Fermentation study of metabolically engineered *Escherichia coli* strains for high-level 1-propanol production

by

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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.

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Abstract

As the current global energy requirements are mainly met through the combustion of petroleum, coal, and natural gas, concerns over the global warming and the sustainability of the future exploitation of fossil fuels are growing. 1-propanol of which carbon chain is longer than the ethanol could be a better biofuel for blending with gasoline. Previously in our lab, a novel alternative 1-propanol biosynthesis pathway was constructed by introducing the sleeping beauty mutase (Sbm) operon into the native *E.coli*. During the anaerobic cultivation of a metabolically engineered *E.coli* strain in a shake flask, approximately 150 mg/L of 1-propanol was produced using glucose as the main carbon source. In this study, we demonstrated high-level production of both 1-propanol and ethanol by fermenting our novel metabolically engineered *E. coli* strains anaerobically with glycerol/glucose as the main carbon source. In a batch fermentation of a wild type *E. coli* stain harboring a low copy number plasmid with an active Sbm operon, low concentration of 1-propanol was detected in the culture media. By knocking out the gene involved in the major carbon flux competing pathway (*ldhA*), the titer of 1-propanol was increased by four-fold. Due to glucose's limited NADH-generating capacity, glycerol, which is a more reduced compound, was used as the main carbon source, and the 1-propanol titer reached 2.15 g/L and 4.12 g/L from 30 g/L and 87 g/L of glycerol, respectively. Further enhancement in the 1-propanol production was achieved by constructing a plasmid-free propanogenic *E. coli* strain that had a faster glycerol dissimilation rate and higher 1-propanol yield. In summary, 7.52 g/L and 35.66 g/L of 1-propanol and ethanol, respectively, was produced from 116 g/L of glycerol in a fed-batch cultivation of the plasmid-free propanogenic *E. coli* strain.

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Table of Contents

Author's declaration ii

Abstract iii

Acknowledgements iv

Table of Contents v

List of Figures vii

List of Tables viii

Chapter 1 Overview 1

1.1 Research backgrounds 1

1.2 Research objectives 6

1.3 Outline of thesis 7

Chapter 2 Literature Review 8

2.1 Biomanufacturing of biofuels 8

2.2 Microorganisms for bioethanol production 13

2.3 Metabolic engineering toward 1-propanol production 17

2.4 The sleeping beauty mutase pathway 22

Chapter 3 Materials and Methods 24

3.1 Bacterial strains, plasmids, and primers 24

3.2 Media and cultivation conditions 28

3.3 HPLC analysis 29

3.4 Calculations 30

Chapter 4 Results 32

4.1 Characterization of 1-propanol production under anaerobic conditions using glucose as the main carbon source 32

4.2 Characterization of 1-propanol production under anaerobic conditions using glycerol as the main carbon source 38

4.3 Fed-batch cultivation for 1-propanol production using glycerol as the main carbon source 42

4.4 Fed-batch cultivation of plasmid-free propanogenic *Escherichia coli* strain for 1-propanol production 46

Chapter 5 Discussion 52

Chapter 6 Conclusions and Recommendations 58

References 61

Appendix A 75

List of Figures

Figure 1: The U.S. annual energy flow chart, 2014 2

Figure 2: Chemical structure of 1-propanol 3

Figure 3: Transesterification of triglyceride with alcohol 12

Figure 4: Ethanol production pathways in microorganisms 16

Figure 5: Cyclical *E. coli* sleeping beauty mutase pathway 23

Figure 6: Time profiles of glucose, biomass, and major metabolites during batch cultivation of A. CPC-CNTRL1 and B. CPC-PrOH1 with glucose as the major carbon source 36

Figure 7: Time profiles of glucose, biomass, and major metabolites during batch cultivation of A. CPC-CNTRL2 and B. CPC-PrOH2 with glucose as the major carbon source 37

Figure 8: Time profiles of glycerol, biomass, and major metabolites during batch cultivation of A. CPC-CNTRL2 and B. CPC-PrOH2 with glycerol as the main carbon source 41

Figure 9: Time profile of glycerol, biomass, and major metabolites during fed-batch cultivation of CPC-PrOH2 with glycerol as the major carbon source 45

Figure 10: Time profile of glycerol, biomass, and major metabolites during fed-batch cultivation of CPC-PrOH3 with glycerol as the major carbon source 51

List of Tables

Table 1: U.S. Renewable Energy Consumption in 2014 3

Table 2: 2014 global ethanol production by country 9

Table 3: Assessment of selected pretreatment processes 11

Table 4: Fermentation performance of industrial xylose-fermenting *S. cerevisiae* strains in lignocellulose hydrolysates 14

Table 5: Fuels energy density and average octane number 18

Table 6: Fermentation performance of metabolically engineered microorganisms 21

Table 7: *E. coli* strains, plasmids, and primers used in this study 26

Table 8: Pathway equations and the theoretical yield of intermediates and final metabolites 30

Table 9: Theoretical yield of final metabolites from glucose and glycerol 31

Table 10: Fermentation profiles of the strain CPC-PrOH1, CPC-CNTRL1, CPC-PrOH2, and CPC- CNTRL2 cultured on glucose as the main carbon source 35

Table 11: Fermentation profiles of the strain CPC-PrOH2 and CPC-CNTRL2 cultured on glycerol as the main carbon source 40

Table 12: Fed-batch fermentation profiles of the strain CPC-PrOH2 cultured on glycerol as the main carbon source 44

Table 13: Fed-batch fermentation profiles of the strain CPC-PrOH3 cultured on glycerol as the main carbon source 49

Chapter 1 Overview

1.1 Research backgrounds

As the current global energy requirements are mainly met through the combustion of petroleum, coal, and natural gas, concerns over the global warming and the sustainability of the future exploitation of fossil fuels are growing. Therefore, an alternative, environmentally friendly and sustainable energy source, biofuels, defined as fuels produced from biological raw materials, has gained significant public and research interest over the past decade (Schubert, 2006; Srirangan et al., 2012). In 2014, approximately 9.8% of total energy consumed in the United States (representing ~9.6 quadrillion Btu's of energy) was derived from the renewable energy, whereas fossil fuels supplied over 80% of total energy consumed in the United States (representing ~80.2 quadrillion Btu's of energy) (Figure 1). Among the renewable energy sources, biofuels provided ~25% of total renewable energy consumed in the United States, representing ~2.1 quadrillion Btu's of energy (Table 1). It is important to note that there are only two types of biofuels that have been large-scale commercialized, bioethanol and biodiesel, and bioethanol is the predominant one which supplied almost 85% of total biofuel-derived energy consumed in the United States in 2014 (The United States Energy Information Administration [EIA], 2014). In terms of the application, vast majority of bioethanol produced were used as the transportation fuel either by blending with gasoline or by itself, whereas only ~1.5% of bioethanol were used for heat and electricity generation in the United States in 2014 (EIA, 2014). Although by adding bioethanol into the motor gasoline could help reducing the emissions of greenhouse gases (e.g., carbon dioxide), extra cost on the maintenance of the vehicle fuel system and fuel infrastructures may be incurred due to some unfavorable properties of bioethanol, such as corrosiveness and high hygroscopicity (Connor & Liao, 2009).

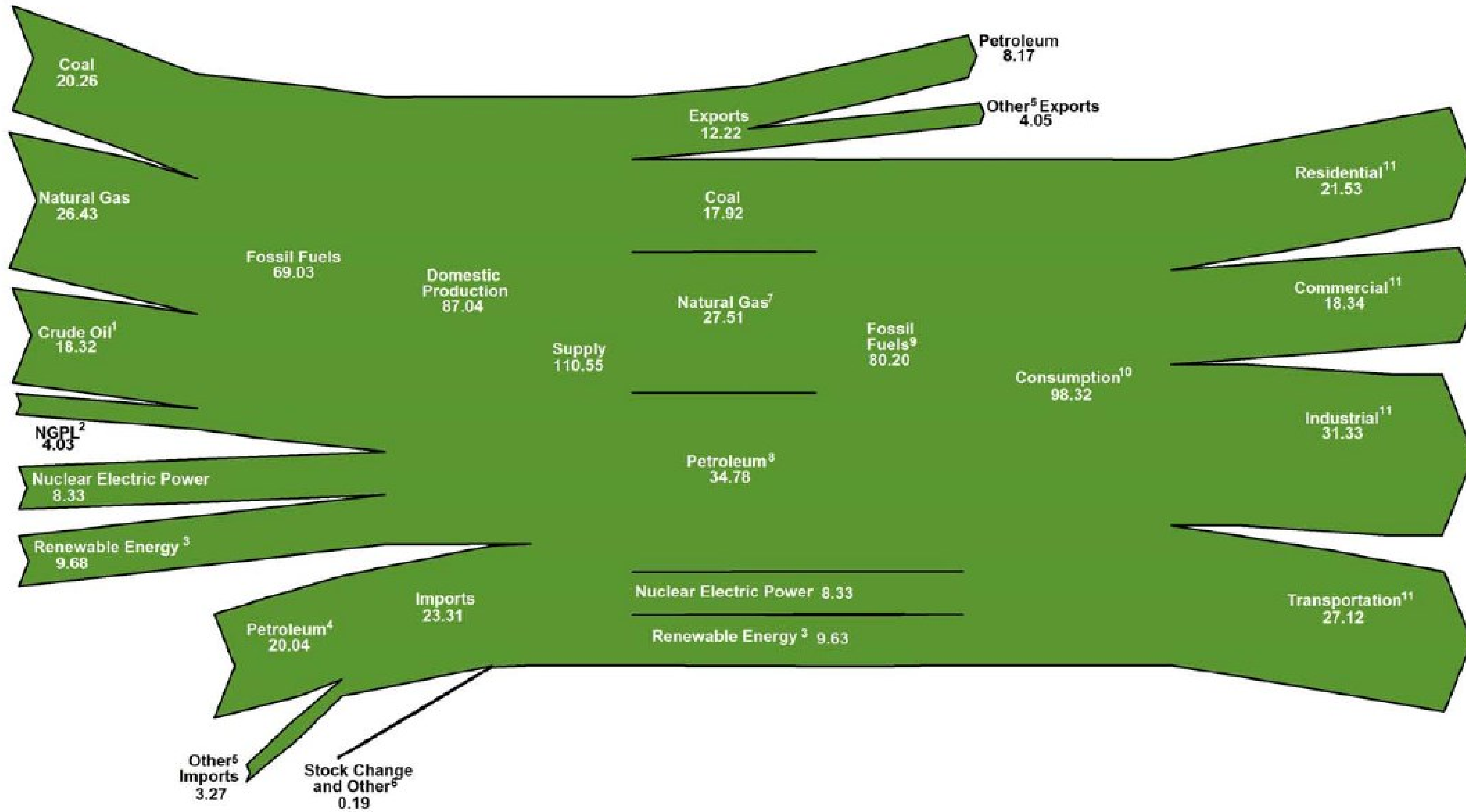


Figure 1: The U.S. annual energy flow chart, 2014

Note. Adapted from "Annual Energy Review," by The U.S. Energy Information Administration, 2014, retrieved from http://www.eia.gov-/totalenergy/data/monthly/pdf/flow/total_energy.pdf

Table 1: U.S. Renewable Energy Consumption in 2014

Renewable Source	U.S. consumption (QBtu)	Yearly Change (%)
Hydroelectric	2.469	-3.6
Geothermal	0.222	+3.7
Solar / PV	0.427	+40.0
Wind	1.734	+8.3
Wood	2.214	+2.0
Waste	0.488	-1.6
Biofuels	2.068	+3.0
Total	9.622	+2.8

Note. Adapted from "Annual Energy Review," by The U.S. Energy Information Administration, 2014, retrieved from http://www.eia.gov/totalenergy/data/monthly/pdf/sec10_3.pdf

Therefore, 1-propanol ($CH_3CH_2CH_2OH$), a 3-carbon alcohol (Figure 2), could be an alternative biofuel which is even better than the bioethanol, since it contains a higher energy density and is less hygroscopic than the ethanol (Connor & Liao, 2009). In addition, 1-propanol is a popular chemical compound that has been widely used in the industrial sector either as solvents in producing commercial products, such as dyes, paints, cosmetics, and pesticides, or as an intermediate for production of other organic derivatives, such as propylene (Ammar & Wang, 2013). However, currently no natural microorganism has been found capable of producing 1-propanol in large quantities, the industrial-scale manufacturing of 1-propanol mainly relies on chemical synthesis; one popular method is hydroformylation of ethene to propionaldehyde followed by hydrogenation of propionaldehyde to 1-propanol. (Shen & Liao, 2008; Srirangan et al., 2013; Kirk & Othmer, 1978-1984).

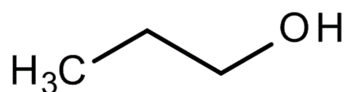


Figure 2: Chemical structure of 1-propanol

Recently, efforts have been made toward developing microbial strains that can produce 1-propanol from renewable biomass through the metabolic engineering approach. It was first reported by Shen and Liao (2008) that 1-propanol production in *Escherichia coli (E.coli)* using glucose as the carbon source was achieved via the microorganism's native amino-acid (threonine) biosynthesis pathway by introducing the promiscuous 2-ketoacid decarboxylase (encoded by *kivd*) from *Lactococcus lactis* (Smit et al., 2005) and alcohol dehydrogenase 2 (encoded by *ADH2*) from *Saccharomyces cerevisiae* into metabolic engineered *E. coli* strains. Later that year, a higher level of 1-propanol production was reported by Atsumi and Liao (2008), as they engineered an alternative route in *E.coli* to produce 2-ketobutyrate, the precursor of 1-propanol production, by introducing an evolved *Methanococcus jannaschii* citramalate synthase (encoded by *cimA*) into the metabolic engineered *E. coli* strains. Choi et al. (2012) combined both threonine pathway and citramalate pathway to increase the 2-ketobutyrate pool by overexpressing the *cimA* gene in a metabolic engineered threonine-overproducing *E.coli* strain, and by overexpressing the *ackA* gene (encoding acetate kinase A) and a mutant *adhE* gene (encoding an aerobically functional alcohol/aldehyde dehydrogenase) in the resulting strain, 2-ketobutyrate was converted into 1-propanol under aerobic conditions, which allowed high-level production of 1-propanol using either glucose or glycerol as the main carbon source. On the other hand, Jain and Yan (2011) developed a new pathway for 1-propanol production in *E.coli* by channeling the carbon flux from glycolysis pathway toward the 1, 2-propanediol synthesis pathway which was further expanded by two additional enzymatic reactions catalyzed by 1, 2-propanediol dehydratase (encoded by *ppdABC* from *Klebsiella oxytoca*) and alcohol dehydrogenase (encoded by *adhE* from *E.coli*) to convert 1, 2-propanediol into 1-propanol. Although *E.coli* is the most popular bacterial host for genetic manipulation, metabolic engineering, and biomanufacturing owing to its manifold advantages, the

production of 1-propanol in other microorganisms, such as *Propionibacterium ferudenreichii, Thermobifida fusca, Shimwellia blattae,* and *Corynebacterium glutamicum,* has also been demonstrated (Ammar et al., 2013; Deng & Fong, 2011; Urano et al., 2015; Siebert & Wendisch, 2015).

Previously in our lab, a novel alternative 1-propanol biosynthesis pathway was constructed by introducing the sleeping beauty mutase (Sbm) operon (*sbm-ygfD-ygfG-ygfH*, encoding Sbm: methylmalonyl-CoA mutase, YgfD: Sbm-interacting protein kinase, YgfG: methylmalonyl-CoA decarboxylase, and YgfH: propionyl-CoA/succinyl-CoA transferase, respectively) into *E.coli* (Srirangan et al., 2013). During the anaerobic cultivation of a metabolically engineered *E.coli* strain in a shake flask, approximately 150 mg/L of 1-propanol was produced using glucose as the main carbon source, which demonstrated that the Sbm operon was functionally expressed in the engineered *E.coli* strain (Srirangan et al., 2013). Although the Sbm operon has been identified in the native *E.coli* genome and the function of the enzymes encoded by the Sbm operon have been revealed (Sbm catalyzes the isomerization of succinyl-CoA to L-methylmalonyl-CoA; YgfG catalyzes the decarboxylation of methylmalonyl-CoA to propionyl-CoA; YgfH transfers the coenzyme A from propionyl-CoA to succinate; YgfD has GTPase activity), the metabolic context of this pathway remains unclear, as the operon is thought to be silent or minimal expressed *in vivo* due to an inactive or weak promoter (Haller et al., 2000; Froese et al., 2009; Srirangan et al., 2013).

1.2 Research objectives

The overall objectives of this thesis include:

1. Utilizing a bench-scale bioreactor to characterize the 1-propanol production in metabolic engineered *E.coli* strains using glucose as the main carbon source under anaerobic conditions, and identifying the constraints which limit the 1-propanol production during the fermentations.
2. Using glycerol as the main carbon source for fermentation of metabolic engineered *E.coli* strains under anaerobic conditions in order to enhance the level of solventogenesis and boost the production of 1-propanol.
3. Performing the fed-batch cultivation of metabolic engineered *E.coli* strains using glycerol as the main carbon source under anaerobic conditions to further characterize the strains' 1-propanol production capacity by utilizing the Sbm pathway.

1.3 Outline of thesis

Chapter 2 reviews (1) the overall biofuel market, especially the bioethanol and biodiesel market, (2) microorganism that have been widely studied and used for laboratory or industrial bioethanol production from a variety of carbon sources, (3) current metabolic and genetic engineering strategies that have been applied to produce 1-propanol in a variety of microorganisms, (4) the identification and characterization of the sleeping beauty mutase (Sbm) pathway and also the *E. coli* genes that are involved in the Sbm pathway. Chapter 3 contains all the materials (e.g., bacterial strains, plasmids, primers, chemical reagents, medium, and instruments) and methods (e.g., bacterial strains construction, plasmids construction, cultivation, fermentation, HPLC analysis, and calculations) that have been used in this study. Chapter 4 presents all the results obtained in this study, including: (1) characterization of 1-propanol production under anaerobic conditions using glucose as the main carbon source, (2) characterization of 1-propanol production under anaerobic conditions using glycerol as the main carbon source, and (3) fed-batch cultivation for 1-propanol production using glycerol as the main carbon source. In Chapter 5, the results presented in Chapter 4 are discussed in detail. Finally, the conclusions of the study and the recommendations for future works are summarized in Chapter 6.

Chapter 2 Literature Review

2.1 Biomanufacturing of biofuels

Bioethanol, which is the ethanol (CH_3CH_2OH) produced from biological materials, received broad interest from the public in the late 20th century when the over-use of fossil fuels caused pollutions, along with a surge in crude oil price, though it has been produced by human since early humanity history as a pivotal ingredient in alcoholic beverages. In the middle 20th century, ethanol was manufactured in large-scale by hydration of ethylene, a petrochemical derived from petroleum by steam cracking (Okafor, 2007). However, after the crude petroleum price shot up, fermentation of biomass (e.g., sugar cane in Brazil or corn in the U.S.) became the dominant way to produce ethanol, which was named as a clean and renewable energy source (Okafor, 2007). Interestingly, ethanol was used as motor fuel in early 20th century but was soon replaced by the cheap petroleum-derived gasoline when there was a boom in the petroleum industry (Mills & Ecklund, 1987). Thus, the upsurge of ethanol production in the late 20th century was most likely due to the fluctuation of the petroleum price, which was not completely controlled by the governments, especially those oil importation countries, and therefore, world governments such as Brazil, the United States, and some Europe countries initiated incentives for the bioethanol production and sales in order to make the price of biofuel more competitive and at the mean time to conserve petroleum resources (Mills & Ecklund, 1987; Okafor, 2007). Thereafter, the bioethanol industry grows in a fast pace and has become a stable, sustainable, and momentous driving force behind the world economic prosperity. In 2014, the global fuel ethanol production reached approximately 24,570 million gallons, the highest level for the past decade, and the United States remained to be the largest ethanol fuel production country with 14,300 million gallons, which accounted for approximately 58% of total global output (Table 2). In the United States, the feedstock for bioethanol production is primarily

derived from corn, which is the most common grain crop grown in the Americas, and certain species of the yeast (e.g., *Saccharomyces cerevisiae*) is used as the workhorse of the biomass fermentation for bioethanol production, owing to its high ethanol productivity and high ethanol tolerance. Since only sugars (e.g., glucose) could be directly consumed by the yeast, feedstock pre-treatments (e.g., milling, liquefaction, and saccharification) are required to extract the starch from corn and then break down the starch into fermentable sugars (Onuki et al., 2008). The second large ethanol production country in the world is Brazil, whose production reached 6,190 million gallons in 2014, accounting for a 25% of global ethanol production (Table 2). Since the country is the worlds' biggest sugarcane producer, the feedstock used for ethanol production in Brazil is mainly derived from this domestically grown sucrose-rich crop. The ethanol production by the other countries in 2014 are also summarized in Table 2.

Table 2: 2014 Global ethanol production by country

Country	Fuel ethanol production (million gallons)	Share of global production (%)
The United States	14,300	58%
Brazil	6,190	25%
Europe	1,445	6%
China	635	3%
Canada	510	2%
Thailand	310	1%
Argentina	160	1%
India	155	1%
Rest of world	865	3%

Note. Adapted from "2015 Ethanol Industry Outlook", by Renewable Fuels Association, 2015, retrieved from http://ethanolrfa.3cdn.net/c5088b8e8e6b427bb3_cwm626ws2.pdf

Other than the corn and sugarcane, bioethanol could be produced from various crops, including sugar beet, sugar sorghum, potato, sweet potato, cassava, and wheat (Power et al., 2008; Billa et al., 1997; Quintero et al., 2008; Sree et al., 1999; Akihiko et al., 2009; Murphy & Power, 2008). These crops could be easily fermented by the yeast without too complicated pretreatments, as they are rich in either starch or sucrose. Currently, most of bioethanol plants are using these food-related sugar-based crops as the fermentation feedstock, which is often refereed as the first generation of bioethanol production, and the selection of feedstock is primarily based on the regional availability and economical efficiency (Baeyens et al., 2015; Onuki et al., 2008). However, there is always controversy over using food-related crops to produce fuel ethanol, as people are concerned that this will rise the food prices as a result of the direct competition between fuel ethanol production and food production. Not only does farming food-related crops for bioethanol production occupy the agricultural lands which are originally used for food production, but also it requires large amount of water resources and fertilizer. Therefore, the second generation of bioethanol production, which uses lignocellulosic raw materials (e.g., corn straw, sugarcane bagasse, and switchgrass) as the feedstock, has received tremendous attention from the public, bio-fuel company, and even government (Slade et al., 2009). Some advantages of the second generation of bioethanol production are listed below: (1) lignocellulosic raw materials are not food-related, (2) lignocellulosic raw materials are cheap and abundant, as they could be obtained from unwanted agricultural waste, and (3) using lignocellulosic raw materials as the feedstock could lead to more CO_2 emission reduction than using food-related crops as the feedstock (Balat, 2011; Li et al., 2010). Nevertheless, there has been a difficulty in commercialization of the second generation of bioethanol production in the past few decades. Although the main process of the second generation of bioethanol production (converting sugars into bioethanol through microbial

fermentation) is similar to that of the first generation of bioethanol production, the pretreatment of lignocellulosic materials is much more complicated and expensive (Balat, 2011). Lignocellulosic materials are mainly composed of three basic biopolymers: cellulose, hemicellulose, and lignin, whereas only cellulose and hemicellulose could be hydrolyzed into fermentable sugars, such as glucose, xylose, and arabinose (Buruiana et al., 2013). Due to the rigid structure of lignocellulose, cellulose and hemicellulose could not be directly hydrolyzed by the enzymes, and therefore, lignocellulosic materials must undergo a thorough delignification process in the first place to release the cellulose and hemicellulose from the lignin-carbohydrate complexes (LCC) for hydrolysis (Buruiana et al., 2013; Nagy, 2009). Table 3 lists a few selected pretreatment processes with a general assessment for each of them. It is important to note that the effectiveness of each pretreatment process could be affected by the type of lignocellulose used, for example, steam explosion is more effective for hardwood but less effective for softwood (Balat, 2011).

Table 3: Assessment of selected pretreatment processes

Pretreatment process	Yield of fermentable sugars	Wastes	Investment
Physical or physic-chemical			
Mechanical	Low	Very low	Low
Steam explosion	High	Low	High
Ammonia fiber explosion (AFEX)	Moderate	Very low	High
Carbonic acid	Very high	Very low	Low
Chemical			
Dilute acid	Very high	High	Moderate
Concentrated acid	Very high	High	High
Alkaline extraction	Very high	High	Low
Wet oxidation	High	Low	Low
Organosolv	Very high	Low	Very high

Note. Adapted from Baeyens et al. (2015)

While bioethanol is dominating the biofuel market, biodiesel, which is also known as the fatty acid methyl esters (FAME), has its own niche in the market. In the United States, 1.63 billion gallons of biodiesel was produced in 2014, and an annual increase of 0.07 billion gallons, 0.1 billion gallons, and 0.1 billion gallons in biodiesel production is expected for years 2015, 2016, and 2017, respectively (The United States Environmental Protection Agency [EPA], 2015). Unlike the bioethanol production which relies on microbial fermentation, biodiesel is mainly produced by a chemical reaction, namely transesterification (Connor, 2010). During the transesterification reaction, fats (e.g., triglyceride) are converted into esters (biodiesel) by reacting with alcohols (e.g., methanol and ethanol) in the presence of a catalyst, with glycerol being produced as a side-product (Connor, 2010). Figure 3 shows a general chemical equation of the transesterification for biodiesel production. The triglycerides used for the biodiesel production are mainly derived from vegetable oils and animal fats. One major advantage of the biodiesel is that the waste vegetable oil (WVO) which is a side-product generated from food industry could be recycled and used as the feedstock for biodiesel production (Nagy, 2009). Pure plant oils (PPO) such as virgin oil, rapeseed, and soybean oil could also be used for biodiesel production and they are currently the major source of the feedstock. However, since pure plant oils are food-related and their production are limited by the agricultural capacity of a given country, the production of biodiesel is also limited, and thus new feedstocks and technologies need to be explored in order to increase the global biodiesel production capacity (Nagy, 2009).

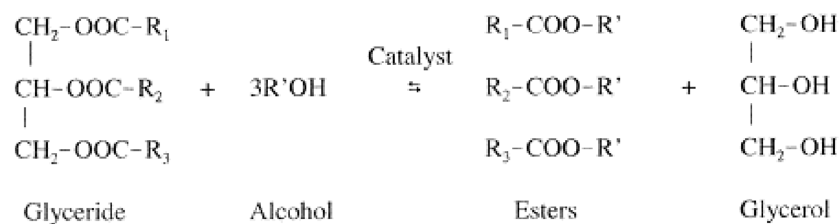


Figure 3: Transesterification of triglyceride with alcohol
Note. Adapted from Ma & Hanna (1999)

2.2 Microorganisms for bioethanol production

There is a long history of the ethanol production through fermentation process in human history, and the brewer's yeast, *Saccharomyces cerevisiae,* is the most popular workhorse for the industrial ethanol production (Okafor, 2007). In order to reduce the overall cost of ethanol production, the microorganism selected for ethanol production should possess the following characteristics: (1) high ethanol yield (greater than 90% of theoretical yield), (2) high ethanol tolerance (more than 40 g/L of ethanol), (3) high ethanol productivity (greater than 1 g L^{-1} h^{-1}), (4) robust growth rate and low growth requirements, (5) resistance to hydrolysate inhibitors, and (6) broad pH and temperature range for cultivation (Dien et al., 2003). Although the current ethanol industry relies heavily on the first generation of ethanol production, a large portion of research efforts in the past few decades have been put on fermenting lignocellulosic materials, the feedstock for second generation of ethanol production. Since the native *S. cerevisiae* is not capable of utilizing pentose sugars (mostly xylose), which is the second most abundant sugars contained in the hydrolysate of lignocellulosic materials, metabolic and genetic engineering strategies are employed to enable the assimilation of pentose sugars in native *S. cerevisiae* (Tantirungkij et al., 1994; Jin, 2002; Wahlbom et al., 2003; Kim, 2007; Cann, 2010; Du, 2011; Hector et al., 2011; Kuhad et al., 2011). However, most of the pentose fermentation study are based on laboratory *S. cerevisiae* strains, which are not suitable for the industrial-scale ethanol production due to a lack of robustness and hydrolysates tolerance compared to the industrial *S. cerevisiae* strains (Sonderegger et al., 2004; Hahn-Hägerdal et al., 2007). Table 4 summarizes the fermentation performance of selected industrial xylose-fermenting *S. cerevisiae* strains in lignocellulose hydrolysates.

Table 4: Fermentation performance of industrial xylose-fermenting *S. cerevisiae* strains in lignocellulose hydrolysates

Strain	Hydrolysate	Detoxification	Setup	Ethanol productivity (g/[g cells·h])	Ethanol yield on total sugar (g/g)	Reference
TMB 3400	Spruce	Nondetoxified	Fed-batch	0.25	0.43	(Hahn-Hägerdal & Pamment, 2004)
TMB 3006	Spruce	Nondetoxified	Fed-batch	0.66	0.37	(Hahn-Hägerdal & Pamment, 2004)
424A (LNH-ST)	Corn stover	Overliming	Batch	-	0.41	(Sedlak & Ho, 2004)
Adapted TMB 3006	Northern spruce 70%	Nondetoxified	Continuous	-	0.41 (on glucose)	(Hahn-Hägerdal et al., 2005)
MT8-1/Xyl/BGL	Wood chip hydrolysate	Overliming	Batch	0.42	0.41	(Katahira et al., 2006)
F 12	Still bottoms fermentation residue (vinasse)	Not known	Batch	0.005-0.24	0.27	(Olsson et al., 2006)
TMB 3400	Corn stover, steam pretreatment	Nondetoxified	Batch SSF	-	0.32	(Öhgren et al., 2006)
TMB 3400	Corn stover, steam pretreatment	Nondetoxified	Fed-batch SSF	-	0.30	(Öhgren et al., 2006)

Note. Adapted from Hahn-Hägerdal et al. (2007)

On the other hand, efforts have been put on identifying native xylose-fermenting yeasts that are capable of producing ethanol as the major end-product, such as *Pachysolen tannophilus*, *Candida shehatae*, *Pichia stipites, and Spathaspora passalidarum* (Jeffries & Kurtzman, 2004; Kuhad et al., 2011; Harner et al., 2015). However, these yeasts normally have a poor fermentation performance (i.e., low ethanol yield) when growing on industrial substrates (e.g., pretreated biomass hydrolysate of lignocellulosic materials) as a result of the competition between xylose and hexoses sugars (e.g., glucose and mannose) utilization and low tolerance to pH, ethanol, and hydrolysate inhibitors (Hahn-Hägerdal et al., 2007).

Except for yeasts, microorganisms such as *Escherichia coli, Zymomonas mobilis,* and Filamentous fungi have also been extensively studied for their xylose-fermenting capacity, whereas *E. coli* is the most promising one (Kuhad et al., 2011; Kim, 2007). Although the native *Z. mobilis* is superior in several aspects, including homo-ethanol production, high ethanol yield and specific productivity, and high ethanol tolerance, it can only ferment a limited types of sugars (glucose, fructose, and fructose) which makes it undesirable for usage in industrial applications (Rogers et al., 2007). On the other hand, *E. coli* has a wide substrate-utilization range, as it is able to ferment both pentose sugars (xylose and arabinose) and hexose sugars (glucose, mannose, galactose, and fructose) (Zaldivar et al., 2001). In addition, owing to its numerous advantageous characteristics such as rapid growth under both aerobic and anaerobic conditions, simple and inexpensive cultivation requirements, well-established genetic manipulation tools and technologies, and clear genetic, proteomic, and metabolic information, *E. coli* has become one of the most popular microorganism in the biomanufacturing industry (Chang et al., 1999; Cirino et al., 2006; Yu et al., 2011). However, one shortage for *E. coli* is that ethanol is not the main fermentation product in the native *E. coli*, so that it is not as effective as other native ethanol

producer such as *Z. mobilis* and *S. cerevisiae*. While *Z. mobilis* and *S. cerevisiae* can convert pyruvate into ethanol with a consumption of only one NADH per each ethanol produced by utilizing pyruvate decarboxylase (PDC), native *E. coli* produces ethanol through the mixed-acid fermentation pathway which requires two NADH for each ethanol produced (Figure 4) (Dien et al., 2003). Therefore, the production of ethanol in the native *E.* coli is always coupled with a coproduction of organic acid (acetate) which are less reduced than the ethanol (Dien et al., 2003). By integrating the genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) derived from *Zymomonas mobilis* into the chromosomal DNA of *E. coli* with a fumarate reductase (encoded by *ftd*) mutation, an approximately 100% theoretical yield of ethanol production was achieved in the resulting *E. coli* KO11 strain by using either glucose (100 g/L) or xylose (80 g/L) as the main carbon source, with a final ethanol yield of 52.8 g/L and 41.6 g/L, respectively (Ohta et al., 1991)

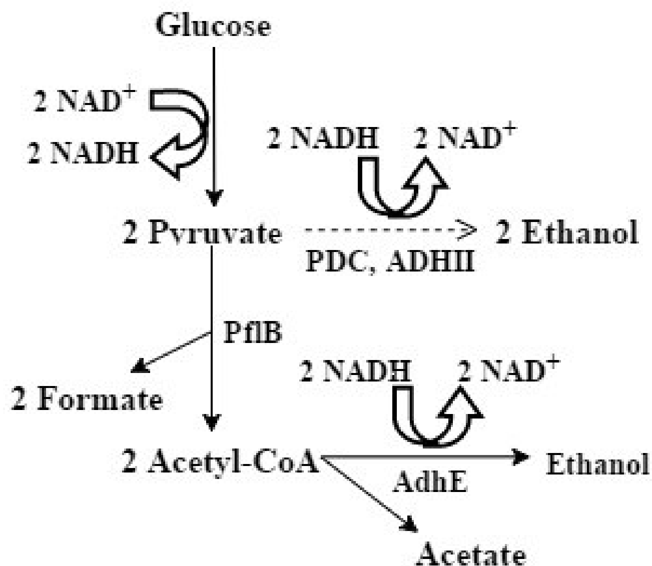


Figure 4: Ethanol production pathways in microorganisms

The broken line shows the ethanol production pathway in *Z. mobilis* and *S. cerevisiae* and the real line shows the ethanol production pathway in *E. coli.* The enzymes involved in the pathways are: pyruvate formate-lyase (PflB), pyruvate decarboxylase (PDC), alcohol dehydrogenase II (ADHII), and aldehyde-alcohol dehydrogenase (AdhE).

2.3 Metabolic engineering toward 1-propanol production

In spite of the advancements and achievements in high-level ethanol production, higher chain alcohol (e.g., 1-propanol, butanol, and isobutanol) remains to be a superior gasoline additive or substitute in terms of the energy density and octane number in contrast to ethanol (Table 5). Among the higher chain alcohols, 1-propanol received limited amount of attention from researchers as no known microorganisms were found capable of producing large quantities of 1-propanol in nature (Shen & Liao, 2008). Eden et al. (2001) detected ~60 mg/L of 1-propanol during the fermentation of yeast and Janssen (2004) reported a production of ~0.46 mg/L of 1-propanol in threonine fermentation of *Clostridium* sp.. Although low level of 1-propanol production has been found in yeast and *Clostridium* sp., the 1-propanol biosynthesis pathway in both microorganisms have not been very well characterized and no further effort has been made to improve the 1-propanol yield. On the other hand, Shen and Liao (2008) constructed a non-native 1-propanol-producing *E. coli* strain by introducing the promiscuous 2-ketoacid decarboxylase (encoded by *kivd*) from *Lactococcus lactis* (Smit et al., 2005) and alcohol dehydrogenase 2 (encoded by *ADH2*) from *Saccharomyces cerevisiae* into a threonine hyper-producing *E. coli* strain. Firstly, the gene *ilvA* (encoding threonine deaminase), *thrA^{fbr}* (encoding a feedback-resistant mutant of aspartate kinase/homoserine dehydrogenase), and *thrBC* (encoding homoserine kinase and threonine synthase) were over-expressed from pSA62 (Atsumi et al., 2008) and pCS49 to direct the metabolic flux towards 2-ketobutyrate, the precursor for 1-propanol production, then by over-expression of *kivd* and *ADH2* from pSA55I, 2-ketobutyrate was converted into 1-propanol (Shen & Liao, 2008). To enhance the production level of 1-propanol, several competing pathways were removed by deleting the gene *metA* (encoding homoserine *O*-succinyltransferase), *tdh* (encoding threonine dehydrogenase), *ilvB* (encoding a large subunit of acetohydroxy acid synthase

I), *ilvI* (encoding a subunit of acetohydroxy acid synthase III), and *adhE* (encoding aldehyde-alcohol dehydrogenase) (Shen & Liao, 2008). The final strain CRS-BuOH 23 (JCL16 Δ*metA,* Δ*tdh,* Δ*ilvB,* Δ*ilvI,* Δ*adhE*/pCS49, pSA62, and pSA55I) produced ~1 g/L of 1-propanol from ~25 g/L of glucose (Shen & Liao, 2008).

Table 5: Fuels energy density and average octane number
AKI - Anti-Knock Index: This octane rating is used in countries like Canada and the United States.
RON - Research Octane Number: This octane rating is used in Australia and most of Europe

Fuel	Energy Density (MJ/L)	Average Octane (AKI rating/RON)
Gasoline	~33	85-96/90-105
Methanol	~16	98.65/108.7
Ethanol	~20	99.5/108.6
Propanol	~24	108/118
Butanol	~30	97/103

Note. Retrieved from http://biofuel.org.uk/bioalcohols.html

In the work presented by Atsumi and Liao (2008), an alternative but shorter 2-ketobutyrate synthesis pathway was constructed by expressing an evolved (*R*)-citramalate synthase (CimA) of *Methanococcus jannaschii* in *E. coli*. The CimA utilizes pyruvate and acetyl-CoA as the substrates to form (*R*)-citramalate which is then converted into 2-ketobutyrate catalyzed by the enzymes LeuBCD (Atsumi & Liao, 2008). The following steps to produce 1-propanol from 2-ketobutyrate are the same as described in the work done by Shen and Liao (2008) where 2-ketoacid decarboxylase (*L. lactis*) and alcohol dehydrogenase 2 (*S. cerevisiae*) were over-expressed in the host cell to carry the reaction. The final strain containing Δ*ilvB* and Δ*ilvI* along with an over-expression of *leuABCD* and the *cimA* mutant in pSA142 and *kivd* and *ADH2* in pSA55 (Atsumi et al., 2008) was able to produce more than 3.5 g/L of 1-propanol from ~20 g/L of glucose (Atsumi & Liao, 2008).

Instead of utilizing keto-acid pathway to produce 1-propanol, Jain and Yan (2011) constructed the 1-propanol biosynthesis pathway by introducing the well-studied 1, 2-propanediol pathway into the native *E. coli*, followed by converting 1, 2-propanediol into 1-propanol through two enzymatic steps catalyzed by the 1, 2-propanediol dehydratase from *Klebsiella oxytoca* and the native *E. coli* alcohol dehydrogenases. However, the final titer of 1-propanol achieved in this study was only ~0.25 g/L which was much lower than those reported by Shen and Liao (2008) and Atsumi and Liao (2008). The main issues with this new 1-propanol synthesis pathway were that the production of 1, 2-propanediol, the precursor of 1-propanol production, was too low (~0.8 g/L without producing 1-propanol) and the conversion of 1, 2-propanediol to 1-propanol was inefficient as approximately 0.46 g/L of 1, 2-propanediol remained unconverted by the end of the fermentation, however, by knocking out genes involved in the competing pathways may help to enhance the production of 1, 2-propanediol and 1-propanol (Jain & Yan, 2011).

Recently, Jain et al. (2015) reported a new study which showed a higher production level of both 1, 2-propandiol and 1-propanol utilizing the same pathway as previously reported. By identifying and over-expressing the optimal minimal set of enzymes for 1, 2-propanediol production, the titer of 1, 2-propanediol reached 1.52 g/L (Jain et al., 2015). To direct more carbon flux toward the 1, 2-propanediol pathway, genes involved in the competing pathways were knocked out, such as *tpiA* (encoding triose phosphate isomerase), *zwf* (encoding glucose 6-phosphate dehydrogenase), *ldhA* (encoding lactate dehydrogenase), *gloA* (encoding glyoxalase I), and *adhE* (encoding alcohol dehydrogenase), which resulted in the production of minimal amount of by-products, however, the 1, 2-propanediol titer was decreased as well (Jain et al., 2015). Further strategies were employed to increase both the NADH availability for 1, 2-propanediol synthesis and the cell growth of the engineered *E. coli* strains, and finally, 5.13 g/L of 1, 2-

propanediol was produced with a yield of 0.48 g/g glucose (Jain et al., 2015). On the basis of this 1, 2-propanediol hyper-producing *E. coli* strain, a production of 2.91 g/L of 1-propanol was achieved by expressing a superior fusion diol dehydratase in the resulting strain and employing a dual strain strategy during the fermentation process (Jain et al., 2015).

Except for *E. coli*, microorganisms like *Propionibacterium ferudenreichii, Thermobifida fusca, Shimwellia blattae,* and *Corynebacterium glutamicum* have also been metabolically or genetically engineered for 1-propanol production (Ammar et al., 2013; Deng & Fong, 2011; Urano et al., 2015; Siebert & Wendisch, 2015). Table 6 summarizes the fermentation performance of several successful metabolically engineered propanogenic microorganisms.

Table 6: Fermentation performance of metabolically engineered microorganisms

Strain	Carbon source (g/L)	Set up	1-propanol Titer (g/L)	1-propanol Yield (g/g)	References
E. coli CRS-BuOH 23	Glucose (~25)	Shake flask	~ 1	~0.04	(Shen & Liao, 2008)
E. coli KS145 (pSA55, pSA142)	Glucose (~20)	Shake flask	3.5	~0.175	(Atsumi & Liao, 2008)
E. coli BW25113 (pRJ11, pYY93)	Glucose	Shake flask	0.25	Unknown	(Jain & Yan, 2011)
Thermobifida fusca B6	Raw switchgrass (20)	Shake flask	0.48	0.06 On glucose	(Deng & Fong, 2011)
E. coli PRO2 (pTacDA-ptac-adhE^{mut}, pBRthrABC-ptac-cimA-ptac-ackA)	Glucose (20)	Batch	1.38	0.069	(Choi et al., 2012)
	Glucose (100)	Fed-batch	10.8	0.107	
	Glycerol (20)	Batch	4.18	0.209	
	Glycerol (40)	Fed-batch	10.3	0.259	
Propionibacterium freudenreichii Pf(adhE)-1	Glucose (20)	Batch	0.32	0.016	(Ammar et al., 2013)
	Glycerol (20)	Batch	0.49	0.0245	
E. coli RJ57 (pRJ70, pRJ58) *E. coli* BW25113 (pRJ79, pRJ58)	Glucose	Shake flask	2.91	Unknown	(Jain et al., 2015)

2.4 The sleeping beauty mutase pathway

The sleeping beauty mutase pathway (Sbm), which is known as the methylmalonyl-CoA mutase (MCM) pathway, plays an important role in the fermentation of native propionate-producing microoraganisms, such as *Propionibacteriaceae shermanii* and *Streptomyces cinnamonensis*, as the redox balance in those microorganisms are maintained by producing propionic acid through the MCM pathway (McKie et al., 1990; Banerjee, 1997). Although the native *E. coli* strain is not capable of producing either propionate or 1-propanol, the *E. coli* genes encoding enzymes that have similar functions as those involved in the MCM pathway have been identified and the enzyme encoded by those genes have been characterized (Haller et al., 2000). In general, three enzymes constitute the *E. coli* sleeping beauty mutase pathway for the conversion of succinyl-CoA to propionate, which are Sbm (methylmalonyl-CoA mutase or sleeping beauty mutase), YgfG (methylmalonyl-CoA decarboxylase), and YgfH (propionyl-CoA: succinyl-CoA transferase) (Figure 5). The first enzyme, Sbm (encoded by *sbm*), catalyzes the conversion of succinyl-CoA to methylmalonyl-CoA in an adenosylcobalamin (coenzyme B12)-dependent manner (Haller et al., 2000). Then the second enzyme, YgfG, which is a member of the crotonase superfamily, catalyzes the conversion of methylmalonyl-CoA to propionyl-CoA through decarboxylation (Haller et al., 2000). Finally, the coenzyme A is transferred from propionyl-CoA to succinate catalyzed by the third enzyme, YgfH, and propionate is produced as the end product while succinyl-CoA is recycled in the pathway (Haller et al., 2000). On the *E. coli* chromosome, all three genes are located in the same operon which is called the sleeping beauty mutase (Sbm) operon, and there is another gene, *ygfD*, which is also in the Sbm operon, however, the role of this gene in the Sbm pathway is not clear (Haller et al., 2000). In a later study reported by Froese et al.

(2009), the enzyme encoded by the *ygfD* was found that it had *in vivo* interaction with the methylmalonyl-CoA mutase and had GTPase activity.

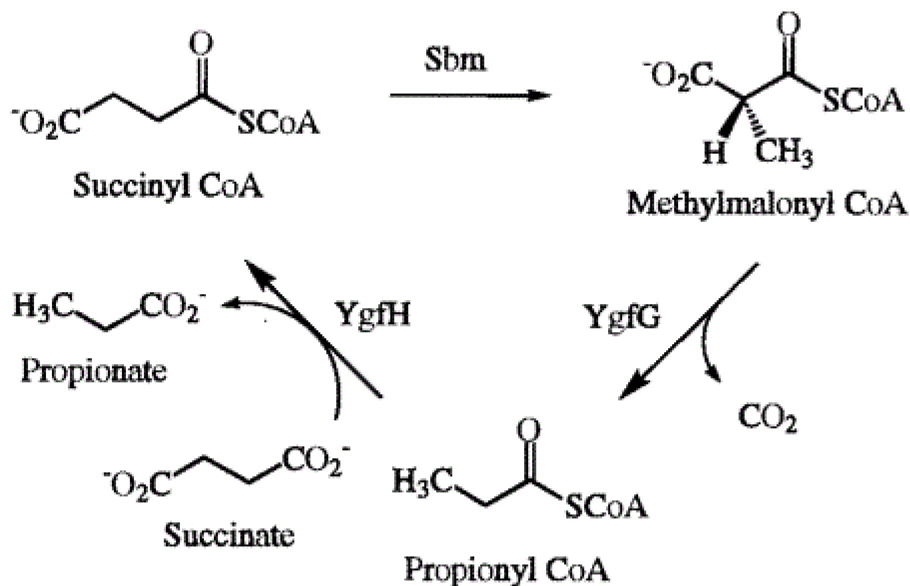


Figure 5: Cyclical *E. coli* sleeping beauty mutase pathway
Note. Adapted from Haller et al. (2000)

Although the function of the enzymes encoded by the sleeping beauty mutase operon have been determined, the metabolic context of the Sbm pathway was unknown as (1) the strain could not use either succinate or propionate as the carbon source with the addition of hydroxocobalamin, (2) the strain does not produce any propionate or 1-propanol when growing on glucose anaerobically (Haller et al., 2000). However, the integrity and functionality of the Sbm operon in the native *E. coli* is evidenced by a detection of the endogenous expression of both Sbm (sleeping beauty mutase) and YgfD in native *E. coli* through SDS-PAGE and immunoblotting (Froese et al., 2009). Therefore, it is hypothesized that the expression level of the Sbm operon in native *E. coli* was too low to form a functional pathway (Froese et al., 2009).

Chapter 3 Materials and Methods

3.1 Bacterial strains, plasmids, and primers

E. coli strains, plasmids and primers used in this study are listed in Table 7. Standard recombinant DNA technologies for molecular cloning were applied (Miller, 1992). *Pfu* and *Taq* DNA polymerases, T4 DNA ligase, and large (Klenow) fragment of DNA Polymerase I were all purchased from New England Biolabs (Ipswich, MA), and all synthesized oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). The Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada) performed all the DNA sequencing of the resulting plasmids.

E. coli BW25141 was used to provide the parental genetic background for 1-propanol production. *E. coli* HST08 was used for molecular cloning purpose. Gene deletions were introduced to BW25141 strains by P1-phage transduction (Miller, 1992) using proper Keio Collection strains (The Coli Genetic Stock Center, Yale University) as donors (Baba et al., 2006). The pCP20 was used to remove the co-transduced Km^R-FRT gene cassette (Datsenko and Wanner, 2000). The genotypes of the resulting knockout strains were confirmed by colony PCR using appropriate primer sets.

A modified λ Red-mediated recombination protocol was used to fuse the strong promoter (P_{trc}) with the Sbm operon in the *E. coli* genome as described by Sukhija et al. (2012). The FRT-Cm^R-FRT cassette was PCR-amplified from pKD3 using the c-frt primer set, whereas the P_{trc} promoter-operator fragment was PCR-amplified from pTrc99a using the c-ptrc primer set. The two DNA fragments were fused by splice overlap extension (SOE) PCR as described by 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 36-bp homology arms of H1 and H2, respectively. To derive the plasmid-free strain of CPC-PrOH3, 0.5 μg of the amplified/purified DNA cassette was electro-transformed, using a Gene Pulser (BioRad Laboratories, Hercules, CA) set at 2.5 kV, 25 μF, and 200 Ω, to WT-Δ*ldhA*-Δ*pykF* harboring the λ-Red recombinase expression plasmid pKD46 for DNA recombination to replace the 204-bp upstream region of the Sbm operon. 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 SOC (super optimal broth with catabolite repression) medium (3.6 g/L glucose, 20 g/L tryptone, 5 g/L yeast extract, 0.6 g/L NaCl, 0.19 g/L KCI, 4.8 g/L $MgSO_4$) (Hanahan, 1983) and recovered 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 plate containing 12 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_{trc} cassette with the Sbm operon was verified by colony PCR using the v-frt:ptrc primer set as well as DNA sequencing.

Table 7: *E. coli* strains, plasmids, and primers used in this study

Name	Description, relevant genotype	Reference
E. coli host strains		
HST08	F-, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ(lacZYA–argF)U169, Δ(mrr – hsdRMS – mcrBC), ΔmcrA, λ –	TaKaRa Bio Inc.
MC4100	F-, [*araD139*]B/r, Del(*argF-lac*)169, λ–-, e14-, *flhD5301*, Δ(*fruK-yeiR*)*725*(*fruA25*), *relA1*, *rpsL150*(strR), *rbsR22*, Del(*fimB-fimE*)*632*(::IS1), *deoC1*	Casadaban, 1976
BW25141	F-, Δ(*araD-araB*)567, Δ*lacZ4787*(::*rrnB-3*), Δ(*phoB-phoR*)*580*, *λ*-, *galU95*, Δ*uidA3*::pir+, *recA1*, *endA9*(del-ins)::FRT, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	Datsenko and Wanner, 2000
BW25113	F-, Δ(*araD-araB*)*567*, Δ*lacZ4787*(::*rrnB-3*), λ-, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	Datsenko and Wanner, 2000
WT-Δ*ldhA*	*ldhA* null mutant of BW25113	Srirangan et al., 2013
WT-Δ*ldhA*-Δ*pykF*	*ldhA*/*pykF* double null mutant of BW25113	This study
CPC-CNTRL1	BW25141/pK184	This study
CPC-CNTRL2	WT-Δ*ldhA*/pK184	This study
CPC-PrOH1	BW25141/pK-scpAKB	This study
CPC-PrOH2	WT-Δ*ldhA*/pK-scpAKB	This study
CPC-PrOH3	WT-Δ*ldhA*-Δ*pykF*, P_{trc}::*sbm* (i.e., with the FRT-Cm^R-FRT-P_{trc} cassette replacing the 204-bp upstream of the Sbm operon)	This study
Plasmids		
pCP20	Flp^+, λ $cI857^+$, λ p_R Rep(pSC101 $ori)^{ts}$, Ap^R, Cm^R	Cherepanov and Wackernagel, 1995
pKD46	$RepA101^{ts}$ ori, Ap^R, *araC*-P_{araB}::*gam-bet-exo*	Datsenko and Wanner, 2000
pTrc99a	ColE1 ori, Ap^R, P_{trc}	Amann et al., 1988
pKD3	R6K-γ ori, Ap^R, FRT-Cm^R-FRT	Datsenko and Wanner, 2000
pK184	p15A ori, Km^R, P_{lac}::*lacZ'*	Jobling and Holmes, 1990
pK-scpAKB	From pK184, P_{lac}::*sbm-ygfD-ygfG*	Srirangan et al., 2013

Primers		
v-ldhA	TCATCAGCAGCGTCAACGGC; ATCGCTGGTCACGGGCTTACCGTT	Srirangan et al., 2013
v-pykF	TAGCAATTGAGCGATGATATATTTATACACCGG; TCGTTGCTCAGCTGGTCAACTTT	This study
c-frt	AGATTGCAGCATTACACGTCTTGAG; CCAGCTGCATTAATGAATCGGGCCATGGTCCATATGAATATCCTCC	This study
c-ptrc	CCGATTCATTAATGCAGCTGG; GGTCTGTTTCCTGTGTGAAATTGTTA	This study
r-frt:ptrc	CTCGATTATGGTCACAAAGTCCTTCGTCAGGATTAAAGATTGCAGCATTACACGTCTTGA; GTTGGCAAGCTGTTGCCACTCCTGCACGTTAGACATGGTCTGTTTCCTGTGTGAAATTGT	This study
v-frt:ptrc	GCGCTCGACTATCTGTTCGTCAGCTC; TCGACAGTTTTCTCCCGACGGCTCA	This study

3.2 Media and cultivation conditions

Glucose, yeast extract, and tryptone used in this study were obtained from BD Diagnostic Systems (Franklin Lakes, NJ), while other media components were obtained from Sigma-Aldrich Co. (St Louis, MO). Certain concentration of the antibiotic (30 μg/mL kanamycin or 12 μg/mL chloramphenicol) was supplemented into the media as required. To start a fermentation experiment, the glycerol stock of a propanogenic or control *E. coli* strain (stored at -80 °C) was streaked onto a LB agar plate with appropriate antibiotic and then incubated at 37 °C for 16 h. A single colony was picked from the LB plate to inoculate an overnight culture that contained 30 mL SB (super broth) medium (32 g/L tryptone, 20 g/L yeast extract, and 5 g/L NaCl) with appropriate antibiotic in a 125 mL conical flask. The overnight culture was incubated at 37 °C in a rotary shaker (New Brunswick Scientific, NJ) at 270 rpm. After approximately 12 hours, 2 mL of the overnight culture was used to inoculate the seed culture that contained 200 mL SB medium with appropriate antibiotic in a 1 L conical flask. The seed culture was incubated at 37 °C in a rotary shaker at 270 rpm for approximately 18 h. To collect the cells for inoculation, seed culture was spun down by centrifugation at 6,000 × g and 20 °C for 15 min. After that, the cell pellets were resuspended in 100-mL fresh LB media and the new culture was used to inoculate a 1-L stirred-tank bioreactor (Omni-Culture, VirTis, NY). The bioreactor was operated anaerobically by constantly sparging nitrogen into the bioreactor at a flow rate of 0.1 vvm, and the temperature and the agitation speed was maintained at 30 °C and 430 rpm, respectively. The production medium in the bioreactor contained 30 g/L of certain carbon sources (i.e., glucose or glycerol), appropriate antibiotics, 0.23 g/L K_2HPO_4, 0.51 g/L NH_4Cl, 100 mg/L $MgCl_2$, 48.1 mg/L K_2SO_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 cyaocobalamin (vitamin B_{12}), trace elements (2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2 \bullet 4H_2O$,

0.222 mg/L $ZnSO_4 \cdot 7H_2O$, 0.39 mg/L $Na_2MoO_4 \cdot 2H_2O$, 79 μg/L $CuSO_4 \cdot 5H_2O$, 49.4 μg/L $Co(NO_3)_2 \cdot 6H_2O$) (Neidhardt et al. 1974). The pH of the fermentation broth was maintained at 7.0 ± 0.1 by adding 30% (v/v) NH_4OH and 30% (v/v) H_3PO_4. The feeding solution for fed-batch cultivation contained 50% (w/v) glycerol only and 50 mL (25 g) of it was added manually into the bioreactor when the glycerol concentration in the fermentation culture fell below 5 g/L. Note that no isopropyl β-D-1-thiogalactopyranoside (IPTG) was supplemented in the cultivation medium for induction purpose since it was observed that IPTG supplementation had negligible effects on the 1-propanol production for all propanogenic strains in this study, and the cyaocobalamin was only supplemented at the beginning of the fed-batch cultivation.

3.3 HPLC analysis

The optical density (OD_{600}) was measured by appropriately diluting the culture samples with 0.15 M sodium chloride solution using a spectrophotometer (DU520, Beckman Coulter, Fullerton, CA). After that, the samples were spun down by centrifugation at 10,000 RPM and 4 °C for 8 min. The pellets were discarded and the cell-free supernatants were collected and filtrated for titer analysis of glucose, glycerol, and all the other carbohydrate compounds using an HPLC system (LC-10AT, Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan) and a chromatographic column (Aminex HPX-87H, Bio-Rad Laboratories, CA, USA). The column was kept in a column oven (CTO-20A, Shimadzu, Kyoto, Japan) at 65 °C and the 5 mM H_2SO_4 (pH 2.0) was used as the mobile phase. The pump was set at a flow rate of 0.6 mL/min under the working conditions. The RID signal was transferred to a desktop computer and the data collected was processed by the Clarity Lite Chromatographic Station (Clarity Lite, DataApex, Prague, The Czech Republic).

3.4 Calculations

The glucose/glycerol equivalent for each metabolite was calculated based on the equation of each metabolite synthesis pathway presented in Table 8 and the corresponding theoretical yield of each final metabolite presented in Table 9. The fraction of dissimilated glucose/glycerol to form a metabolite is defined as the ratio of the glucose/glycerol equivalent of a metabolite to the sum of the glucose/glycerol equivalent of each metabolite. The glycerol efficiency is calculated as the ratio of the sum of the glycerol equivalent of each metabolite to overall glycerol consumption.

Table 8: Pathway equations and the theoretical yield of intermediates and final metabolites

	Equation of Metabolic Pathways	Theoretical yield ($g_{product}/g_{substrate}$)
1	0.5 glucose → pyruvate + NADH + 1 ATP	0.98
2	0.5 glucose → PEP + NADH	1.87
3	glycerol → pyruvate + 2 NADH + 2 ATP	0.96
4	glycerol → PEP + 2 NADH + ATP	1.82
5	pyruvate + 2 NADH → ethanol + formate	0.52
6	pyruvate → acetate + formate + ATP	0.67
7	pyruvate + NADH → lactate	1.02
8	PEP + CO_2 + 2 NADH → succinate	0.70
9	PEP + 2 NADH → propionate	0.44
10	PEP + 4 NADH → 1-propanol	0.36

Table 9: Theoretical yield of final metabolites from glucose and glycerol

Metabolite	Theoretical yield conversion ($g_{product}/g_{substrate}$)	
	Glucose	Glycerol
Succinate	2.60	1.28
Lactate	2.00	0.98
Acetate	1.30	0.62
Propionate	1.64	0.80
Ethanol	1.02	0.50
Propanol	1.34	0.65

Chapter 4 Results

4.1 Characterization of 1-propanol production under anaerobic conditions using glucose as the main carbon source

The strain CPC-PrOH1, which contains an active Sbm operon (i.e., *sbm-ygfD-ygfG*) on a low-copy number plasmid (pK184), was fermented anaerobically using glucose as the main carbon source. As can be seen from the Figure 6, the glucose dissimilation rate of the strain CPC-PrOH1 was similar to that of the control strain CPC-CNTRL1 harboring a dummy pK184 plasmid with a total cultivation time of 14 hours and 16 hours, respectively. Both batch fermentations were finished with the glucose being completely dissimilated at the end of the cultivation. 1-propanol and propionate were produced solely by the strain CPC-PrOH1 with a titer of 0.11 g/L and 0.09 g/L, respectively (Figure 6 and Table 10). As shown in the Figure 6 and Table 10, other fermentation metabolites produced by the strain CPC-PrOH1 and its control strain CPC-CNTRL1 includes: lactate (24.97 g/L and 17.08 g/L, respectively), succinate (1.96 g/L and 2.40 g/L, respectively), acetate (4.80 g/L and 5.38 g/L, respectively), and ethanol (3.26 g/L and 3.73 g/L, respectively), while lactate was the dominant metabolite for both stains and the titer was 32% higher in the strain CPC-PrOH1 as compared to the control strain CPC-CNTRL1. Notably, the combined titers of all organic acids being produced at the end of fermentation were 31.82 g/L for the strain CPC-PrOH1 and 24.86 g/L for the control strain CPC-CNTRL1, accounting for approximately 84% and 79% of dissimilated glucose, respectively. However, the biomass titer for the strain CPC-PrOH1 (1.96 g/L) was approximately 35% lower than that of the control strain (3 g/L) (Table 10), implying that overproduction of organic acids (i.e., lactate, acetate, and succinate) can potentially harm the cell growth and viability due to their toxicity at high concentration (Warnecke and Gill, 2005). As a result, in order to reduce the lactate production and redirect the

carbon flux toward the sleeping beauty mutase (Sbm) pathway, the *ldhA* gene (encoding lactate dehydrogenase) knocked-out strain CPC-PrOH2 was selected for fermenting.

The *ldhA* mutant strain CPC-PrOH2 contains the same plasmid as its parental strain CPC-PrOH1, which has been shown capable of producing 1-propanol through the extensive Sbm pathway. Fermentation of the strain CPC-PrOH2 and its control *ldhA* mutant strain CPC-CNTRL2 harboring a dummy pK184 plasmid were performed under the same conditions using glucose as the carbon source, and the results are shown in Figure 7 and Table 10. As expected, the lactate titer for the strain CPC-PrOH2 (2.27 g/L) and CPC-CNTRL2 (1.35 g/L) was decreased by approximately 90% and 92% as compared to that of the strain CPC-PrOH1 (24.97 g/L) and CPC-CNTRL1 (17.08 g/L), respectively (Table 10). The remaining level of lactate could be produced through an alternative pathway (i.e., methylglyoxal degradation) (Kalapos, 1999). The strain CPC-PrOH2 produced 0.55 g/L of 1-propanol and 0.41 g/L of propionate, representing a 400% and 456% increase in production compared to the strain CPC-PrOH1, respectively (Table 10). Except for 1-propanol and propionate (neither were detected in the culture of the strain CPC-CNTRL2), the distribution of fermentation metabolites (i.e., acetate, succinate, lactate, and ethanol) for the control strain CPC-CNTRL2 were similar to those obtained with the strain CPC-PrOH2 (Table 10). Although lactate level was significantly reduced and 1-propanol production was increased by four-fold, the amount of glucose being used toward 1-propanol production remained low (2.73% of dissimilated glucose) (Table 10). On the other hand, acetate production for the strain CPC-PrOH2 was increased from 4.80 g/L to 9.33 g/L, which accounted for approximately 48% of dissimilated glucose (Table 10). Even though the fraction of dissimilated glucose being used for the organic acids production (i.e., acetate, lactate, succinate, and propionate) was reduced from 84% to 63%, the biomass titer (1.81 g/L) for the strain CPC-PrOH2 was less than that obtained

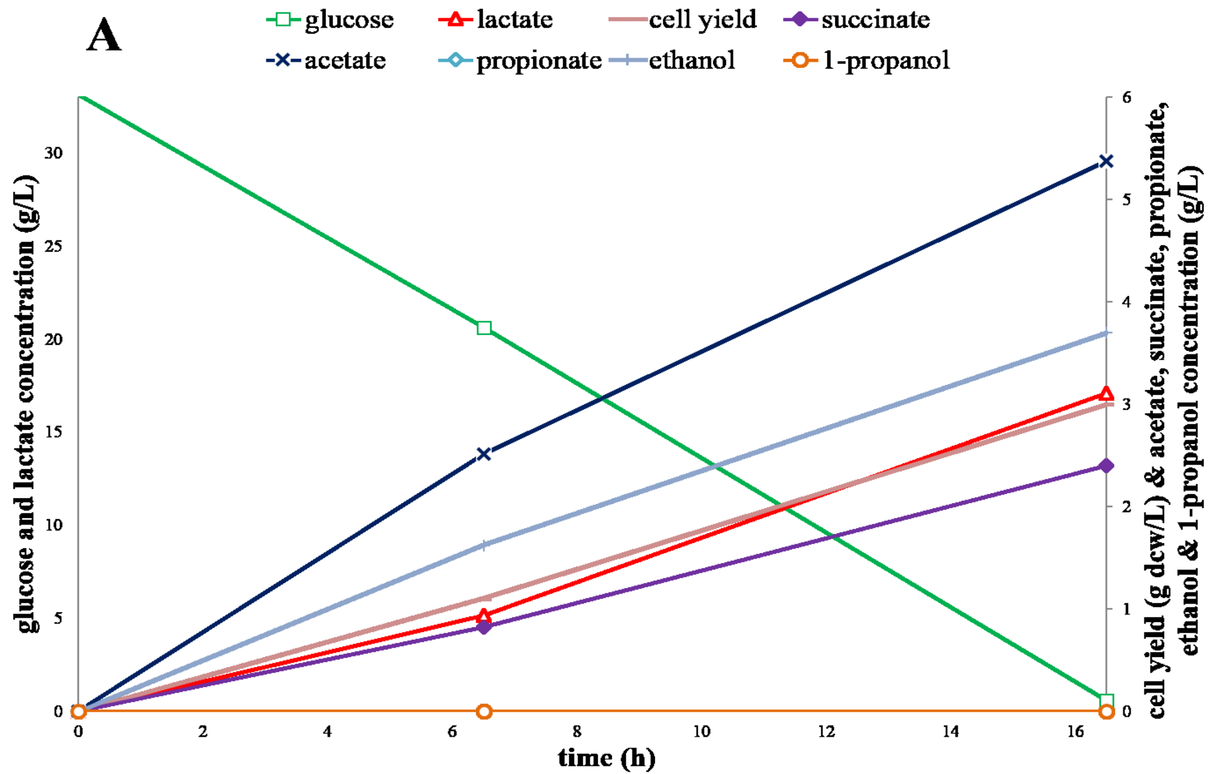
with the strain CPC-PrOH1 (Table 10), implying that acetate is more potent than the lactate. Nevertheless, it should be noted that the combined solvent production (i.e., ethanol at 5.77 g/L and 1-propanol at 0.55 g/L) was increased from 3.77 g/L to 6.32 g/L (representing approximately 37% of dissimilated glucose), suggesting that the carbon flux was partially redirected from acidogenesis to solventogenesis (Table 10).

Table 10: Fermentation profiles of the strain CPC-PrOH1, CPC-CNTRL1, CPC-PrOH2, and CPC-CNTRL2 cultured on glucose as the main carbon source

Overall glucose consumption and final biomass and metabolite concentrations of the strain CPC-PrOH1, CPC-CNTRL1, CPC-PrOH2, and CPC-CNTRL2 in a bioreactor under anaerobic conditions using glucose as the carbon source. The glucose equivalent for each metabolite and the metabolite distribution (i.e., the fraction of dissimilated glucose to form a metabolite) are calculated as described in Section 3.4.

	Glucose	Biomass	Succinate	Lactate	Acetate	Propionate	Ethanol	1-Propanol
CPC-PrOH1								
Concentration[a] (g/L)	37.32	1.96	1.96	24.97	4.80	0.09	3.26	0.11
Glucose equivalent[b] (g/L)	-	-	1.50	24.97	7.32	0.11	6.38	0.17
Metabolite distribution[c] (%)	-	-	3.70	61.73	18.10	0.28	15.77	0.42
CPC-CNTRL1								
Concentration[a] (g/L)	32.55	3.00	2.40	17.08	5.38	ND	3.73	ND
Glucose equivalent[b] (g/L)	-	-	1.83	17.08	8.20	-	7.29	-
Metabolite distribution[c] (%)	-	-	5.31	49.62	23.82	-	21.19	-
CPC-PrOH2								
Concentration[a] (g/L)	36.41	1.81	2.32	2.27	9.33	0.41	5.31	0.55
Glucose equivalent[b] (g/L)	-	-	1.77	2.29	14.23	0.50	10.38	0.82
Metabolite distribution[c] (%)	-	-	5.89	7.65	47.45	1.66	34.62	2.73
CPC-CNTRL2								
Concentration[a] (g/L)	37.35	1.67	2.94	1.35	9.50	ND	5.77	ND
Glucose equivalent[b] (g/L)	-	-	2.24	1.36	14.82	-	11.54	-
Metabolite distribution[c] (%)	-	-	7.62	4.63	49.31	-	38.43	-

[a] initial glucose concentration, biomass concentration (g-DCW/L), and major metabolite concentrations
[b] calculated based on theoretical yield of each metabolite to glucose
[c] represents the fraction of dissimilated glucose
ND not detected



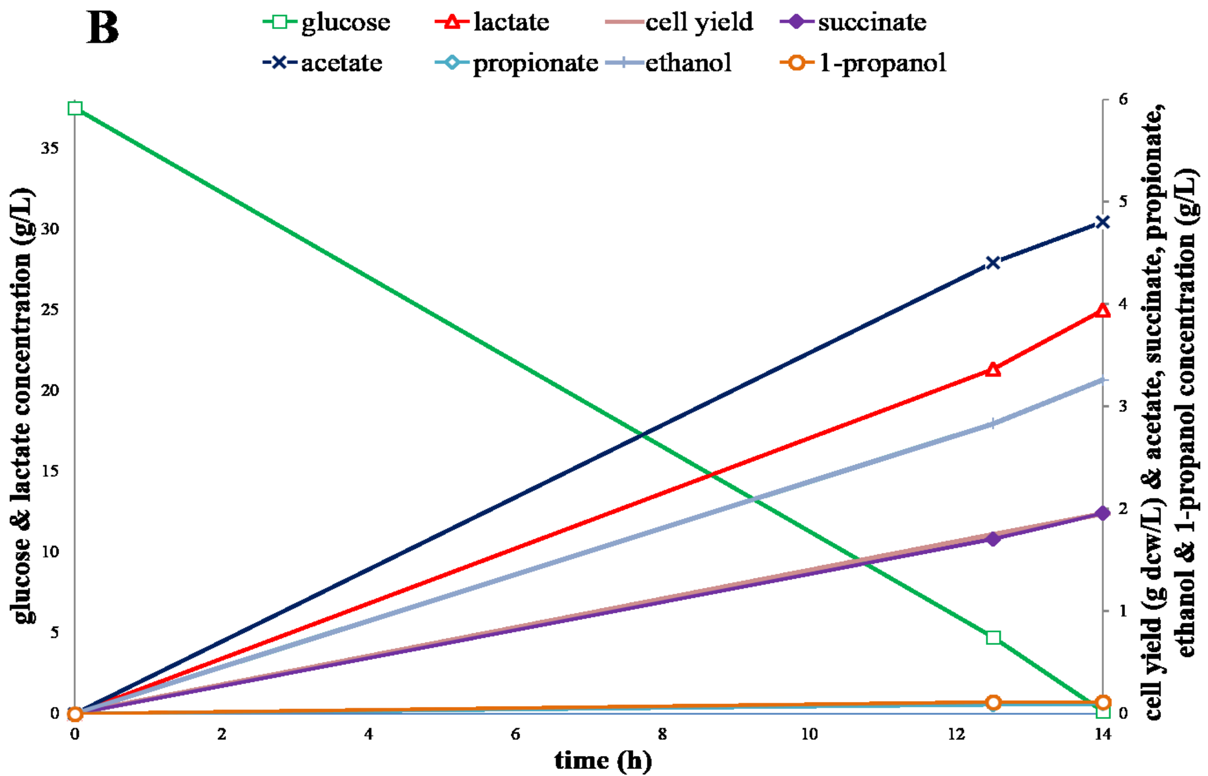
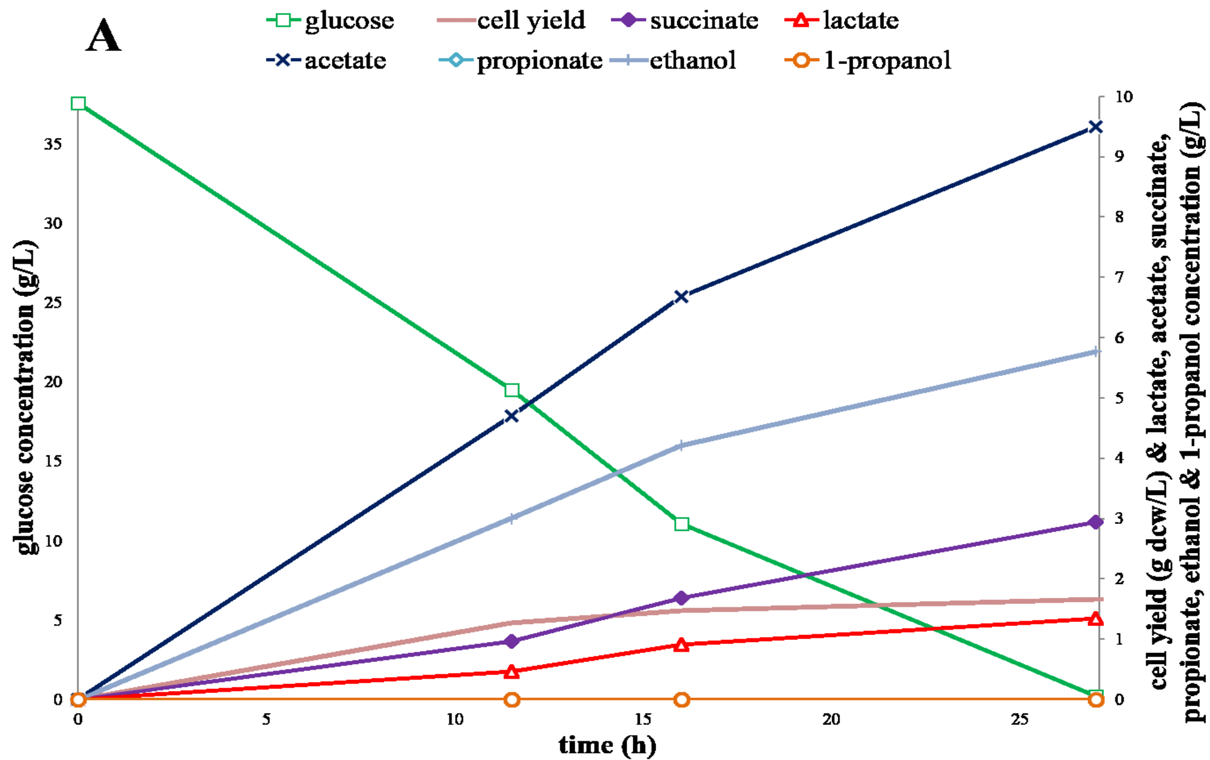


Figure 6: Time profiles of glucose, biomass, and major metabolites during batch cultivation of A. CPC-CNTRL1 and B. CPC-PrOH1 with glucose as the major carbon source



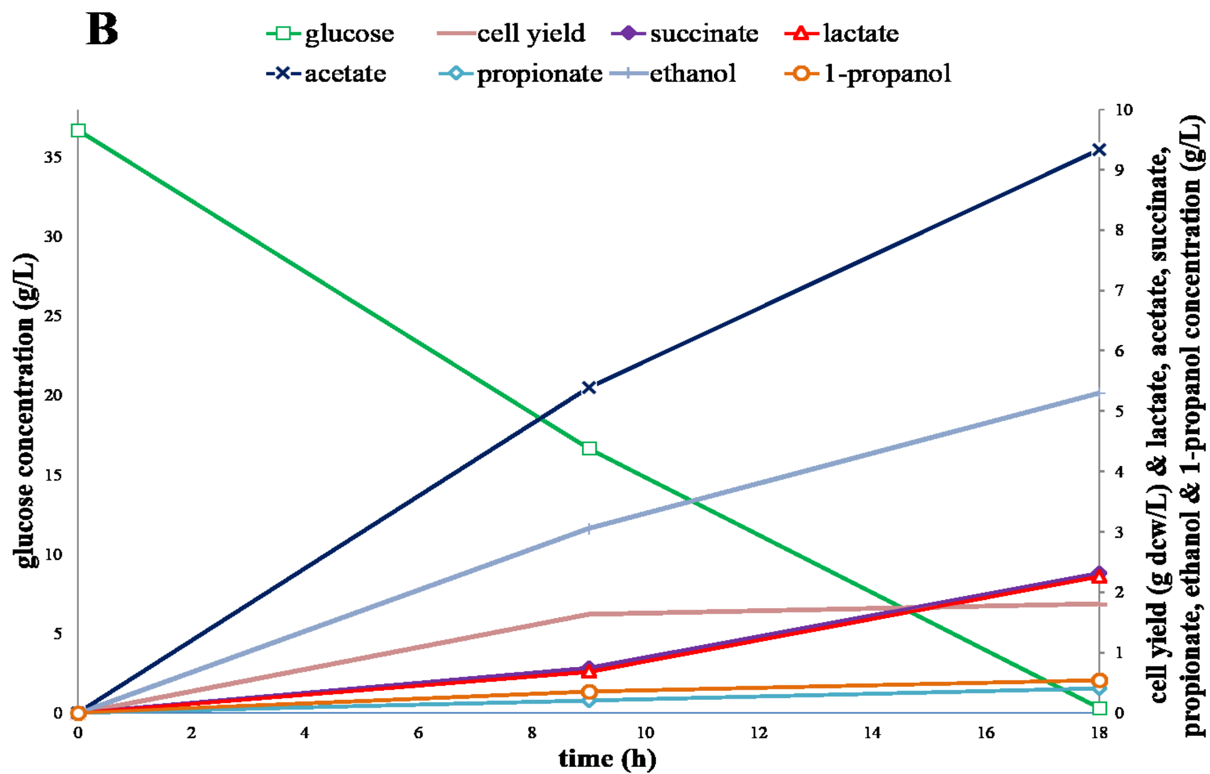


Figure 7: Time profiles of glucose, biomass, and major metabolites during batch cultivation of A. CPC-CNTRL2 and B. CPC-PrOH2 with glucose as the major carbon source

4.2 Characterization of 1-propanol production under anaerobic conditions using glycerol as the main carbon source

Due to the high-level of acidogenesis for fermentations under anaerobic conditions using glucose as the carbon source, the effect of using glycerol, which is a more reduced compound compared to glucose (Clomburg and Gonzalez, 2013), as an alternative carbon source was investigated. The glycerol fermentation of the strain CPC-PrOH2 and CPC-CNTRL2 were performed under the same condition as those glucose fermentations. As shown in the Figure 8B and Table 11, 2.15 g/L of 1-propanol were produced by the strain CPC-PrOH2, representing an almost three-fold increase compared with that obtained by using glucose as the carbon source (0.55 g/L). However, ethanol became the dominant by-product as its titer was increased from 5.31 g/L to 9.31 g/L, while acetate titer was decreased from 9.33 g/L to 3.92 g/L and succinate titer was decreased from 2.32 g/L to 0.62 g/L (Figure 8B and Table 11). Propionate titer was also increased from 0.41 g/L to 0.89 g/L, representing a more than two-fold increase compared to that obtained with glucose (Table 11). Interestingly, lactate was totally eliminated in the culture of both the strain CPC-PrOH2 and CPC-CNTRL2 by using glycerol as the carbon source (Figure 8 and Table 11). More importantly, the combined titer of all organic acids produced by the strain CPC-PrOH2 (i.e., acetate, succinate, and propionate) accounted for approximately 26% of the dissimilated glycerol, whereas the combined titer of all solvents (i.e., ethanol and 1-propanol) accounted for 74% of the dissimilated glycerol (Table 11). This result shows the effectiveness of using glycerol as the main carbon source in driving the carbon flux toward solventogenesis instead of acidogenesis. However, the dissimilation rate of glycerol in the strain CPC-PrOH2 was much slower than that of the glucose, with a total cultivation time of 85.5 hours and 18 hours, respectively (Figure 8B & 7B). The prolonged cultivation time might be related to glycerol's highly reduced state ($\kappa \approx 4.67$, where

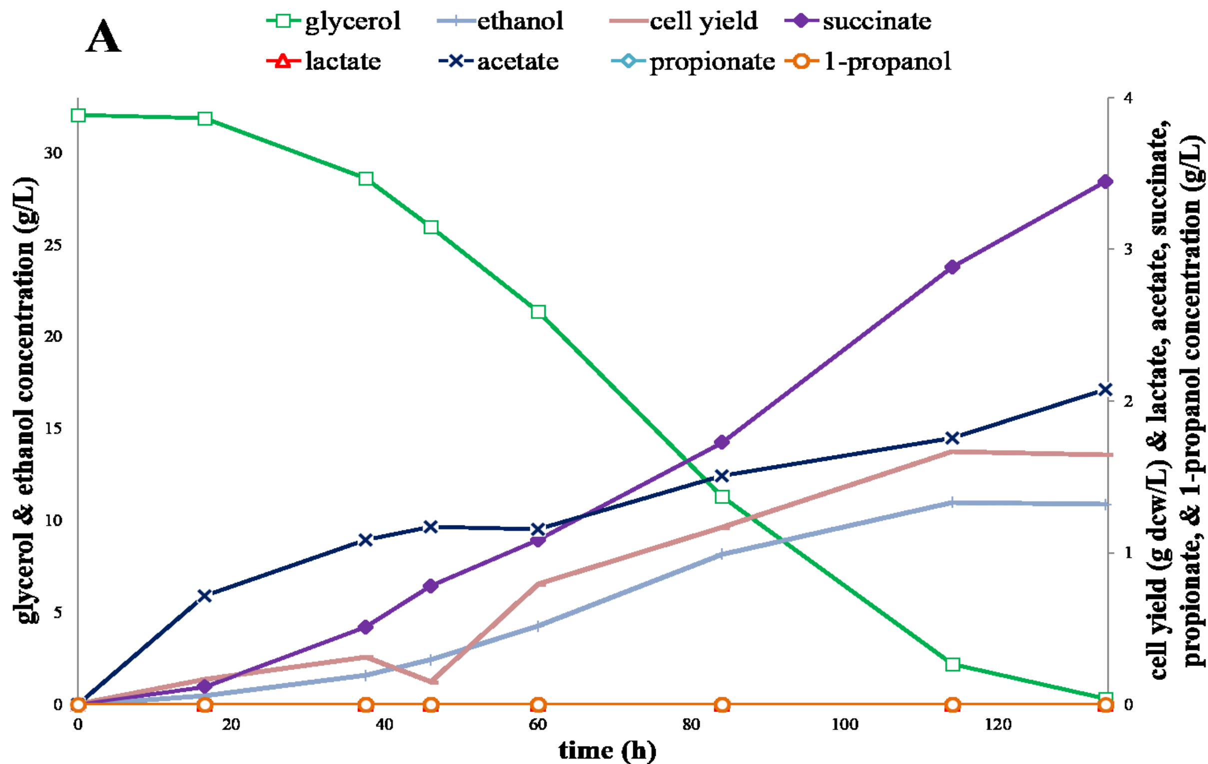
κ is the degree of reduction per carbon atom in the compound) (Villadsen et al., 2011) and less ATP generated by the acetate synthesis pathway (Table 8). As shown in the Fig 8A and Table 11, the control strain CPC-CNTRL2 without an active Sbm operon took 134 hours to completely consume all the glycerol, and the biomass titer (1.64 g/L) was 28% lower than that of the strain CPC-PrOH2 (2.27 g/L). This result suggests that the extensive Sbm pathway can not only facilitate the dissimilation of glycerol but also help the cell growth under fermentative conditions. As expected, both 1-propanol and propionate were not detected in the culture of the strain CPC-CNTRL2 by using glycerol as the main carbon source (Figure 8A and Table 11). Ethanol was the dominate metabolite with a titer of 10.89 g/L, representing approximately 79% of dissimilated glycerol (Table 11). Compared to the strain CPC-PrOH2, the control strain CPC-CNTRL2 produced 456% more succinate at 3.45 g/L and 47% less acetate at 2.08 g/L by using glycerol as the main carbon source (Table 11). Clearly, the succinate accumulation in the strain CPC-CNTRL2 was due to the lack of the Sbm pathway, which can facilitate the conversion of succinate into 1-propanol. In addition, the acetate accumulation in the culture of the strain CPC-PrOH2 by using glycerol as the carbon source might be due to the fact that for each mole of succinate converted to 1 mol of 1-propanol, 1 net mol of ATP is consumed (Table 8), so that more acetate needs to be produced in order to compensate this net ATP consumption. Surprisingly, the glycerol efficiency (i.e., ratio of the sum of the glycerol equivalents associated with all metabolites to overall glycerol consumption) for the strain CPC-PrOH2 (96.29%) was higher than that of the control strain CPC-CNTRL2 (87.19%) (Table 11).

Table 11: Fermentation profiles of the strain CPC-PrOH2 and CPC-CNTRL2 cultured on glycerol as the main carbon source

Overall glucose consumption and final biomass and titer of final metabolites of the strain CPC-PrOH2, and CPC-CNTRL2 in a bioreactor under anaerobic conditions using glycerol as the main carbon source. The glycerol equivalent, glycerol efficiency, and the metabolite distribution (i.e., the fraction of dissimilated glycerol to form a metabolite) are calculated as described in Section 3.4.

	Glycerol	Biomass	Succinate	Lactate	Acetate	Propionate	Ethanol	1-Propanol
CPC-PrOH2								
Concentration[a] (g/L)	30.76	2.27	0.62	ND	3.92	0.89	9.31	2.15
Glycerol equivalent[b] (g/L)	-	-	0.47	-	6.12	1.12	18.61	3.30
Metabolite distribution[c] (%)	-	-	1.58	-	20.66	3.77	62.84	11.15
Glycerol efficiency[d] (%)	96.29	-	-	-	-	-	-	-
CPC-CNTRL2								
Concentration[a] (g/L)	31.77	1.64	3.45	ND	2.08	ND	10.89	ND
Glycerol equivalent[b] (g/L)	-	-	2.69	-	3.24	-	21.77	-
Metabolite distribution[c] (%)	-	-	9.72	-	11.69	-	78.59	-
Glycerol efficiency[d] (%)	87.19	-	-	-	-	-	-	-

[a] initial glycerol concentration, biomass concentration (g-DCW/L), and major metabolite concentrations
[b] calculated based on theoretical yield of each metabolite to glycerol
[c] represents the fraction of dissimilated glycerol
[d] ratio of the sum of the glycerol equivalents associated with all metabolites to overall glycerol consumption
ND not detected



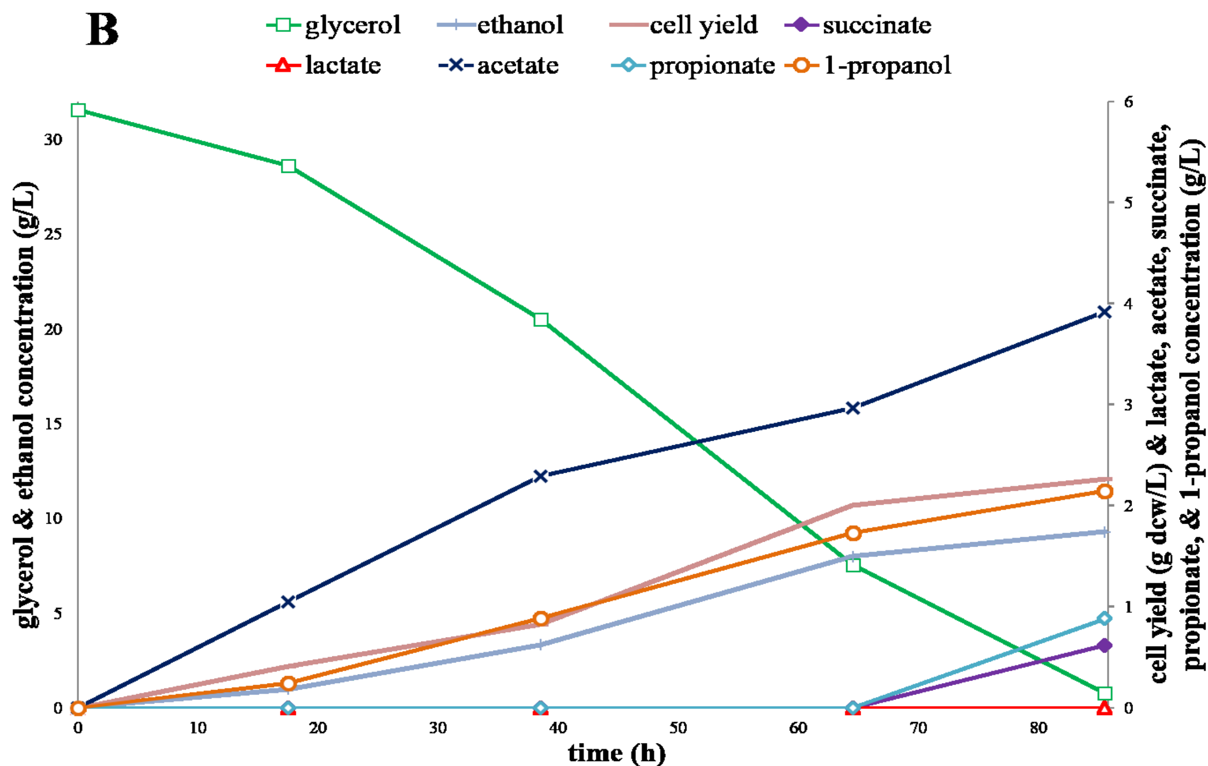


Figure 8: Time profiles of glycerol, biomass, and major metabolites during batch cultivation of A. CPC-CNTRL2 and B. CPC-PrOH2 with glycerol as the main carbon source

4.3 Fed-batch cultivation for 1-propanol production using glycerol as the main carbon source

In order to characterize the 1-propanol production capacity of our propanogenic strain, the fed-batch cultivation of the strain CPC-PrOH2 was performed following the first batch cultivation of the strain CPC-PrOH2 using glycerol as the carbon source (Figure 8B) by periodically adding approximately 50 mL of the 50% (w/v) glycerol stock solution into the bioreactor when the glycerol concentration in the bioreactor fell near to 1 g/L. The fed-batch fermentation profile of the strain CPC-PrOH2 are shown in the Figure 9 and Table 12. Due to the dilution effect resulted from addition of either glycerol stock solution or acidic and alkaline solution and sampling of fermentation cultures for the HPLC analysis, the working volume of the bioreactor varied between stages, which made the metabolite concentrations underestimated from Stage II to Stage IV. As shown in the Figure 9, there was always a sudden drop in the metabolite concentration after the addition of the glycerol stock solution, which implied the existence of such dilution effect. Therefore, the metabolite distribution (i.e., the fraction of dissimilated glycerol to form a metabolite) and glycerol efficiency (i.e., ratio of the sum of the glycerol equivalents associated with all metabolites to overall glycerol consumption) were analyzed based on each individual stage's cultivation performance to avoid this dilution effect (Table 12). Ethanol was the dominant metabolite in all four stages, and the fraction of dissimilated glycerol used for ethanol production was increased from 62.84% in Stage I to 74.4% in Stage IV. However, 1-propanol production was hampered in all the stages after, with 9.51% and 5.62% of dissimilated glycerol being used for 1-propanol production in Stage II and Stage III, respectively, and almost no 1-propanol was accumulated in the last stage (Table 12). On the other hand, the fraction of dissimilated glycerol used for succinate production was increased from 1.58% in Stage I to 7.59% in Stage II, 8.32% in

Stage III, and 6.7% in Stage IV, implying that the activity of the Sbm pathway diminished over time. Interestingly, 0.39 g/L of lactate was produced from 13.84 g/L of glycerol in the Stage IV. The high-level of solventogenesis was maintained throughout the whole fed-batch cultivation, with ~73%-77% of dissimilated glycerol being used toward the co-production of ethanol and 1-propanol. Overall, 4.12 g/L of 1-propanol and 26.97 g/L of ethanol were produced from approximately 87 g/L of glycerol at the end of the fed-batch fermentation, while other major metabolites, such as acetate, succinate, and propionate, was produced at a titer of 8.98 g/L, 5.37 g/L, and 1.33 g/L, respectively (Table 12). Notably, the glycerol efficiency in Stage II (92.74%), Stage III (89.92%), and Stage IV (87.21%) was all lower than the stage before, whereas the Stage I (96.26%) had the highest glycerol efficiency (Table 12). In addition, the biomass yield was much lower in Stage III (0.014 g/g) and was negative in Stage IV (-0.006 g/g) as compared with that obtained in Stage I (0.074 g/g) and Stage II (0.032 g/g) (Table 12). In summary, these results indicate that the cell growth was hampered in the last two stages (i.e., Stage III and Stage IV) as a result of the accumulation of both acetate and ethanol at a relatively high level, which might impose a certain degree of environmental stress onto the cell culture.

Table 12: Fed-batch fermentation profiles of the strain CPC-PrOH2 cultured on glycerol as the main carbon source

Overall glycerol consumption and final biomass and titer of final metabolites for each stage of the fed-batch cultivation of the strain CPC-PrOH2 under anaerobic conditions using glycerol as the main carbon source. The glycerol equivalent for each metabolite, glycerol efficiency, and the metabolite distribution are calculated as described in Section 3.4.

CPC-PrOH2		Glycerol	Biomass	Succinate	Lactate	Acetate	Propionate	Ethanol	1-Propanol
Stage I	Concentration[a] (g/L)	30.76	2.27	0.62	ND	3.92	0.89	9.31	2.15
0-85.5 h	Glucose equivalent[b] (g/L)	-	-	0.47	-	6.12	1.12	18.61	3.30
	Metabolite distribution[c] (%)	-	-	1.58	-	20.66	3.77	62.84	11.15
	Glycerol efficiency[d] (%)	96.26	-	-	-	-	-	-	-
Stage II	Concentration[a] (g/L)	23.01	0.73	1.83	ND	2.01	0.11	7.21	1.32
85.5-137 h	Glucose equivalent[b] (g/L)	-	-	1.62	-	3.13	0.14	14.42	2.03
	Metabolite distribution[c] (%)	-	-	7.59	-	14.65	0.67	67.57	9.51
	Glycerol efficiency[d] (%)	92.74	-	-	-	-	-	-	-
Stage III	Concentration[a] (g/L)	19.65	0.27	1.88	ND	1.98	0.16	5.96	0.65
137-196 h	Glucose equivalent[b] (g/L)	-	-	1.47	-	3.09	0.20	11.92	0.99
	Metabolite distribution[c] (%)	-	-	8.32	-	17.49	1.11	67.47	5.62
	Glycerol efficiency[d] (%)	89.92	-	-	-	-	-	-	-
Stage IV	Concentration[a] (g/L)	13.84	-0.08	1.04	0.39	1.07	0.17	4.49	0.00
196-245.5 h	Glucose equivalent[b] (g/L)	-	-	0.81	0.40	1.66	0.21	8.98	0.01
	Metabolite distribution[c] (%)	-	-	6.70	3.29	13.77	1.76	74.40	0.05
	Glycerol efficiency[d] (%)	87.21	-	-	-	-	-	-	-

[a] total concentration of glycerol consumption, biomass concentration (g-DCW/L), and major metabolite concentrations for each specific stage of the fed-batch culture
[b] calculated based on theoretical yield of each metabolite to glycerol
[c] represents the fraction of dissimilated glycerol
[d] ratio of the sum of the glycerol equivalents associated with all metabolites to overall glycerol consumption
ND not detected

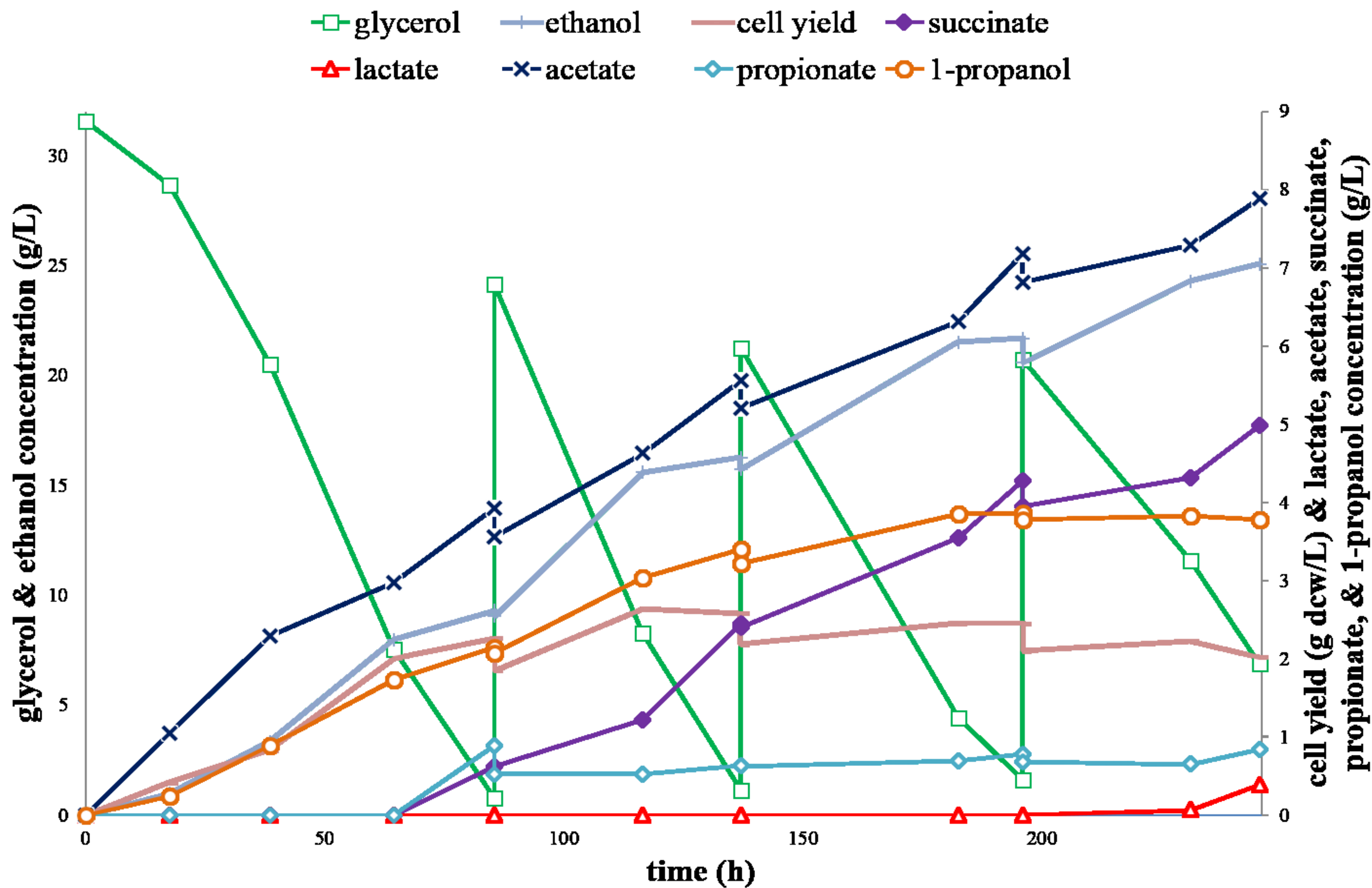


Figure 9: Time profile of glycerol, biomass, and major metabolites during fed-batch cultivation of CPC-PrOH2 with glycerol as the major carbon source

4.4 Fed-batch cultivation of plasmid-free propanogenic *Escherichia coli* strain for 1-propanol production

Although the carbon flux has been successfully channelled toward solventogenesis by using glycerol as the carbon source, the dissimilation rate of glycerol is rather slow as compared to that of the glucose. It has been previously studied by Togna, Shuler, and Wilson (1993), Makrides (1996) and Martinez et al. (1999) that using plasmid system could potentially harm the cell growth rate due to the metabolic burden imposed on the host cell. To surmount this limitation, a plasmid-free propanogenic strain, CPC-PrOH3, was derived, which had a strong *trc*-promoter (P_{trc}) being integrated upstream of the chromosomal Sbm operon (Srirangan et al., 2014). Fed-batch cultivation of the strain CPC-PrOH3 was then performed to test its 1-propanol production capacity under anaerobic conditions using glycerol as the carbon source. These results were summarized in Table 13 and Figure 10. As expected, the cultivation time of the strain CPC-PrOH3 in Stage I was reduced significantly from 85.5 hours to 42.5 hours (Figure 10 & 9). In addition, the biomass titer was increased from 2.27 g/L to 2.79 g/L with the dissimilation of a similar amount of glycerol (30.76 g vs 30.73 g, respectively) in Stage I (Table 12 & 13), implying that the plasmid-free strain CPC-PrOH3 had a much better cell growth and faster glycerol dissimilation rate than the strain CPC-PrOH2. Also, a similar effect was observed in other stages (i.e., Stage II, III, and IV) as well. 1-propanol was produced at 2.44 g/L in Stage I as compared to 2.15 g/L in the strain CPC-PrOH2 (Table 13 & 12). More interestingly, the fraction of dissimilated glycerol used for 1-propanol production was steadily increased in Stage II (12.95 %) and Stage III (13.84%) as compared to Stage I (12.29 %) (Table 13), suggesting that the plasmid-free strain CPC-PrOH3 was superior to the strain CPC-PrOH2 in maintaining the functional expression of the gene of interest (i.e., Sbm operon) overtime. However, the 1-propanol production was hampered in late stages (Stage IV and

Stage V), as only 7.90% and 6.31% of dissimilated glycerol was used toward the 1-propanol production, respectively (Table 13). Again, ethanol was the dominant metabolite in all five stages. The fraction of dissimilated glycerol used to produce ethanol was around 70% in the middle three stages (i.e., Stage II, III, and IV), which were higher than the 62.34% in Stage I and 67.81% in Stage V (Table 13). Over 80% of the carbon flux was directed toward solventogenesis in Stage II (84.89%) and Stage III (84.27%), but showed a downward trend in Stage IV (77.98%) and Stage V (74.12%). On the other hand, the fraction of dissimilated glycerol used for acetate production was lower in Stage II (12.06%) and Stage III (13.31%) compared with Stage I (20.64%), primarily due to an enhancement in solventogenesis level. However, when the co-production of ethanol and 1-propanol was hampered in Stage IV and Stage V, acetate started to accumulate again and approximately 18% and 23 % of the dissimilated glycerol, respectively, was used toward the acetate production. Notably, succinate was produced minimally in the strain CPC-PrOH3 as compared to the strain CPC-PrOH2, as only ~1-3% of dissimilated glycerol was used toward the succinate production throughout the whole fed-batch fermentation, implying that succinate was continuously converted to 1-propanol catalyzed by the extensive Sbm pathway (Figure 9 & 10). Similar to the fed-batch cultivation of the strain CPC-PrOH2, the biomass yield was decreased dramatically after Stage I and was negligible in the last two stages (i.e., Stage IV and V) (Table 13). The glycerol efficiency was maintained at a high level in Stage I (99.34%) and Stage II (105.06%), but was drastically lower in Stage III (87.16%), Stage IV (81.41%), and Stage V (73.92%), which might be due to the utilization of carbon source for cell maintenance under environmental stress as a result of the accumulation of high concentration of acetate and ethanol in the cell culture. In summary, 7.52 g/L of 1-proapnol and 35.66 g/L of ethanol were produced

from 115.82 g/L of glycerol at the end of the fed-batch fermentation, along with 1.65 g/L of propionate, 2.25 g/L of succinate, and 11.63 g/L of acetate being produced.

Table 13: Fed-batch fermentation profiles of the strain CPC-PrOH3 cultured on glycerol as the main carbon source

Overall glycerol consumption and final biomass and metabolite concentrations for each stage of the fed-batch cultivation of the strain CPC-PrOH3 under anaerobic conditions using glycerol as the main carbon source. The glycerol equivalent for each metabolite, glycerol efficiency, and the metabolite distribution (i.e., the fraction of dissimilated glycerol to form a metabolite) are calculated as described in Section 3.4

CPC-PrOH3		Glycerol	Biomass	Succinate	Lactate	Acetate	Propionate	Ethanol	1-Propanol
Stage I	Concentration[a] (g/L)	30.73	2.79	0.59	ND	4.04	0.78	9.51	2.44
0-42.5 h	Glucose equivalent[b] (g/L)	-	-	0.46	-	6.30	0.99	19.03	3.75
	Metabolite distribution[c] (%)	-	-	1.51	-	20.64	3.23	62.34	12.29
	Glycerol efficiency[d] (%)	99.34	-	-	-	-	-	-	-
Stage II	Concentration[a] (g/L)	22.69	0.38	0.34	ND	1.84	0.37	8.57	2.01
42.5-72 h	Glucose equivalent[b] (g/L)	-	-	0.26	-	2.88	0.46	17.15	3.09
	Metabolite distribution[c] (%)	-	-	1.10	-	12.06	1.94	71.94	12.95
	Glycerol efficiency[d] (%)	105.56	-	-	-	-	-	-	-
Stage III	Concentration[a] (g/L)	18.51	0.65	0.30	ND	1.38	0.12	5.68	1.45
72-94 h	Glucose equivalent[b] (g/L)	-	-	0.24	-	2.15	0.16	11.36	2.23
	Metabolite distribution[c] (%)	-	-	1.46	-	13.31	0.96	70.43	13.84
	Glycerol efficiency[d] (%)	87.16	-	-	-	-	-	-	-
Stage IV	Concentration[a] (g/L)	25.67	0.10	0.85	ND	2.39	0.17	7.33	1.07
94-144 h	Glucose equivalent[b] (g/L)	-	-	0.66	-	3.73	0.21	14.65	1.65
	Metabolite distribution[c] (%)	-	-	3.16	-	17.86	1.00	70.08	7.90
	Glycerol efficiency[d] (%)	81.41	-	-	-	-	-	-	-

Stage V	Concentration[a] (g/L)	18.22	0.02	0.17	ND	1.98	0.21	4.57	0.55
144-210.5 h	Glucose equivalent[b] (g/L)	-	-	0.13	-	3.09	0.26	9.14	0.85
	Metabolite distribution[c] (%)	-	-	0.99	-	22.94	1.95	67.81	6.31
	Glycerol efficiency[d] (%)	73.92	-	-	-	-	-	-	-

[a] total concentration of glycerol consumption, biomass concentration (g-DCW/L), and major metabolite concentrations for each specific stage of the fed-batch culture
[b] calculated based on theoretical yield of each metabolite to glycerol
[c] represents the fraction of dissimilated glycerol
[d] ratio of the sum of the glycerol equivalents associated with all metabolites to overall glycerol consumption
ND not detected

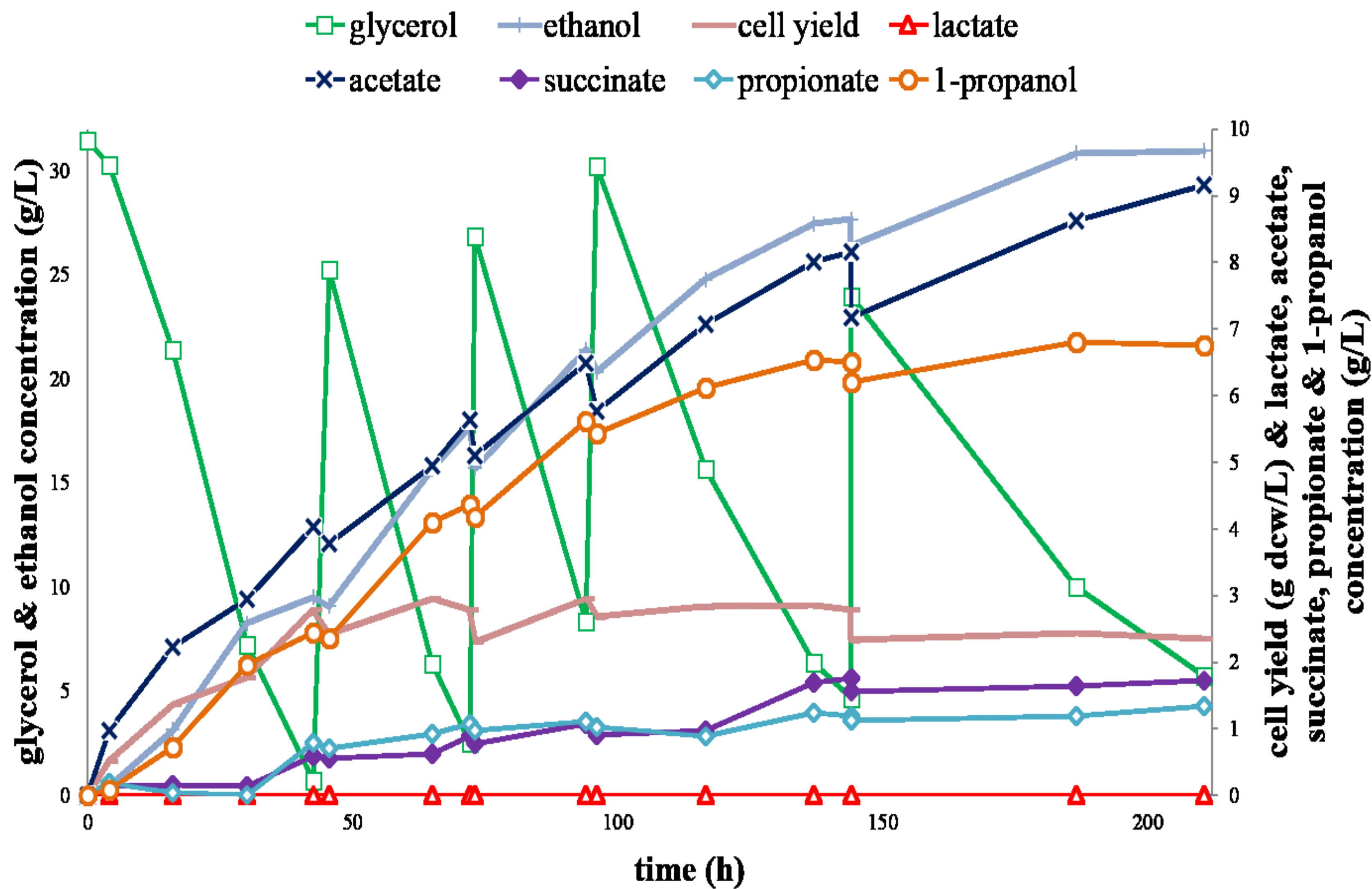


Figure 10: Time profile of glycerol, biomass, and major metabolites during fed-batch cultivation of CPC-PrOH3 with glycerol as the major carbon source

Chapter 5 Discussion

It has been long argued that the combustion of fossil fuels is not only environmental unfriendly, which produces air pollutants that contribute to climate change, smog, and acid-rain, but also unsustainable due to its limited global reserves. By blending renewable fuels (e.g., ethanol) into the traditional petroleum fuels, emissions of greenhouse gases and air pollutants can be significantly reduced. In Canada, up to 10% of ethanol can be blended into gasoline for usage in internal combustion engines. However, as a potential renewable fuel, 1-propanol is superior to ethanol in terms of energy density and RON (research octane number). Several studies have reported the heterologous production of 1-propanol in metabolically engineered *Escherichia coli* strains (Atsumi and Liao, 2008; Shen and Liao, 2008; Jain and Yan, 2011; Lee et al., 2012; Jain et al., 2015). Recently, our group constructed a new 1-propanol biosynthesis pathway in *E.coli* by expanding the native succinate pathway via overexpression of the native *E.coli* Sbm operon (Srirangan et al., 2013). In that work, 150 mg/L of 1-propanol was produced by a metabolically engineered *E.coli* stain in shake flasks under anaerobic conditions using glucose as the main carbon source. Due to low 1-propanol yield and poorly defined cultivation conditions in shake flasks, the performance of our metabolically engineered propanogenic strains could be further improved by scaling up from the shake flask to the bioreactor.

Anaerobic fermentation of wild type *E. coli* strains using glucose as the carbon source mainly produce lactate, acetate, ethanol, formate, and succinate as the end products, which is known as the mixed-acid fermentation (Clark, 1989). By introducing a low copy plasmid pK184 containing an active Sbm operon, the wild type *E.coli* strain was capable of converting succinate into 1-propanol via propionyl-CoA intermediate (Figure 6 and Table 10). Even though the sleeping

beauty mutase pathway worked as expected, only a small portion of succinate was dissimilated. This might be due to the fact that 1-propanol possesses a higher degree of reduction per carbon atom ($\kappa = 6$) compared with the glucose ($\kappa = 4$) (Villadsen et al., 2011), so that the 1-propanol production from glucose will result in net NADH consumption which can cause the redox imbalance in the host cell. Based on the concept described by Roels (1983), the redox level of glucose and 1-propanol is 24 and 18, respectively, and for each mole of 1-propanol produced from 0.5 mol of glucose (Table 8), there is a net consumption of 3 mol of reducing equivalents (i.e., NADH = 2 H), which is calculated based on the calculation: $-(0.5 \times 24) + 18 = 6$. In addition, for each mole of 1-propanol converted from 1 mol succinate via the Sbm pathway, one mole of ATP is consumed (Table 8), which makes the synthesis of 1-propanol also energetically unfavorable when compared to the synthesis of succinate. On the other hand, lactate synthesis from glucose represents a perfect redox-balanced reaction. Since the redox level of lactate is 12 and each mole of lactate is converted from 0.5 mol of glucose (Table 8), based on the calculation: $-(0.5 \times 24) + 12 = 0$, there are neither net consumption nor net production of the reducing equivalents in the reaction. Furthermore, 2 mol of ATP are generated for each mole of glucose converted to 2 mol of lactate, whereas for each mole of 1-propanol produced, there is a net consumption of 1 mol ATP. Consequently, lactate was produced as the dominant product in the batch culture of both the control strain CPC-CNTRL1 and the propanogenic strain CPC-PrOH1 (Figure 6 and Table 10). This result is consistent with the findings reported by Stokes (1949), Alam and Clark (1989), and Mat-Jan, Alam, and Clark (1989) that the fermentative production of lactate in wild type *E.coli* is induced by anaerobic conditions with glucose as the main carbon source. In order to channel carbon flux toward the Sbm pathway and conserve NADH for 1-propanol production, the *ldhA* gene was deleted. In the resulting mutant strain, CPC-PrOH2, 1-propanol production was increased by four-

fold along with minimal amount of lactate being produced compared to that obtained with the strain CPC-PrOH1 (Figure 6B & 7B and Table 10). However, 2.32 g/L of succinate remained unconverted and both acetate and ethanol were overproduced by the end of the fermentation (Figure 7B). This metabolites shift caused by the disruption of the lactate synthesis pathway suggests that the carbon flux is mainly driven toward the pyruvate catabolism rather than the reductive arm of the TCA cycle (oxaloacetate → malate → fumarate → succinate) via the phosphoenolpyruvate (PEP) node when using glucose as the carbon source, as lactate, acetate, and ethanol are all produced via the pyruvate node. Again, the NADH availability might be the limiting factor that prevents the carbon flux being driven toward the 1-propanol production via the PEP node. While only 2 mol of NADH could be generated from each mole of glucose dissimilated via the glycolysis pathway, the production of each mole of 1-propanol requires a net consumption of 3 mol NADH, and thus 1.5 mol of glucose are minimally required for production of each mole of 1-propanol in order to maintain the overall redox balance. Therefore, glucose is an unfavorable substrate for 1-propanol production in our metabolically engineered propanogenic strains due to its limited NADH-generating capacity.

Glycerol, which has a higher degree of reduction per carbon atom ($\kappa \approx 4.67$) as compared to the glucose ($\kappa \approx 4$), has been extensively studied in microbial fermentations as an inexpensive feedstock for biofuels and chemicals production during the past decade (Dharmadi et al., 2006; Murarka et al., 2008; Ganesh et al., 2012; Bauer and Hulteberg, 2013; Mattam et al., 2013). As illustrated in the Table 8, for each mole of glycerol converted to one mol of pyruvate or PEP, 2 mol of NADH are produced, which are twice as much of that generated from 0.5 mol of glucose. Thus, using glycerol as the main carbon source should be able to direct more carbon flux toward the reductive arm of the TCA cycle via the PEP node and subsequently toward the sleeping beauty

mutase pathway for 1-propanol production. The results from the glycerol batch fermentation of the strain CPC-PrOH2 confirmed this hypothesis, as the titer of 1-propanol was increased from 0.55 g/L to 2.15 g/L, which accounted for more than 11% of dissimilated glycerol compared to 2.73% with glucose (Table 10 & 11). Moreover, only 0.62 g/L of succinate remained unconverted at the end of the fermentation, while 2.32 g/L of succinate was accumulated with glucose (Table 10 & 11). Since the succinate/1-propanol ratio was much lower when glycerol was used as the main carbon source, the excess amount of the reducing equivalents generated by the glycerol dissimilation was able to facilitate the conversion of succinate to 1-propanol via the Sbm pathway. However, Table 10 and Table 11 shows that the fraction of dissimilated glucose/glycerol being used for the coproduction of succinate and 1-propanol is only increased from 8.62% to 12.73%, suggesting that the carbon flux toward the pyruvate node remained dominant over the reductive arm of the TCA cycle via the PEP node. It was thought that the usage of PEP as an intermediate to convert dihydroxyacetone into glycerine phosphate during the glycerol assimilation and the lack of net ATP production limited the conversion of PEP into oxaloacetate catalyzed by the native *E.coli* PEP carboxylase (encoded by *ppc*) (Jin et al., 1983; Gonzalez et al., 2008; Blankschien et al., 2010; Zhang et al., 2010). Furthermore, as illustrated in the Table 8, there is a net consumption of 1 mol ATP per mole 1-propanol produced, which makes the reaction energetically unfavorable. Thus, in order to compensate the shortage of ATP caused by the accumulation of 1-propanol, an increase in the acetate production was found in the strain CPC-PrOH2 compared with the control strain CPC-CNTRL2 (Table 11). On the other hand, synthesis of ethanol, which is the dominant fermentative product accumulated during the anaerobic glycerol fermentation, is not only redox-balanced but also capable of generating 1 mol ATP per mole of ethanol produced. Thus, it is concluded that this metabolically engineered 1-propanol synthesis pathway is not able to compete

with the native fermentative pathway by simply switching the carbon source from glucose to glycerol.

Fed-batch fermentation of the propanogenic strain CPC-PrOH2 using glycerol as the main carbon source was then performed to further characterize the strain's 1-propanol production capacity. As shown in the Table 13, approximately 4 g/L of 1-propanol was produced from 87 g/L of glycerol by the end of the fed-batch fermentation. However, after the Stage I, succinate started to accumulate at a faster pace while 1-propanol production rate was decreased over time, suggesting that the conversion of succinate into 1-propanol via the Sbm pathway was hindered. It is reasoned that this might be due to a gradually decrease in the catalytic activity of the enzymes encoded by the Sbm operon, as the metabolites distribution in the last stage was similar to that of the batch fermentation of the control strain CPC-CNTRL2 (Table 8B). Although the strain CPC-PrOH2 was constructed by introducing a low copy number plasmid (pK184) that contained an activate Sbm operon, which should impose a minimal level of metabolic load onto the host cell as compared to the high or middle copy number plasmids, certain amounts of cellular energy were still required to maintain the presence and expression of the foreign plasmid in the host cell (Glick, 1995). Moreover, it was reported that the plasmid stability was sensitive to the dissolved oxygen level, as the fluctuation in dissolved oxygen level (i.e., dissolved oxygen shock) might cause the cell to lose the plasmid (Hopkins et al., 1987; Ryan et al., 1989; Khosravi et al., 1990). Since the glycerol was manually added into the bioreactor by using serological pipette during the fed-batch fermentation, the bioreactor was kept open for a few seconds during the addition of glycerol solution, and therefore, the fresh air might be accidentally introduced into the bioreactor by either pipetting or self-diffusion, which could potentially cause the cell culture being exposed to the dissolved oxygen shock.

Thus, in order to improve the unstable performance of the propanogenic strain which is constructed with the plasmid system, a metabolically engineered plasmid-free propanogenic strain, CPC-PrOH3 (Srirangan et al., 2014), was then derived for glycerol fermentation. Throughout the whole fed-batch cultivation, the glycerol dissimilation rate was greatly improved in the resulting strain, and about 7.5 g/L of 1-propanol was produced from ~115.8 g/L of glycerol with a shorter period of total cultivation time (210.5 h) compared with that obtained with the strain CPC-PrOH2 (245.5 h) (Table 12 & 13). These results suggest that the metabolic burden placed upon the host cell is alleviated by applying the chromosomal engineering strategy. Further analysis of the metabolites distribution in each specific stage shows that the catalytic activity of the enzymes encoded by the Sbm operon are maintained throughout the whole fed-batch fermentation, especially in the first three stages in which the fraction of dissimilated glycerol toward 1-propanol production is even gradually increased over time. In addition, only a small amount of the succinate remained unconverted in each specific stage, which also suggests that the native Sbm operon is functionally expressed as expected. However, a drastic drop in the glycerol efficiency was observed during the late stages of the cultivation, which might be related to the high-level accumulation of both acetate and ethanol in the culture medium. It has been reported that the accumulation of acetate to a certain level in the fermentation broth could inhibit the cell growth by reducing the intracellular pH and forming toxic intermediate homocysteine (Luli and Strohl, 1990; Irvine, 2005). On the other hand, the accumulation of ethanol in the medium could harm the cell by inducing nucleotides and ion leakage which could eventually result in the cell lysis (Ingram and Vreeland, 1980; Eaton, Tedder and Ingram, 1982; Dombek and Ingram, 1984). Thus, it is reasoned that the cell needs to consume more carbon source and energy to maintain the cell viability under these physiological stresses.

Chapter 6 Conclusions and Recommendations

Although today's economy relies heavily on the traditional petroleum industry for our daily energy supply, more and more novel technology have been developed and implemented to generate energy from renewable sources, such as solar power, wind power, and nuclear power. However, in light of the high cost, low efficiency, or high risk of utilizing these new energy sources, it would be meaningful to produce renewable energy from agricultural and biochemical waste though the microbial fermentation. Here, we reported a bench-scale bioreactor study for 1-propanol production by metabolically engineered *Escherichia coli* strains. Two carbon sources, glucose and glycerol, were investigated in this study. When glucose was used as the main carbon source, by knocking out one of the genes that were involved in lactate production (*ldhA*), minimal amount of lactate was produced and the level of solventogenesis was slightly enhanced, though acetate became the dominant metabolite and large amount of succinate remained unconverted in the culture medium. The results reveal that our metabolically engineered strain's 1-propanol production capacity is limited when using glucose as the main carbon source due to limited NADH availability. Therefore, in order to facilitate the conversion of succinate into 1-propanol via the extensive Sbm pathway, excess amount of NADH are required. When glycerol, which has a higher degree of reduction per carbon atom compared with the glucose, was used as the main carbon source, the 1-propanol production in our metabolically engineered strain was greatly improved and the overall metabolite distribution shifted from acidogenesis to solventogenesis. Hence, glycerol is a more suitable carbon source for 1-propanol production in our propanogenic *E. coli* strain. During the fed-batch cultivation of our propanogenic strain with glycerol, there was an inconsistency in 1-propanol production in later stages. The results imply that the functional expression of the Sbm operon on a plasmid might not be stable. By inserting a strong promoter

upstream of the native *E. coli* Sbm operon, a plasmid-free propanogenic strain was derived. The fed-batch fermentation of the resulting strain with glycerol allowed faster glycerol dissimilation rate and higher level of 1-propanol production with a final titer of 7.52 g/L from 115.82 g/L of glycerol.

However, the production of pyruvate derived fermentative metabolites (i.e., lactate, acetate, and ethanol) is overwhelming during the anaerobic fermentation of both glucose and glycerol, even with the Sbm operon being functionally expressed. Further metabolic engineering strategies that could be employed to prevent these by-products formation include knocking out genes that are involved in each metabolite synthesis pathway (e.g., *pta*, encoding phosphate acetyltransferase, *ackA*, encoding acetate kinase, *poxB*, encoding pyruvate oxidase, and *gloAB*, encoding glyoxalase I&II) and deleting genes that facilitate the conversion of PEP into acetyl-CoA via the pyruvate intermediate (e.g., *pfkFA*, encoding pyruvate kinase I&II, *pflB*, encoding pyruvate formate-lyase, and *ptsI,* encoding PTS enzyme I). On the other hand, by overexpressing *ppc* (encoding phosphoenolpyruvate carboxylase) or *pyc* (encoding pyruvate carboxylase), which drives the conversion of PEP or pyruvate, respectively, into the oxaloacetate, more carbon flux could be channeled toward the reductive arm of the TCA cycle instead of the pyruvate node, so that the production of pyruvate derived fermentative metabolites should be minimized.

Since glycerol is a superior carbon source for 1-propanol production in terms of the NADH-generating capacity and the cost compared to the glucose, it is worthwhile to put effort into optimizing the glycerol utilization pathways. By replacing the native *E.coli dhaK* gene (encoding the PEP-dependent dihydroxyacetone kinase) with the heterologous *C. freundii dhaK* gene (encoding the ATP-dependent dihydroxyacetone kinase), the PEP pool could be reserved

(Clomburg and Gonzalez, 2011). A similar effect could also be obtained by deleting the *gldA* (encoding glycerol dehydrogenase) and *dhaK* (encoding dihydroxyacetone kinase).

Except for the genetic and metabolic engineering strategies, cultivation strategies, such as medium optimization and cultivation conditions optimization, could also be explored to achieve a higher the 1-propanol productivity and yield. It is also recommended to test the 1-propanol-producing capacity of our metabolically engineered propanogenic strains by using other carbon sources (e.g., crude glycerol, lignocellulose biomass, and corn), which are more economically efficient, which has already been used or can be potentially used for the industrial-scale biological manufacturing of biofuels, compared with the high cost cell culture grade glucose and glycerol that are currently used in this study.

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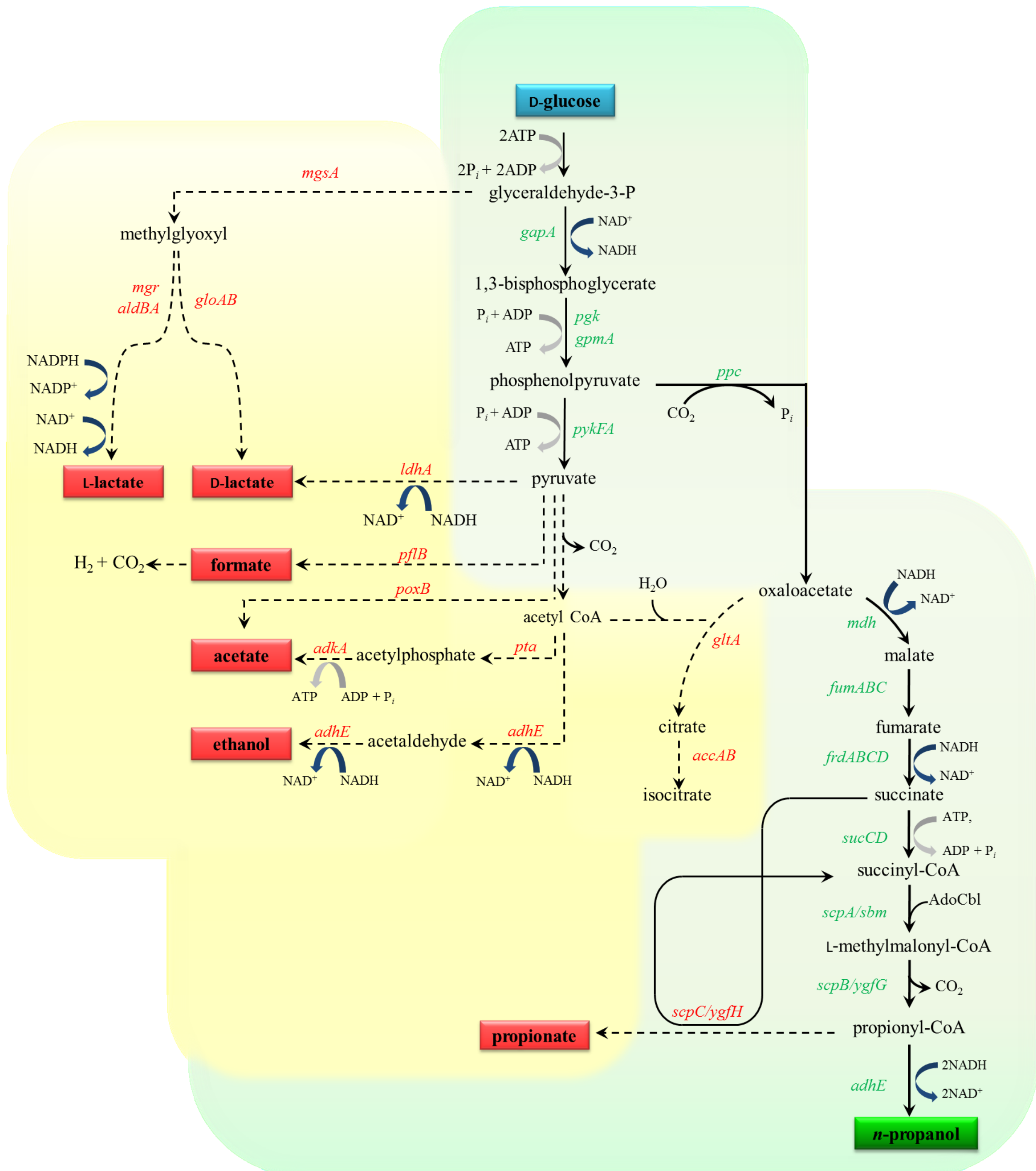
Appendix A



Supplementary Figure 1: The genetically engineered central metabolic pathway under anaerobic conditions showing the activation of the Sbm operon (*sbm*, *ygfD*, and *ygfG*)
Note. Adapted from Srirangan et al. (2013)