabs LongAmp Taq DNA Polymerase (Ipswich, MA, USA) or Finnzymes Phusion Polymerase (Espoo, Finland) according to the manufacturers’ instructions. All oligonucleotides were custom-made and purified by Integrated DNA Technologies (Coralville, IA, USA). The PCR products were digested with appropriate NEB restriction enzymes, purified and cloned into pK184 (Jobling and Holmes, 1990) for expression under the regulation of the inducible P\textsubscript{lac} promoter. E. coli strain DH5\textalpha was used for all molecular cloning purposes. Standard recombinant DNA technologies for molecular cloning were applied (Miller, 1992; Sambrook et al., 1989). T4 DNA ligase and the large (Klenow) fragment of DNA Polymerase I were obtained from New England Biolabs. DNA sequencing of the resulting plasmids pK-glpK, pK-glpD, pK-glpABC, pK-gldA, pK-dhaKLM and pK-ptsI was conducted by the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada).
### Table 5: List of plasmids and primers used in this study

Notation for primers: v- verification primer, r- recombineering primer and c- cloning primer. Underlined sequences within the primers denote restriction recognition sites and homology arms for recombineering are in bold print.

<table>
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<th>Name</th>
<th>Description, relevant genotype or primer sequence (5’→3’)</th>
<th>Reference</th>
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<td><strong>Plasmids</strong></td>
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<td>p15A ori, KmR, Pₐₐₐ::lacZ’</td>
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<td>pK-dhaKLM</td>
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<td>pK-ptsI</td>
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E. coli strains used in this study are listed in Table 6. E. coli BW25113 was used to provide the wild-type genetic background for propionate production. Gene knockouts (i.e. ldhA, glpK, glpD, glpA, gldA, dhaK, and ptsI) were introduced into BW25113 and its propionogenic derivatives by P1 phage transduction (Miller, 1992) using the appropriate Keio Collection strains (The Coli Genetic Stock Center, Yale University, New Haven, CT, USA) as donors (Baba et al., 2006). To eliminate the co-transduced FRT-KmR-FRT cassette, the mutants were transformed with pCP20 (Cherepanov and Wackernagel, 1995), a temperature sensitive plasmid expressing a flippase (Flp) recombinase. Upon Flp-mediated excision of the KmR cassette, a single Flp recognition site (FRT “scar site”) was left behind. pCP20 was then removed by growing the cells at 42 °C. The genotypes of derived knockout strains were confirmed by whole-cell colony PCR using the appropriate “verification” primers sets listed in Table 5.
**Table 6: E. coli strains used in this study**

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<td>F&lt;sup&gt;−&lt;/sup&gt;, endA1, glnV44, thi-1, recA1, relA1, gyrA96, deoR, nupG ϕ80d lacZΔ M15, Δ(lacZYA – argF) U169, hsdR17(rK-mK+)</td>
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</table>
To activate the Sbm operon, a strong promoter ($P_{trc}$) was fused with the native Sbm operon in the *E. coli* BW-ΔldhA genome using a modified λ Red-mediated recombination protocol (Sukhija et al., 2012). The FRT-Cm$^R$-FRT cassette from pKD3 was PCR-amplified using the primer set c-frt, whereas the promoter-operator region was PCR-amplified using the c-ptrc primer set. The two DNA amplicons were then fused together by splice overlap-extension (SOE) PCR (Jones and Barnard, 2005) using the forward primer of the c-frt primer set and the reverse primer of the c-ptrc primer set to generate the FRT-Cm$^R$-FRT-$P_{trc}$ cassette. To generate the DNA cartridge for genomic integration, the FRT-Cm$^R$-FRT-$P_{trc}$ cassette was PCR-amplified using the r-frt:ptrc primer set containing the 5′ and 3′ 36-bp homology arms, respectively. The homology arms were chosen so as to insert the FRT-CmR-FRT-$P_{trc}$ cassette precisely upstream of the native and silent Sbm operon; the 5′ and 3′ homology arms correspond to nucleotides 3060611–3060646 and 3060885–3060851, respectively, from the *E. coli* MG1655 genome (Genbank accession no. NC_000913). To derive the plasmid-free propionogenic strain SbmCTRL, 0.1 µg of the amplified/purified DNA cassette was electro-transformed, using a Gene Pulser (BioRad Laboratories, Hercules, CA, USA) set at 1.8 kV, 25 µF, and 200 Ω, to BW-ΔldhA harboring the λ-Red recombinase expression plasmid pKD46 for DNA recombination. Expression of the λ-Red recombination enzymes and preparation of competent cells were carried out as described by Datsenko and Wanner (2000). After electroporation, cells were resuspended in 500 µL of super optimal broth with catabolite repression (SOC) medium (3.6 g/L glucose, 20 g/L tryptone, 5 g/L yeast extract , 0.6 g/L NaCl, 0.19 g/L KCl, 4.8 g/L MgSO$_4$) (Hanahan, 1983) and recuperated at 37 °C for 1 h in a rotatory shaker at 250 rpm (New Brunswick Scientific, NJ). Cells were then plated on lysogeny broth (LB) agar containing 17 µg/mL chloramphenicol for incubation at 37 °C for 16 h to select chloramphenicol-resistant recombinants. The fusion of the FRT-Cm$^R$-FRT-
P_{\text{rc}} cassette with the Sbm operon was verified by colony PCR using the v-frt:ptrc primer set as well as DNA sequencing. Removal of the FRT-Cm^{R}-FRT cassette from the chromosome was achieved by transforming the isolated mutants with pCP20. Strains were cured of pCP20 and pKD46 by growth at 42 °C.

3.2 Media and cultivation

All media components were obtained from Sigma-Aldrich Co. (St Louis, MO, USA) except glucose, yeast extract, and tryptone which were obtained from BD Diagnostic Systems (Franklin Lakes, NJ). Media was supplemented with antibiotics as required (30 µg/mL kanamycin and 12 µg/mL chloramphenicol). For propionate production, the propionogenic E. coli strains (stored as glycerol stocks at -80 °C) were streaked on LB agar plates with appropriate antibiotics and incubated at 37 °C for 16 h. Single colonies were picked from LB plates to inoculate 30-mL SB medium (32 g/L tryptone, 20 g/L yeast extract, and 5 g/L NaCl) with appropriate antibiotics in 125 mL conical flasks. Overnight cultures were shaken at 37 °C and 280 rpm in a rotary shaker (New Brunswick Scientific, NJ) and used as seed cultures to inoculate 200 mL SB media at 1% (v/v) with appropriate antibiotics in 1 L conical flasks. This second seed culture was shaken at 37 °C and 280 rpm for approximately 16 h. Cells were then harvested by centrifugation at 6,000 × g and 20 °C for 15 min and resuspended in 100-mL fresh LB media. The suspended culture was used to inoculate a 1-L stirred-tank bioreactor (CelliGen 115, Eppendorf AG, Hamburg, Germany) operated anaerobically or microaerobically at 30 °C and 430 rpm. The semi-defined production medium in the bioreactor contained 30 g/L glycerol, 0.23 g/L K_{2}HPO_{4}, 0.51 g/L NH_{4}Cl, 49.8 mg/L MgCl_{2}, 48.1 mg/L K_{2}SO_{4}, 1.52 mg/L FeSO_{4}, 0.055 mg/L CaCl_{2}, 2.93 g/L NaCl, 0.72 g/L tricine, 10 g/L yeast extract, 10 mM NaHCO_{3}, 0.2 µM
cyanocobalamin (vitamin B_{12}) and trace elements (2.86 mg/L H_{3}BO_{3}, 1.81 mg/L MnCl_{2}•4H_{2}O, 0.222 mg/L ZnSO_{4}•7H_{2}O, 0.39 mg/L Na_{2}MoO_{4}•2H_{2}O, 79 µg/L CuSO_{4}•5H_{2}O, 49.4 µg/L Co(NO_{3})_{2}•6H_{2}O) (Neidhardt et al., 1974), appropriate antibiotics, and supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Anaerobic conditions were maintained by constant bubbling of nitrogen (~0.1 vvm). Microaerobic conditions were maintained by purging air into the headspace at 0.1 vvm. The pH of the production culture was maintained at 7.0 ± 0.1 with 30% (v/v) NH_{4}OH and 15% (v/v) HNO_{3}.

3.3 Analyses

Culture samples were appropriately diluted with saline for measuring the optical cell density (OD_{600}) using a spectrophotometer (DU520, Beckman Coulter, Fullerton, CA). Cell-free supernatant was collected and filter sterilized for titre analysis of glycerol and the various end-fermentation metabolites using an HPLC (LC-10AT, Shimadzu, Kyoto, Japan) with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan) and a chromatographic column (Aminex HPX-87H, Bio-Rad Laboratories, CA, USA). The column temperature was maintained at 65 °C and the mobile phase was 5 mM H_{2}SO_{4} (pH 2.0) running at 0.6 mL/min. Data acquisition and analysis were performed using the Clarity Lite Chromatographic Station (Clarity Lite, DataApex, Prague, Czech Republic).

3.4 Calculations

The fraction of dissimilated glucose/glycerol to form a metabolite as reported in Tables 7-9 is defined as the ratio of the glucose/glycerol equivalent of a metabolite to the total amount (in g) of glycerol consumed. The glucose/glycerol equivalent for each metabolite was calculated based on the corresponding theoretical yield of the conversions presented Table 7. The glycerol
efficiency toward metabolite synthesis is calculated as the sum of all metabolite fractions.

**Table 7: Theoretical yield of metabolites**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Theoretical yield conversion (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5Glucose</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.30</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.00</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.65</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.82</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.51</td>
</tr>
<tr>
<td>Propanol</td>
<td>0.67</td>
</tr>
</tbody>
</table>
Chapter 4- Results

4.1 Determination of cultivation conditions for propionate production

Given that the native *E. coli* Sbm operon is inherently silent, a functional Sbm pathway was established in the engineered strain SbmCTRL using a previously developed bacteriophage λ (λ-Red) genomic recombineering-based method (Sukhija et al., 2012). Specifically, a synthetic DNA fusion containing a strong P<sub>trc</sub> promoter-operator along with a chloramphenicol-resistance cat cassette flanked by two FRT sites was used to replace a 204-bp region upstream of the Sbm operon. The chloramphenicol-resistance marker was excised using Flp-mediated excision and the strain was cured of all episomal plasmids for recombineering, creating the markerless and plasmid-free strain of SbmCTRL.

SbmCTRL was first characterized for its ability to produce propionate and culture performance under various culture conditions is presented in Table 8 and Figure 8. Anaerobic conditions were chosen given that propionate is a fermentative product. Furthermore, a putative Fnr binding site upstream of sbm in the *E. coli* chromosome was identified (Salmon et al., 2003) suggesting that the expression of the Sbm operon is positively regulated by anaerobiosis. While the activated Sbm operon was competent for propionate production, the propionate titer was limited with ~1.23 g/L and ~0.17 g/L being produced at the end of the strict anaerobic cultivations using glucose and glycerol, respectively, as a carbon source.

Under anaerobic conditions, cultivation performance varies significantly with the carbon source. First, complete dissimilation of 30 g/L glucose and glycerol occurred at 11 h and 56 h, respectively (Appendix D-1). Both cell growth rate and biomass yield were significantly higher for anaerobic glucose culture. In addition, the two cultures showed rather different profiles of
metabolite production. For the anaerobic glucose culture, the majority of carbon flux was directed toward the C2-fermentative pathway, with a C3:C2 product ratio equal to 0.15 (Figure 8B). Anaerobic cultivation using glycerol selectively favored solvent production, with the sum of ethanol and 1-propanol titers accounting for 87% of dissimilated glycerol (an overall acid:solvent ratio of 0.13), but also with limited propionate production. In light of limited cultivation performance, particularly low propionate production, as well as major diversion of carbon flux into the C2-fermentative pathway under strict anaerobic conditions, we explored microaerobic cultivation for which air was purged into the headspace of the bioreactor at 0.1 vvm.

Switching from anaerobic to microaerobic conditions for glucose culture slightly reduced the carbon flux into the C2-fermentative pathway with acetate and ethanol still being the two dominant metabolites. However, the switch had no major effect on propionate production. On the other hand, for glycerol culture, the introduction of microaerobic conditions significantly increased both the glycerol dissimilation rate and biomass yield by ~100% compared to the corresponding anaerobic operation. The change to a microaerobic environment resulted in a dramatic decrease in ethanol production with a simultaneous increase in acetate production. With respect to the fraction of dissimilated glycerol, ~24% was directed towards succinate, acetate and propionate and ~22% towards ethanol and 1-propanol, a nearly equal ratio. More importantly, the propionate titre reached 1.60 g/L, representing a more than seven-fold increase compared to the anaerobic glycerol culture. However, note that introduction of microaerobic conditions reduced the overall efficiency of dissimilation of carbon sources (i.e. carbon loss for both glucose and glycerol) presumably due to the formation of carbon dioxide. Overall, based on these SbmCTRL cultures, it appears that microaerobic cultivation using glycerol as the major carbon source is most suitable for propionate production as both the C3:C2 product ratio (propionate + 1-
propanol: acetate + ethanol) and the propionate: acetate ratio in this culture were the highest, 0.54 and 0.39, respectively (Figure 8B). Therefore, all subsequent cultivations were conducted under this culture condition.
**Table 8:** Culture performance of SbmCTRL
Overall glycerol or glucose consumption and final biomass and metabolite concentrations of SbmCTRL in a bioreactor under anaerobic and microaerobic cultivation conditions. The metabolite distribution (i.e. the fraction of dissimilated glycerol to form a metabolite) was calculated as described in Section 3.4.

<table>
<thead>
<tr>
<th></th>
<th>Glucose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Glycerol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Biomass&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Succinate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Lactate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Acetate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Propionate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Ethanol&lt;sup&gt;c&lt;/sup&gt;</th>
<th>1-Propanol&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anaerobic cultivation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SbmCTRL – Glucose</td>
<td>30.37 ± 0.35 (81.77%)</td>
<td>- 3.77 ± 0.05 (4.24%)</td>
<td>1.72 ± 0.05 (4.00%)</td>
<td>0.43 ± 0.14 (1.39%)</td>
<td>8.27 ± 0.11 (40.85%)</td>
<td>1.23 ± 0.01 (4.85%)</td>
<td>4.07 ± 0.08 (25.80%)</td>
<td>0.96 ± 0.01 (4.64%)</td>
<td></td>
</tr>
<tr>
<td>SbmCTRL – Glycerol</td>
<td>- 27.58 ± 0.74 (100.02%)</td>
<td>- 2.16 ± 0.04 (1.77%)</td>
<td>0.63 ± 0.00 (0.00%)</td>
<td>ND 1.90 ± 0.13 (0.76%)</td>
<td>0.17 ± 0.03 (10.62%)</td>
<td>10.63 ± 0.19 (76.40%)</td>
<td>1.93 ± 0.08 (10.64%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Microaerobic cultivation</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SbmCTRL – Glucose</td>
<td>28.41 ± 0.89 (62.94%)</td>
<td>- 3.37 ± 0.06 (4.62%)</td>
<td>1.72 ± 0.06 (3.39%)</td>
<td>0.97 ± 0.12 (33.97%)</td>
<td>6.34 ± 0.40 (33.97%)</td>
<td>1.29 ± 0.03 (5.53%)</td>
<td>1.74 ± 0.30 (11.99%)</td>
<td>0.65 ± 0.06 (3.44%)</td>
<td></td>
</tr>
<tr>
<td>SbmCTRL – Glycerol</td>
<td>- 31.89 ± 0.91 (47.00%)</td>
<td>- 4.20 ± 0.08 (0.63%)</td>
<td>0.26 ± 0.00 (0.00%)</td>
<td>ND 3.53 ± 0.08 (17.28%)</td>
<td>1.60 ± 0.10 (6.32%)</td>
<td>2.06 ± 0.06 (12.93%)</td>
<td>2.04 ± 0.13 (9.83%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> glucose or glycerol consumption (g/L), glucose or glycerol efficiency is presented in parentheses under the carbon source consumption value (%)

<sup>b</sup> biomass accumulation (g-DCW/L)

<sup>c</sup> metabolite concentrations (g/L), the fraction of dissimilated glycerol is presented in parentheses under each titre

ND not detected
Figure 8: Major metabolites titers during batch cultivation of SbmCTRL under anaerobic or microaerobic conditions using glucose or glycerol as a carbon source (A), and ratios of C3:C2 fermentative products (propionate + 1-propanol: acetate + ethanol), overall acid:solvent production (propionate + acetate: propanol + ethanol) as well as propionate:acetate production for each of the batch cultivation conditions, ratios are calculated from the fractions of dissimilated glycerol (B).
4.2 Inactivation of the respiratory pathway for glycerol dissimilation

In light of the effectiveness of glycerol as a carbon source for propionate production, we aimed to re-engineer the propionogenic strain SbmCTRL by targeting glycerol metabolism in an attempt to identify the link between glycerol dissimilation and propionate production. Six genes associated with glycerol dissimilation, either via the respiratory or fermentative pathway, were manipulated by gene knockout or episomal overexpression to observe their individual effects on cultivation performance under microaerobic conditions, particularly cell growth, relative levels of acidogenesis and solventogenesis, metabolite profile, and propionate production were evaluated.

The respiratory pathway of glycerol dissimilation includes glycerol kinase, encoded by glpK, and two glycerol-3-phosphate dehydrogenases, encoded by glpD (aerobic) and glpABC (anaerobic), respectively (Figure 7). This ATP-dependent glycerol dissimilation pathway starts with aerobic phosphorylation of glycerol to G3P, followed by the oxidation of G3P to DHAP (Figure 3). While the oxidation step is primarily carried out by aerobic GlpD, the minor pathway via anaerobic GlpABC can be functional when oxygen is unavailable. Microaerobic cultivations of three single-knockout mutants, i.e. Sbm-ΔglpK, Sbm-ΔglpD, and Sbm-ΔglpA, using glycerol as the major carbon source were conducted and the results are summarized in Table 9 and Figure 9. While the overall glycerol dissimilation rate for Sbm-ΔglpK and Sbm-ΔglpD was slightly slower than that of the control strain SbmCTRL (taking more than 30 h to consume 30 g/L glycerol for the two mutants and 26 h for SbmCTRL) (Appendix D-2), implying slightly defective glycerol dissimilation associated with these mutations under microaerobic conditions, the biomass yield for these two mutants was significantly higher than that of SbmCTRL.
However, the Sbm-ΔglpA mutant had approximately the same glycerol dissimilation rate and biomass yield as those of SbmCTRL.

The various single-gene knockouts associated with the respiratory pathway of glycerol dissimilation also resulted in major changes in metabolite production. In comparison to SbmCTRL, solventogeneis was significantly inhibited for Sbm-ΔglpD and was even completely abolished for Sbm-ΔglpK. This was not the case for Sbm-ΔglpA, which had a solventogenesis level similar to that of SbmCTRL (Figure 9). On the other hand, acidogenesis was significantly enhanced particularly for Sbm-ΔglpK and Sbm-ΔglpD, and their propionate titers (i.e. 6.67 g/L and 5.22 g/L, respectively) were more than three-fold that of SbmCTRL (i.e. 1.60 g/L). However, the propionate titer for Sbm-ΔglpA (i.e. 1.97 g/L) was only slightly increased compared to SbmCTRL though acetate titer was significantly increased. Apart from a higher level of acidogenesis, the overall cultivation performance of Sbm-ΔglpA was more or less the same as that of SbmCTRL.

4.3 Inactivation of the respiratory pathway for glycerol dissimilation

Alternatively, glycerol dehydrogenase and dihydroxyacetone kinase (encoded by gldA and dhaKLM, respectively) comprise the fermentative pathway of glycerol dissimilation in E. coli (Figure 7). Active during anaerobic conditions, this pathway mediates the conversion of glycerol to DHA which is subsequently phosphorylated to DHAP using a PTS-like phosphorelay system (Figure 3) (Gutknecht et al., 2001). Microaerobic cultivations of three single-knockout mutants, i.e. Sbm-ΔgldA, Sbm-ΔdhaK, and Sbm-ΔptsI, using glycerol as the major carbon source were conducted and the results are summarized in Table 9 and Figure 10. While the overall glycerol dissimilation rate was slightly reduced by these knockouts (taking more than 30 h to consume 30
g/L glycerol for the three mutants and 26 h for SbmCTRL) (Appendix D-3), the biomass yield for all three mutants had a ~50% increase compared to SbmCTRL. Similar to the respiratory-pathway knockout mutants, minimal solventogenesis and high acidogenesis were observed for the fermentative-pathway knockout mutants. More importantly, the high-level acidogenesis preferentially favoured propionate production over acetate (Figure 10B), leading to a high propionate titre of more than 11 g/L for Sbm-ΔgldA and Sbm-ΔdhaK, accounting for up to 50% of dissimilated glycerol and representing a seven-fold increase over the propionate titre of the microaerobic SbmCTRL culture. Inactivation of the alternative phosphorelay system in Sbm-ΔptsI also significantly increased the propionate titer to 8.74 g/L. A comparison of the C3:C2 product ratio as well as the propionate:acetate ratio of SbmCTRL and the fermentative pathway mutants suggest the effectiveness of inactivating the fermentative pathway of glycerol dissimilation for enhancing propionate production (Figures 9 and 10).
**Table 9: Culture performance of glycerol pathway mutant strains**

Overall glycerol consumption and final biomass and metabolite concentrations of respiratory pathway mutants (Sbm-ΔglpK, Sbm-ΔglpD, and Sbm-ΔglpA) and fermentative pathway mutants (Sbm-ΔgldA, Sbm-ΔdhaK and Sbm-ΔptsI). The metabolite distribution (i.e. the fraction of dissimilated glycerol to form a metabolite) was calculated as described in Section 3.4.

<table>
<thead>
<tr>
<th></th>
<th>Glycerol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Biomass&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Succinate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Lactate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Acetate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Propionate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Ethanol&lt;sup&gt;c&lt;/sup&gt;</th>
<th>1-Propanol&lt;sup&gt;c&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td><strong>Respiratory pathway mutants</strong></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>SbmΔglpK</td>
<td>31.35</td>
<td>8.41</td>
<td>0.00</td>
<td>ND</td>
<td>8.62</td>
<td>6.67</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>(± 0.92)</td>
<td>-</td>
<td>± 0.00</td>
<td>-</td>
<td>-</td>
<td>± 0.52</td>
<td>± 0.09</td>
<td>± 0.00</td>
<td>± 0.00</td>
</tr>
<tr>
<td>(69.78%)</td>
<td>-</td>
<td>(0.00%)</td>
<td>(0.00%)</td>
<td>(42.95%)</td>
<td>(26.83%)</td>
<td>(0.00%)</td>
<td>(0.00%)</td>
<td>(0.00%)</td>
</tr>
<tr>
<td></td>
<td>30.14</td>
<td>6.70</td>
<td>0.49</td>
<td>ND</td>
<td>7.88</td>
<td>5.22</td>
<td>1.03</td>
<td>0.98</td>
</tr>
<tr>
<td>SbmΔglpD</td>
<td>± 0.12</td>
<td>-</td>
<td>± 0.10</td>
<td>-</td>
<td>± 0.16</td>
<td>± 0.04</td>
<td>± 0.10</td>
<td>± 0.04</td>
</tr>
<tr>
<td>(75.73%)</td>
<td>-</td>
<td>(1.27%)</td>
<td>(0.00%)</td>
<td>(40.80%)</td>
<td>(21.82%)</td>
<td>(6.84%)</td>
<td>(5.00%)</td>
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</tr>
<tr>
<td></td>
<td>27.21</td>
<td>3.98</td>
<td>0.52</td>
<td>ND</td>
<td>6.07</td>
<td>2.29</td>
<td>1.97</td>
<td>1.86</td>
</tr>
<tr>
<td>SbmΔglpA</td>
<td>± 0.83</td>
<td>-</td>
<td>± 0.04</td>
<td>-</td>
<td>± 0.09</td>
<td>± 0.07</td>
<td>± 0.04</td>
<td>± 0.23</td>
</tr>
<tr>
<td>(71.88%)</td>
<td>-</td>
<td>(1.49%)</td>
<td>(0.00%)</td>
<td>(34.78%)</td>
<td>(10.62%)</td>
<td>(14.48%)</td>
<td>(10.50%)</td>
<td></td>
</tr>
<tr>
<td><strong>Fermentative pathway mutants</strong></td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SbmΔgldA</td>
<td>31.74</td>
<td>6.29</td>
<td>0.05</td>
<td>ND</td>
<td>5.01</td>
<td>11.39</td>
<td>0.12</td>
<td>0.21</td>
</tr>
<tr>
<td>(± 0.66)</td>
<td>-</td>
<td>± 0.06</td>
<td>-</td>
<td>-</td>
<td>± 0.07</td>
<td>± 0.39</td>
<td>± 0.03</td>
<td>± 0.06</td>
</tr>
<tr>
<td>(71.76%)</td>
<td>-</td>
<td>(0.11%)</td>
<td>(0.00%)</td>
<td>(24.63%)</td>
<td>(45.23%)</td>
<td>(0.76%)</td>
<td>(1.02%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30.03</td>
<td>6.56</td>
<td>0.48</td>
<td>ND</td>
<td>4.44</td>
<td>11.83</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>SbmΔdhaK</td>
<td>± 0.05</td>
<td>-</td>
<td>± 0.09</td>
<td>-</td>
<td>± 0.16</td>
<td>± 0.09</td>
<td>± 0.00</td>
<td>± 0.00</td>
</tr>
<tr>
<td>(73.94%)</td>
<td>-</td>
<td>(1.24%)</td>
<td>(0.00%)</td>
<td>(23.04%)</td>
<td>(49.66%)</td>
<td>(0.00%)</td>
<td>(0.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29.50</td>
<td>6.56</td>
<td>0.78</td>
<td>ND</td>
<td>4.61</td>
<td>8.74</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>SbmΔptsI</td>
<td>± 0.79</td>
<td>-</td>
<td>± 0.03</td>
<td>-</td>
<td>± 0.05</td>
<td>± 0.08</td>
<td>± 0.00</td>
<td>± 0.00</td>
</tr>
<tr>
<td>(63.77%)</td>
<td>-</td>
<td>(2.07%)</td>
<td>(0.00%)</td>
<td>(24.36%)</td>
<td>(37.35%)</td>
<td>(0.00%)</td>
<td>(0.00%)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> glycerol consumption (g/L), glucose or glycerol efficiency is presented in parentheses under the glycerol consumption value (%)

<sup>b</sup> biomass accumulation (g-DCW/L)

<sup>c</sup> metabolite concentrations (g/L), the fraction of dissimilated glycerol is presented in parentheses under each titre

ND not detected
Figure 9: Major metabolites titers during microaerobic batch cultivation of respiratory pathway mutants (Sbm-ΔglpK, Sbm-ΔglpD and Sbm-ΔglpA) using glycerol as a carbon source (A), and ratios of C3:C2 fermentative products, overall acid:solvent production as well as propionate:acetate production for each cultivation, ratios are calculated from the fractions of dissimilared glycerol (B).
Figure 10: Major metabolites titers during microaerobic batch cultivation of fermentative pathway mutants (Sbm-ΔgldA, Sbm-ΔdhaK and Sbm-ΔptsI) using glycerol as a carbon source (A), and ratios of C3:C2 fermentative products, overall acid:solvent production as well as propionate:acetate production for each cultivation, ratios are calculated from the fractions of dissimilated glycerol (B).
4.4 Overexpression of the respiratory pathway genes for glycerol dissimilation

Microaerobic cultivations of strains episomally overexpressing the genes encoding each of the enzymes in the respiratory pathway of glycerol dissimilation, i.e. Sbm-glpK, Sbm-glpD and Sbm-glpABC (Figure 3), were conducted and their culture performance is presented in Table 10 and Figure 11. In comparison to SbmCTRL, overexpression of glpD and glpABC had a minor effect on the overall rate of glycerol dissimilation, whereas glpK overexpression slightly decreased the overall glycerol dissimilation rate (Appendix D-4). While the biomass yield of Sbm-glpD was similar to SbmCTRL, Sbm-glpK and Sbm-glpABC had significantly lower and higher biomass yields, respectively.

Metabolite profiles of Sbm-glpK, Sbm-glpD and Sbm-glpABC varied considerably. Acidogenesis was generally enhanced in all three mutant strains, accounting for more than 55% of dissimilated glycerol. Note that Sbm-glpK, though with an ΔldhA genetic background, had an unusually high lactate titre potentially associated with its low glycerol dissimilation rate, biomass yield, and even propionate titre. Overexpression of glpD and glpABC resulted in improved propionate production potentially due to enhanced acidogenesis and less carbon loss (i.e. higher glycerol efficiency) (Figure 11B).

4.5 Overexpression of the fermentative pathway genes for glycerol dissimilation

Alternatively, microaerobic cultivations of strains episomally overexpressing the genes encoding each of the enzymes in the fermentative pathway of glycerol dissimilation, i.e. Sbm-gldA, Sbm-dhaKLM and Sbm-ptsI, (Figure 3) were conducted and their culture performance is presented in Table 10 and Figure 12. The biomass yields of the three mutant strains were similar to that of SbmCTRL. Compared to SbmCTRL, the glycerol dissimilation rates of Sbm-gldA and
Sbm-dhaKLM were slightly reduced (taking more than 30 h to consume 30 g/L glycerol for the two mutants and 26 h for SbmCTRL), whereas that of Sbm-ptsI was significantly slowed (taking more than 45 h to consume 30 g/L glycerol) (Appendix D-5).

Overexpression of gldA resulted in a substantial shift in the metabolite profile, compared to SbmCTRL, with high acetate and succinate titres accompanied by the loss of propionate and 1-propanol synthesis. The results imply that gldA overexpression completely inactivated the Sbm pathway by stalling the dissimilated carbon flux at the succinate node (Figure 7). On the other hand, overexpression of dhaKLM or ptsI resulted in enhanced solventogenesis with a propionate titre either similar to (for Sbm-dhaKLM) or lower than (for Sbm-ptsI) that of SbmCTRL. These results suggest that carbon flux should be preferentially channeled through the respiratory pathway, but not the fermentative pathway, of glycerol dissimilation for effective propionate production.
Table 10: Culture performance of strains overexpressing the glycerol dissimilation genes

Overall glycerol consumption and final biomass and metabolite concentrations of the respiratory pathway overexpression strains (Sbm-glpK, Sbm-glpD, and Sbm-glpA) and the fermentative pathway overexpression strains (Sbm-gldA, Sbm-dhaK and Sbm-ptsI). The metabolite distribution (i.e. the fraction of dissimilated glycerol to form a metabolite) was calculated as described in Section 3.4.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Respiratory pathway overexpression strains</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Glycerol⁴</td>
<td>Biomass⁵ [g/L]</td>
<td>Succinate⁶ [g/L]</td>
<td>Lactate⁶ [g/L]</td>
<td>Acetate⁶ [g/L]</td>
<td>Propionate⁶ [g/L]</td>
<td>Ethanol⁶ [g/L]</td>
<td>1-Propanol⁶ [g/L]</td>
</tr>
<tr>
<td>Sbm-glpK</td>
<td>30.08 ± 1.45</td>
<td>1.98</td>
<td>1.09</td>
<td>6.38</td>
<td>6.22</td>
<td>0.87</td>
<td>2.87</td>
</tr>
<tr>
<td>(82.52%)</td>
<td>(2.83%)</td>
<td>(21.72%)</td>
<td>(32.33%)</td>
<td>(3.62%)</td>
<td>(19.19%)</td>
<td>(2.83%)</td>
<td></td>
</tr>
<tr>
<td>Sbm-glpD</td>
<td>29.12 ± 0.16</td>
<td>4.58</td>
<td>0.51</td>
<td>ND</td>
<td>7.10</td>
<td>4.45</td>
<td>1.49</td>
</tr>
<tr>
<td>(76.46%)</td>
<td>(1.35%)</td>
<td>(0.00%)</td>
<td>(38.01%)</td>
<td>(19.26%)</td>
<td>(10.24%)</td>
<td>(7.60%)</td>
<td></td>
</tr>
<tr>
<td>Sbm-glpABC</td>
<td>30.79 ± 1.01</td>
<td>8.01</td>
<td>0.26</td>
<td>ND</td>
<td>8.41</td>
<td>5.67</td>
<td>0.29</td>
</tr>
<tr>
<td>(70.90%)</td>
<td>(0.66%)</td>
<td>(0.00%)</td>
<td>(42.59%)</td>
<td>(23.21%)</td>
<td>(1.90%)</td>
<td>(2.54%)</td>
<td></td>
</tr>
<tr>
<td>Sbm-gldA</td>
<td>30.87 ± 0.59</td>
<td>4.72</td>
<td>4.07</td>
<td>ND</td>
<td>9.24</td>
<td>0.00</td>
<td>2.35</td>
</tr>
<tr>
<td>(72.15%)</td>
<td>(10.30%)</td>
<td>(0.00%)</td>
<td>(46.65%)</td>
<td>(0.00%)</td>
<td>(15.19%)</td>
<td>(0.00%)</td>
<td></td>
</tr>
<tr>
<td>Sbm-dhaKLM</td>
<td>30.25 ± 0.08</td>
<td>4.93</td>
<td>0.63</td>
<td>ND</td>
<td>4.61</td>
<td>1.61</td>
<td>4.80</td>
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<td>(74.12%)</td>
<td>(1.63%)</td>
<td>(0.00%)</td>
<td>(23.75%)</td>
<td>(6.71%)</td>
<td>(31.71%)</td>
<td>(10.33%)</td>
<td></td>
</tr>
<tr>
<td>Sbm-ptsI</td>
<td>26.09 ± 0.73</td>
<td>3.52</td>
<td>0.12</td>
<td>ND</td>
<td>2.59</td>
<td>0.40</td>
<td>6.29</td>
</tr>
<tr>
<td>(76.14%)</td>
<td>(0.35%)</td>
<td>(0.00%)</td>
<td>(15.44%)</td>
<td>(1.93%)</td>
<td>(48.22%)</td>
<td>(10.21%)</td>
<td></td>
</tr>
</tbody>
</table>

⁴ glycerol consumption (g/L), glucose or glycerol efficiency is presented in parentheses under the carbon source consumption value (%)
⁵ biomass accumulation (g-DCW/L)
⁶ metabolite concentrations (g/L), the fraction of dissimilated glycerol is presented in parentheses under each titre
ND not detected
Figure 11: Major metabolites titers during microaerobic batch cultivation of respiratory pathway overexpression strains (Sbm-glpK, Sbm-glpD and Sbm-glpABC) using glycerol as a carbon source (A), and ratios of C3:C2 fermentative products, overall acid:solvent production as well as propionate:acetate production for each cultivation, ratios are calculated from the fractions of dissimilated glycerol (B).
Figure 12: Major metabolites titers during microaerobic batch cultivation of respiratory pathway overexpression strains (Sbm-gldA, Sbm-dhaKLM and Sbm-ptsI) using glycerol as a carbon source (A), and ratios of C3:C2 fermentative products, overall acid:solvent production as well as propionate:acetate production for each cultivation, ratios are calculated from the fractions of dissimilared glycerol (B).
Chapter 5- Discussion

While propionate biosynthesis has primarily been conducted by anaerobic cultivation of Gram-positive native producers, such as *Propionibacterium* and *Clostridium* (Barbirato et al., 1997; Himmi et al., 2000; Zhang and Yang, 2009; Zhu et al., 2010), or by anaerobic *E. coli* cultivation but with limited propionate titres (Choi et al., 2012; Kandasamy et al., 2013), we present a novel approach for high-level propionate production through activation of the endogenous Sbm operon of *E. coli*.

Though glucose can be fast dissimilated by propionogenic *E. coli*, it was deemed an unsuitable carbon source primarily due to acetate overproduction. If the rate of consumption of the carbon source is too high, the cell’s capacity to re-oxidize generated reducing equivalents is impacted (Wolfe, 2005). In other words, the NADH turnover is stalled. Formation of acetate, a common C2-fermentative product for *E. coli* cultivation, is often associated with carbon flux overflow (Majewski and Domach, 1990; Vemuri et al., 2006), characterized by an increase in the intracellular NADH:NAD\(^+\) ratio above the critical threshold of 0.06, resulting in transcriptional repression of respiratory genes of the TCA cycle (Vemuri et al., 2006) and potential limitation of dissimilated carbon flux into the C3-fermentative pathway. Additionally, production of acetate is coupled with ATP formation, which is critical for cell growth. Under anaerobic conditions, expression of the Sbm operon appears to be more active when glycerol is used as the major carbon source rather than glucose, resulting in less succinate accumulation and more diversion of dissimilated carbon flux into the Sbm pathway (Srirangan et al., 2014). Nevertheless, glycerol can be a recalcitrant carbon source particularly under strict anaerobic conditions for *E. coli*.
cultivation. Due to its higher degree of reductance, solventogenesis often dominates during anaerobic cultivations on glycerol and, as a result, propionate production becomes limited.

Microaerobic conditions can offer low levels of oxygen as an electron acceptor, leading to improved cell growth and even biomass yield, particularly when glycerol is used as the major carbon source. Compared to 1-propanol which normally requires strict anaerobiosis for effective production, propionate is a less reduced C3-fermentative product which can be effectively produced under microaerobic conditions. While cell growth improved and glycerol dissimilation was more effective under microaerobic conditions for SbmCTRL, acetate was to some extent overproduced, potentially due to carbon flux overflow and stalling of intracellular NADH turnover (Wolfe, 2005), and therefore propionate production was limited. Since AdhE is normally repressed in the presence of oxygen, the persistent solventogenesis during microaerobic cultivation of SbmCTRL with glycerol was likely associated with the increase in the NADH/NAD\(^+\) ratio (Leonardo et al., 1996).

Based on the hypothesis of carbon flux overflow, we explored potential slowing of glycerol dissimilation under microaerobic conditions by inactivating various genes involved in the glycerol dissimilation pathway. Inactivation of the glucose uptake system for slower glucose uptake/dissimilation was an effective strategy to limit carbon flux overflow and therefore alleviate acetate overproduction during *E. coli* cultivation (Cheng et al., 2014; Chou et al., 1994; Sigüenza et al., 1999; Wong et al., 2008). Note that all single-knockout mutants investigated in this study, except Sbm-ΔglpD, cannot be cultivated anaerobically using glycerol as the major carbon source (data not shown). Though Sbm-ΔglpD can be cultivated anaerobically, its glycerol dissimilation rate was much lower than that of SbmCTRL, as
dissimilation of 30 g/L glycerol took 65 h (data not shown) and 30 h for Sbm-ΔglpD and SbmCTRL, respectively. The result suggests the coordinated and synergistic roles of all enzymes involved in both respiratory and anaerobic pathways for glycerol dissimilation under anaerobic conditions, including GlpD which is identified as an aerobic glycerol-3-phosphate dehydrogenase (Durnin et al., 2009; Murarka et al., 2008; Zhang et al., 2010). Nevertheless, such defective glycerol dissimilation under anaerobic conditions associated with various gene knockouts can be prevented by adopting microaerobic cultivation, suggesting that both the respiratory and fermentative pathways of glycerol dissimilation play a significant role in microaerobic utilization of glycerol (Durnin et al., 2009).

Note that, given a slightly reduced overall glycerol dissimilation rate, all single-knockout mutants except Sbm-ΔglpA had a biomass yield at least 50% higher than that of SbmCTRL and the increased biomass yield occurred consistently with high-level acidogenesis (accounting for 60-73% of dissimilated glycerol) and suppressed solventogenesis, resulting in enhanced propionate production. The results also suggest that propionate production is growth-associated. With the introduction of these knockouts, redox constraints are relaxed as carbon flow is uncoupled from the necessity to maintain redox balance through synthesis of highly reduced metabolites (e.g. alcohols). Therefore, the oxidized pathways are activated, allowing for enhanced synthesis of organic acids (Durnin et al., 2009). The lower glycerol dissimilation rate of these mutants than that of SbmCTRL and their suppressed solventogenesis might imply a reduced NADH/NAD⁺ ratio and carbon flux overflow resulting from these gene knockouts. On the other hand, the similar overall cultivation performance for SbmCTRL and Sbm-ΔglpA (in terms of glycerol dissimilation
rate, biomass yield, and metabolite production) suggests that under microaerobic cultivation conditions, the biological role of GlpABC is slight. The genes encoding the anaerobic and aerobic glycerol-3-phosphate dehydrogenase (\textit{glpABC} and \textit{glpD}, respectively) are not on the same operon in the \textit{E. coli} chromosome and, as such, are under different regulation (Luchi et al., 1990). Specifically, \textit{glpABC} expression is induced by the anaerobic regulator FNR which is deactivated in the presence of oxygen; as a result, the anaerobic respiratory pathway plays a minor role in glycerol dissimilation under microaerobic conditions.

Knocking out genes involved in glycerol dissimilation (except \textit{glpA}) can potentially shift more dissimilated carbon flux toward the C3-fermentative pathway, resulting in an increased propionate/acetate ratio (i.e. 0.62 for Sbm-\textit{ΔglpK}, 0.53 for Sbm-\textit{ΔglpD}, 1.84 for Sbm-\textit{ΔagldA}, 2.16 for Sbm-\textit{ΔdhaK}, 1.53 for Sbm-\textit{ΔptsI}) compared to the ratio of 0.37 for SbmCTRL (Figures 9B and 10B). Furthermore, genes involved in the fermentative pathway of glycerol dissimilation appear to be the best targets for knockout since not only solventogenesis was minimized but also propionate was preferentially produced over acetate under high-level acidogenesis conditions. The results suggest not only the effectiveness of this gene manipulation strategy but also the importance of the respiratory pathway of glycerol dissimilation for enhancing propionate production under microaerobic conditions. The observed high propionate titres upon inactivation of the fermentative pathway can be associated with the release of the pathway’s dependence on PEP as a phosphate donor for DHAP synthesis, increasing the PEP pool for its subsequent conversion to oxaloacetate (OAA) and then propionate (Figure 7). Zhang et al. (2010) previously reported that the \textit{ptsI} knockout enhanced succinate production during anaerobic fermentation of glycerol, crediting
the knockout effect on conserving PEP. Conversely, when *gldA* and *dhaK* were episomally overexpressed, a decrease in succinate production was observed presumably due to a reduced PEP pool (Mazumdar et al., 2010).

Alternatively, the effects of overexpression of genes involved in glycerol dissimilation on propionate production were also explored. Generally, overexpression of genes involved in the respiratory pathway of glycerol dissimilation (i.e. *glpD* and *glpABC*), but not the fermentative pathway, enhanced propionate production. The results are consistent with the above knockout results that the dissimilated carbon flux should be channeled through the respiratory pathway for effective propionate production under microaerobic conditions. Interestingly, overexpression of *glpK* impaired cell growth and propionate production with an unusual lactate accumulation even though the *ldhA* gene of the propionogenic strain was inactivated. The redirection of carbon flux toward lactate can potentially result from activation the methylglyoxal pathway, a bypass pathway to glycolysis at the DHAP node (Figure 7). Generally associated with a loss of regulation of carbon uptake resulting in an increased DHAP pool, the physiological role of this pathway is to replenish intracellular inorganic phosphate when its concentration is low, as conversion of DHAP to methylglyoxal releases an inorganic phosphate (Hopper and Cooper, 1971). However, methylglyoxal is toxic and, therefore, its synthesis must be balanced by detoxification through its subsequent enzymatic conversion to lactate (Totemeyer et al., 1998). In Sbm-glpK, this pathway was potentially activated as an effective means for ATP generation to sustain the heightened ATP-dependent phosphorylation of glycerol to G3P.

Similar to the previous observation by Mazumdar (2010), overexpression of genes
involved in the fermentative pathway of glycerol dissimilation may cause a decrease in the PEP pool, since phosphorylation of DHA to DHAP is dependent on PEP as the phosphate donor, resulting in less flux into the TCA cycle and Sbm pathway for propionate production. In particular, overexpression of glycerol dehydrogenase (gldA) caused a severe hindrance of the Sbm pathway with a high succinate accumulation and no production of propionate and 1-propanol. The complete inactivation of the Sbm pathway arising from gldA overexpression can be associated with the resulting toxicity or inhibition of the key enzymes (e.g. Sbm or YgfG) though no molecular or biochemical evidence has been documented.
Chapter 6 - Conclusions and Recommendations

While biological platforms for propionate production have been limited to anaerobic native microbial producers, such as *Propionibacterium* and *Clostridium*, the alternative approach of using engineered *E. coli* cultivated under microaerobic conditions is an effective strategy for high-level microbial production of propionate. Glycerol serves as a more desirable carbon source due to the reduced acetate overflow effect as well as the apparent increased activity of the Sbm operon during glycerol cultivation. The introduction of microaerobic cultivation conditions improves culture performance; allowing for enhanced acidogenesis and heightened propionate titres, particularly with glycerol as a carbon source. Furthermore, the results suggest that microaerobically, the respiratory and fermentative glycerol dissimilation pathways are complementary in the engineered strain. Knocking out genes involved in glycerol dissimilation (except *glpA*) can improve glycerol efficiency with less carbon loss and a higher biomass yield, in addition, these knockouts minimize solventogenesis and shift more dissimilated carbon flux toward the C3-fermentative pathway. Upon suppressed solventogenesis with minimal production of highly reduced alcohols, the alternative NADH-consuming route associated with propionate synthesis can be critical for more flexible redox balancing. The comparative analysis presented in this work suggests that glycerol should be preferentially channeled through the respiratory pathway of glycerol dissimilation for effective propionate production as inactivation of the fermentative pathway of glycerol dissimilation resulted in improved propionate titres, up to ~11 g/L. Studies related to overexpression of the genes of the respiratory glycerol dissimilation pathway confirm that
the respiratory pathway is important for glycerol dissimilation to propionate, though inactivation of the alternative pathway is more effective for high-level propionate production.

The derivation of various propionogenic *E. coli* strains in this study has also offered a unique opportunity for investigating “flux competition” behaviors between the C2 and C3-fermentative pathways under different genetic backgrounds and cultivation conditions. The biochemical grounds associated with the preferential carbon flux channeling through the respiratory pathway warrant in-depth exploration. As previously discussed, we hypothesize the reason for heightened propionate production via the respiratory pathway is related to preservation of a high PEP pool as the phosphorylation of glycerol to G3P is not PEP-dependent in contrast to the phosphorylation of DHA to DHAP by PtsI/HPr/DhaKLM. Further investigations related to the availability of PEP and its significance to propionate production are recommended. Overexpression of *ppc*, phosphoenolpyruvate carboxylase, the enzyme which catalyzes the carboxylation of PEP to OAA or alternatively *pyc*, pyruvate carboxylase, the enzyme which catalyzes the carboxylation of pyruvate to OAA can be explored using various background strains of this study. Specifically, overexpression of these genes should stimulate the C3-fermentative pathway and result in reduced acetate and ethanol production particularly when the fermentative pathway is disabled and the PEP pool is theoretically highest.

Strategies for strain engineering should be developed toward reducing production of acetate, the most abundant byproduct, though knocking out genes involved in the fermentative pathway has been shown effective in increasing the propionate:acetate ratio in this work. Some recommended strategies include targeted knockouts of the genes encoding
phosphotransacetylase (pta), acetate kinase (ackA) or pyruvate oxidase (poxB) which have all been successful means of reducing acetate production in E. coli. Alternatively, reducing the acetyl-CoA pool by inactivation of pyruvate kinase (pykFA), pyruvate formate lyase (pflB), or the pyruvate dehydrogenase complex (aceEF/lpdA) can augment C3-fermentative pathway product formation by obstructing production of C2-fermentative products.

To identify the link between the glycerol dissimilation pathways and the propionate-producing Sbm pathway, full transcriptome analysis, by means of DNA microarrays, is recommended. Analyses of the relative changes in gene expression of thousands of gene at the whole-cell level, under various cultivation conditions and after genetic manipulations, has the potential to identify key conversion bottlenecks in the pathway, and provide metabolic context in order to develop a more comprehensive understanding of the engineered network.

Finally, the high propionyl-CoA pool which was achieved via the various biochemical, genetic and metabolic engineering strategies of this work presents an opportunity to harness propionyl-CoA metabolism for production of other industrially relevant chemicals in E. coli. Further strain engineering can lend itself to the creation of competent production platforms for a variety of target products using propionyl-CoA as an intermediate. Examples include 3-hydroxypropionate, 2-hydroxybutyrate, methylethyl ketone, and 2-butanol.
References


of *Escherichia coli* W3110 for the conversion of sugar to redox-neutral and oxidized products: Homoacetate production. PNAS. 100, 825-832.


encoding anaerobic glycerol-3-phosphate dehydrogenase and the GlpD operon encoding aerobic glycerol-3-phosphate dehydrogenase in *Escherichia coli*: Further characterization of respiratory control. J Bacteriol. 172, 179-84.


Supplementary Figure 1: Schematic representation of the genomic engineering method used to generate SbmCTRL and all derived strains. In order to activate the naturally silent Sbm operon with the strong promoter (P_{trc}), the FRT-CmR-FRT-P_{trc} fragment was PCR amplified using the primer set of r-frt:ptrc with homology extensions (H1 and H2). Next, λ-Red-mediated recombination was used to replace the unessential 204-bp region upstream of the operon (between the H1 and H2 sites). The primer set of v-frt:ptrc was used to PCR-verify the genotype of SbmCTRL, with the wildtype junction being 606 bps, and the mutant junction being 1658 bps, and the markerless mutant junction (with Cm^{R} excised) being 998 bps. Note: genes and regulatory elements [operator (O), terminator (T) and ribosome binding site (RBS)] are not to scale.
Appendix B

Supplementary Figure 2: Sample chromatogram of a sample drawn at the midpoint of microaerobic glycerol cultivation. Analysis was performed using the Clarity Lite Chromatographic Station software.
Appendix C

Supplementary Figure 3: Sample metabolite calibration curve. The linear relationship between peak size (area) and propionate concentration is shown with an $R^2$ value of 0.9998.
Appendix D-1

Supplementary Figure 4: Time profiles of glycerol/glucose, biomass, and major metabolites during batch cultivation of SbmCTRL under (A) anaerobic conditions using glucose as a carbon source, (B) anaerobic conditions using glycerol as a carbon source, (C) microaerobic conditions using glucose as a carbon source, (D) microaerobic conditions using glycerol as a carbon source.
Supplementary Figure 5: Time profiles of glycerol, biomass, and major metabolites during microaerobic batch cultivation of (A) Sbm-ΔglpK (B) Sbm-ΔglpD and (C) Sbm-ΔglpA.
Supplementary Figure 6: Time profiles of glycerol, biomass, and major metabolites during microaerobic batch cultivation of (A) Sbm-ΔgldA (B) Sbm-ΔdhaK and (C) Sbm-ΔptsI.
Supplementary Figure 7: Time profiles of glycerol, biomass, and major metabolites during microaerobic batch cultivation of (A) Sbm-glPK (B) Sbm-glPD and (C) Sbm-glPA.
Appendix D-5

Supplementary Figure 8: Time profiles of glycerol, biomass, and major metabolites during microaerobic batch cultivation of (A) Sbm-gldA (B) Sbm-dhaK and (C) Sbm-ptsI.