Metabolic Engineering of Propionibacteria for Enhanced Propionic Acid and n-Propanol Fermentative Production

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Ehab Mohamed Ammar

The Ohio State Biochemistry Program (OSBP)

The Ohio State University

2013

Dissertation Committee:

Professor Shang-Tian Yang, Adviser

Professor Ross Dalbey

Professor Jeffrey J. Chalmers
Propionibacteria are widely used in industry for manufacturing of Swiss cheese, vitamin B$_{12}$ and propionic acid. However, little is known about their genetics and only a few reports are available on their metabolic engineering aiming at enhancing production of vitamin B$_{12}$ and propionic acid. The latter is widely used as a chemical intermediate in various manufacturing processes, and its salts are common food preservatives. It is currently produced via petrochemicals, but there is increasing interest in its fermentative production from renewable biomass. However, current propionic acid fermentation process suffers from low product yield and productivity. n-Propanol is a common solvent, an intermediate in many industrial applications and a promising biofuel. To date, no wild-type microorganism is known to produce n-propanol in sufficient quantities for industrial purposes. However, some attempts to produce n-propanol in *E. coli* and *S. cerevisiae* have been reported, but the yields and titers are still unsatisfactory.

In this study, a bifunctional aldehyde/alcohol dehydrogenase (*adhE*) was cloned from *E. coli* and expressed in *P. freudenreichii*. The mutants expressing the *adhE* gene converted propionyl-CoA, which is the precursor for propionic acid biosynthesis, to n-propanol. The production of n-propanol was limited by NADH availability, which was improved
significantly by using glycerol as the carbon source. Interestingly, the improved propanol production was accompanied by a significant increase in propionic acid productivity, indicating a positive effect of n-propanol biosynthesis on propionic acid fermentative production. To our best knowledge, this is the first report on producing n-propanol by metabolically engineered propionibacteria, which offers a novel route to produce n-propanol from renewable feedstock, and possibly a new way to boost propionic acid fermentation.

In this work, the gene encoding phosphoenolpyruvate carboxylase (PPC) was cloned from \textit{E. coli} and expressed in \textit{Propionibacterium freudenreichii}. PPC catalyzes the conversion of phosphoenolpyruvate to oxaloacetate with the fixation of one \( \text{CO}_2 \). Its expression in \textit{P. freudenreichii} thus showed profound effects on propionic acid fermentation. Compared to the wild type, the mutant expressing the \textit{ppc} gene grew significantly faster on glycerol, consumed more glycerol and produced propionate to a higher final titer at a faster rate. These effects could be attributed to changes in the flux distributions in the dicarboxylic acid pathway.

Promoters are key elements in metabolic engineering as they control the level of gene expression. Some strong promoters from \textit{P. freudenreichii} have been reported. To our best knowledge, no report is available on analyzing promoter strengths from \textit{P. acidipropionici}. Therefore, eleven promoters from \textit{P. acidipropionici} ATCC 4875 were cloned into a shuttle vector with a promoterless reporter gene and transformed into the host. Promoter strengths were analyzed as function of the reporter gene activity. Results showed that these promoters were able to enforce expression of the reporter gene to
different levels in propionibacteria. In addition, sequence analysis showed that promoters from \textit{P. acidipropionici} were more complicated than those from regular prokaryotes, and hence more investigations are needed in this area. These findings should contribute towards improving future metabolic engineering of \textit{P. acidipropionici} for enhanced propionic acid fermentation.
Dedication

This document is dedicated to my family.
Acknowledgment

I would like to acknowledge my adviser, Prof. Shang-Tian Yang, for his continuous guidance, support and patience through my PhD work. He has a kind personality that makes you feel he is a family member of yours. I have learned a lot of things about life from him, let alone benefiting from his vast scientific knowledge.

I want to thank Dr. Ross Dalbey, Dr. Jeffrey Chalmers and Dr. Hamdy Hassanain for serving on my committee. Their easy and quick communication, guidance and scientific inputs through my research are invaluable.

I am very grateful to all my lab members who maintained a family-like atmosphere around. Truly, I owe all of them especially those who helped me at my very early beginnings including, but not limited to, Saju Varghese, Dr. Mingrui Yu, Dr. Michelle Nauerth and Brandon Crowe.

This work was supported in part by The Dow Chemical Company. The Egyptian Government’s overseas graduate study scholarship (General Mission) is highly acknowledged. Also, I wish to thank Dr. Murooka of Osaka University, Japan and Dr. Dag Anders Brede of Norwegian University of Life Sciences, Norway for providing key plasmids used in my research work.
Last but not least, I will always be indebted to my parents, sisters, wife, kids and all family members for their endless and unconditioned love, support, patience and prayers. Likewise, I am indeed very grateful to all my friends for their continuous encouragement, sincere support and belief in me.
Vita

<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>9th June 1978</td>
<td>Born in Egypt</td>
</tr>
<tr>
<td>June 2000</td>
<td>BSc of Pharmacy, Cairo University, Egypt</td>
</tr>
<tr>
<td>January 2007</td>
<td>MSc of Genetic Engineering and Biotechnology (Pharmaceutical Industries), GEBRI, Minoufiya University, Egypt</td>
</tr>
<tr>
<td>January 2009 - July 2013</td>
<td>Graduate Research Associate, OSBP, The Ohio State University, USA</td>
</tr>
</tbody>
</table>

Publications


Provisional Patent


Field of study

Major Field: Biochemistry

Specialty: Metabolic and Bioprocess Engineering
# Table of Contents

Abstract ......................................................................................................................... ii
Dedication ...................................................................................................................... v
Acknowledgment ......................................................................................................... vi
Vita ............................................................................................................................... viii
Table of Contents ......................................................................................................... x
List of Figures ................................................................................................................ xiv
List of Tables ................................................................................................................ xvi

## CHAPTER 1

INTRODUCTION ............................................................................................................. 1

Objectives ....................................................................................................................... 7
References ....................................................................................................................... 10

## CHAPTER 2

LITERATURE REVIEW ................................................................................................. 15

Propionic Acid ................................................................................................................ 15
Propanol .......................................................................................................................... 17
Propionibacteria ............................................................................................................ 19
Metabolic Pathway ........................................................................................................ 20
Transformation of Propionibacteria ............................................................................ 21
Metabolic Engineering of Propionibacteria ................................................................ 23
Metabolic Engineering of E. coli for Propanol Production ........................................ 26
Metabolic Flux Analysis .............................................................................................. 27
Fermentation Process ................................................................................................... 28
Carbon Source ............................................................................................................. 30
CHAPTER 3

METABOLIC ENGINEERING OF PROPIONIBACTERIUM FREUDENREICHII FOR N-PROPANOL PRODUCTION

Summary ........................................................................ 49
Introduction.................................................................... 51
Materials and Methods......................................................... 53
  Bacterial Strains, Plasmids, and Media.......................... 53
  Construction of aad and adhE Expression Vectors ........ 54
  Transformation of Propionibacteria.............................. 55
  Fermentation Kinetics............................................... 56
  Stoichiometric Analysis of Carbon Flux Distribution .... 56
  Analytical Methods................................................... 57
  Statistical Analysis................................................... 57
Results............................................................................ 58
  Cloning and Expressing adhE and aad in Propionibacteria .... 58
  Effect of adhE Expression on Cell Growth.................. 59
  Batch Fermentation Kinetics with Glucose as Carbon Source ................................................. 59
  Batch Fermentation Kinetics with Glycerol as Carbon Source ............................................. 60
  Effects of adhE Expression and n-Propanol Biosynthesis on Flux Distribution ..... 61
  Effects of Propanol on Propionic Acid Fermentation................................................................. 63
Discussion..................................................................... 64
References.................................................................... 70

CHAPTER 4..................................................................... 86
METABOLIC ENGINEERING OF PROPIONIBACTERIUM FREUDENREICHII: EFFECT OF EXPRESSING PPC FROM E. COLI ON PROPIONIC ACID PRODUCTION

Summary ......................................................................................................................... 86
Introduction ..................................................................................................................... 88
Materials and Methods ................................................................................................ 90
  Bacterial Strains, Plasmids, and Media ......................................................................... 90
  Construction of ppc Expression Vector and Transformation ...................................... 91
  PPC Enzyme Activity Assay ....................................................................................... 91
  Fermentation Kinetics .................................................................................................. 92
  Stoichiometric Analysis of Carbon Flux Distribution .................................................. 92
  Analytical Methods ..................................................................................................... 93
  Statistical Analysis ....................................................................................................... 93
Results ................................................................................................................................ 93
  Cloning and Expressing ppc in Propionibacteria ......................................................... 93
  PPC Enzyme Activity ................................................................................................... 94
  Effect of Propionic Acid on Growth ........................................................................... 95
  Batch Fermentation Kinetics with Glucose as Carbon Source in Serum Bottles .... 95
  Batch Fermentation Kinetics with Glycerol as Carbon Source in Serum Bottles .... 96
  Batch Fermentation Kinetics in Bioreactor with pH Controlled at 6.5 ................. 97
  Effects of ppc Expression on Flux Distribution ......................................................... 98
Discussion ....................................................................................................................... 99
References ....................................................................................................................... 103

CHAPTER 5 ............................................................................................................. 119

CLONING AND FUNCTIONAL ANALYSIS OF CONSTITUTIVE PROMOTERS FROM PROPIONIBACTERIUM ACIDIPROPIONICI ATCC 4875

Summary ......................................................................................................................... 119
Introduction ..................................................................................................................... 121
Materials and Methods ................................................................................................ 123
  Bacterial Strains and Plasmids ................................................................................... 123
Culture Media ................................................................. 123
DNA Manipulations .......................................................... 124
Cloning Promoters by PCR Amplification ................................ 124
Construction of the Expression Vectors .................................... 125
Transformation of Propionibacteria ......................................... 125
Checking Prospective Mutants ............................................. 126
β-Galactosidase Assay ....................................................... 126
SDS-PAGE Analysis .......................................................... 127

Results ............................................................................. 128
Confirmation of Propionibacteria Mutants ............................... 128
Promoter Activity ............................................................ 128
SDS-PAGE Analysis .......................................................... 128
Promoter Sequence Analysis .............................................. 129

Discussion ........................................................................ 129

References ........................................................................ 133

CHAPTER 6 ........................................................................ 143

CONCLUSIONS AND RECOMMENDATION ............................... 143

6.1 Metabolic Engineering for n-Propanol Production .................. 143
Further research in the following areas is recommended. .......... 144

6.2 Metabolic Engineering for Propionic Acid Production ............ 145
Further research in the following areas is recommended. .......... 147

6.3 Analysis of Promoters from P. acidipropionici ATCC 4875 .......... 148
Further research in the following areas is recommended. .......... 148

BIBLIOGRAPHY ................................................................ 150

APPENDIX: SUPPLEMENTARY FIGURES .................................. 161
List of Figures

Figure 2.1 Dicarboxylic acid pathway in propionibacteria ................................................................. 46
Figure 2.2 Conversion of 2-ketoacid to propanol in E. coli (Shen and Liao 2008) ......................... 47
Figure 2.3 Engineering E. coli to produce n-propanol (Choi et al. 2012) ................................. 48
Figure 3.1 Dicarboxylic acid pathway and the expression of heterologous adhE for n-propanol biosynthesis in propionibacteria. ............................................................................. 78
Figure 3.2 Construction of pKHEM04-adhE .................................................................................... 79
Figure 3.3 PCR confirmation of adhE-mutants (a) Culture PCR using prospective mutants (b) Culture PCR using E. coli (c) PCR using pKHEM04-adhE. ......................................................... 80
Figure 3.4 Cell growth kinetics on glucose (a) and glycerol (b) for the wild type (WT) and mutant Pf(adhE). (c) Comparison of the specific growth rates of the WT and mutant......................................................................................................................... 81
Figure 3.5 Kinetics of batch fermentation of glucose by P. freudenreichii wild type and mutant strains Pf(adhE)-1 and Pf(adhE)-2 expressing adhE. ............................................................ 82
Figure 3.6 Kinetics of batch fermentation of glycerol by P. freudenreichii wild type and mutant strains Pf(adhE)-1 and Pf(adhE)-2 expressing adhE. ............................................................ 83
Figure 3.7 Kinetics of batch fermentation of glycerol by mutant strain Pf(adhE)-2 expressing adhE in the bioreactor at pH 6.5. ............................................................................................................................ 84
Figure 3.8 Effects of propanol on cell growth (a), glycerol consumption (b) and propionic acid production (c) in batch fermentations of P. freudenreichii wild type grown on glycerol in the presence of various concentrations of propanol ......................................................... 85
Figure 4.1 Dicarboxylic acid pathway for propionic acid biosynthesis in propionibacteria. Some propionibacteria such as P. acidipropionici ATCC 4875 has pyruvate carboxylase (PYC) that can convert pyruvate to oxaloacetate with CO2 fixation. ........................... 111
Figure 4.2 Construction of pKHEM04-ppc ................................................................. 112
Figure 4.3 Comparison of PPC enzyme activity in P. freudenreichii DSM 20271 (wild
type, WT) and mutant Pf(ppc) expressing ppc................................................................. 113
Figure 4.4 Effect of propionic acid on cell growth on glycerol without CaCO₃ for pH
buffering. The initial medium pH was ~6.5. WT: wild type; Pf(ppc): mutant
expressing ppc...................................................................................................................... 114
Figure 4.5 Batch fermentation kinetics with glucose in serum bottles with and without
CaCO₃ for pH buffering.......................................................................................................... 115
Figure 4.6 Batch fermentation kinetics with glycerol in serum bottles with and without
CaCO₃ for pH buffering.......................................................................................................... 116
Figure 4.7 Batch fermentation kinetics in bioreactor with pH controlled at ~6.5. ....... 117
Figure 4.8 Propionate yields from glucose and glycerol in serum bottles with CaCO₃
(~pH 5) and in bioreactor (pH 6.5). .................................................................................... 118
Figure 5.1 Construction of expression vectors. ................................................................. 137
Figure 5.2 Some constructed promoter plasmids and their corresponding double digestion
pattern with BglII and EcoRV............................................................................................. 138
Figure 5.3 PCR of chl gene using plasmids extracted from propionibacteria mutants... 139
Figure 5.4 β-Galactosidase activity in different propionibacteria mutants.................... 140
Figure 5.5 SDS-PAGE analysis of the β-galactosidase expression................................. 141
Figure 5.6 Promoter sequence analysis............................................................................... 142
Figure A.1 Serum bottles used in anaerobic fermentation.............................................. 161
Figure A.2 Custom made bioreactor .................................................................................. 162
Figure A.3 Examples for calculation of some fermentation kinetics parameters .......... 163
Figure A.4 GC chromatogram showing n-propanol peak.............................................. 164
Figure A.5 HPLC chromatogram showing peaks of carbon source and products. ....... 165
List of Tables

Table 3.1 Bacterial strains, plasmids and primers used in this study. ..............................73
Table 3.2 Stoichiometric equations and constraints used in the metabolic carbon flux
distribution analysis. ....................................................................................................74
Table 3.3 Kinetic parameters of WT, Pf(adhE)-1 and Pf(adhE)-2 on different carbon
sources..........................................................................................................................75
Table 3.4 Stoichiometric analysis of carbon flux distributions in wild type and adhE-
mutant grown on glucose and glycerol in batch fermentations................................76
Table 3.5 Effects of n-propanol on propionic acid fermentation by P. freudenreichii WT
grown on glycerol in serum bottles...........................................................................77
Table 4.1 Bacterial strains, plasmids and primers used in this study. .........................107
Table 4.2 Stoichiometric equations and constraints used in the metabolic carbon flux
distribution analysis. .................................................................................................108
Table 4.3 Fermentation kinetics parameters from glucose and glycerol batches. .........109
Table 4.4 Stoichiometric analysis of carbon flux distributions in wild type and ppc-
mutant grown on glucose and glycerol in batch fermentations in serum bottles with
CaCO$_3$ (~pH 5.0).........................................................................................................110
Table 5.1 Bacterial strains and plasmids.........................................................................135
Table 5.2 Primers for promoters cloning.......................................................................136
CHAPTER 1

INTRODUCTION

Propionibacteria are gram positive, rod shaped and facultative anaerobic bacteria widely used in industry for the production of Swiss cheese for eye formation and flavor development (Thierry et al. 2004). Because of their GRAS (Generally Recognized as Safe) status granted by the FDA (United States Food and Drug Administration), propionibacteria are also the preferred and widely used organisms for propionic acid (food preservative) and vitamin B\textsubscript{12} production (Kośmider et al. 2011; Piao et al. 2004b; Suwannakham and Yang 2005; Wang and Yang 2013; Zhang and Yang 2009a; 2009b).

There have been extensive studies on the growth and metabolic properties of propionibacteria (Hettinga and Reinbold 1972a; 1972b; 1972c; Playne 1985). Metabolically, propionibacteria synthesize propionic acid through the dicarboxylic acid pathway together with other acids, mainly acetate and succinate.

Propionic acid is also called propanoic acid and its salts and/or esters are called either propionates or propanoates. Propionic acid is commonly used as a chemical intermediate in various industrial processes such as manufacturing of polymers, pesticides, and
pharmaceuticals. Propionates are widely used as food preservatives at concentrations ranging from 0.1 to 1% per weight due to their anti-mold activity (Boyaval and Corre 1995). In addition, they are used as solvents and as flavoring agents because of their fruity odors.

According to a report from ICIS Chemical Business on propionic acid market in the US, propionic acid consumption in the US in 2008 was estimated to 42 million pounds, and demand on propionic acid is generally connected to the food industry. The demand on propionic acid was 233, 240 and is expected to be 260 million lbs in 2007, 2008 and 2012, respectively. The market growth through (2003-2008) was 2.9% per year; the expected future growth through 2012 was 2.3% per year. Price used to be around 85 cents/lb through (2003-2008) and increased to 92-97 cents/lb in 2009 (web.ebscohost.com).

Currently, propionic acid is mainly produced in industry from petrochemicals. Recent increases in oil prices, environmental pollution associated with using petrochemicals, and rising demands for natural ingredients in different products by consumers, have led to increasing interest in fermentative production of propionic acid from bio-renewable resource. However, fermentative production is not yet satisfactory for industrial purposes and more enhancements are still required to make it competitive to petrochemicals processes.

In theory, the maximum yield of propionic acid from glucose is 0.548 g/g based on Fitz equations (Boyaval and Corre 1995; Leaver et al. 1955). However, actual propionic acid
yields are less than theoretical values because some of the carbon flux is directed to cell growth (Lewis and Yang 1992b; Obaya et al. 1994). Of the major downsides of propionic acid fermentation is the presence of byproducts, mainly acetic acid, which makes downstream processing more difficult and render the entire process costly. The lower the propionate/acetate (P/A) ratios, the higher the downstream processing cost and vice versa. Another factor that influences propionic acid fermentation negatively is the inhibitory effect of propionic acid on growth and product formation (Blanc and Goma 1987; Gu et al. 1998; Herrero 1983; Ibragimova et al. 1969; Lewis and Yang 1992a; Nanba et al. 1983; Neronova et al. 1967; Ozadali et al. 1996; Paik and Glatz 1994; Yang et al. 1994). For the previously mentioned reasons, there have been continuous efforts from scientists to enhance propionic acid fermentation through improving the final concentration, yield and productivity (Ammar et al. 2013; Dishisha et al. 2013; Kagliwal et al. 2013; Liu et al. 2012; Wang and Yang 2013).

1-Propanol is a primary alcohol mainly used as a solvent, a carrier for a variety of natural products and synthetic polymers, and an intermediate in many manufacturing processes such as propylene and propyl derivatives. It is also used in pharmaceuticals and cosmetics, both as a solvent and as an anti-microbial agent. Like propionic acid, propanol is mainly produced via petrochemical processes. Although propanol production was detected in Clostridium spp. from threonine catabolism (Janssen 2004), in beer fermentation by yeast (Eden et al. 2001), in propionic acid fermentation by Propionibacterium acidipropionici and P. freudenreichii ssp. shermanii (Himmi et al. 2000; Liu et al. 2011), wild-type microorganism cannot produce propanol in amounts
satisfactory for industrial applications. Current research efforts to improve bio-propanol production focus on genetic engineering of *E. coli* and *S. cerevisiae* and up to 4 and 10 g/L was reached from glucose and glycerol, respectively (Atsumi and Liao 2008; Choi et al. 2012; Matsuda et al. 2011; Shen and Liao 2008). However, further improvements in product titer, yield and productivity are necessary for industrial applications.

Engineering the metabolic pathway for propionate production in bacteria is an approach that can be used to improve propionic acid yield and/or switch the pathway towards propanol. However, not much is known about the genetics of propionibacteria with few whole genome sequences are publicly available in the GenBank (Falentin et al. 2010; Horváth et al. 2012; Meuricea et al. 2004; Parizzi et al. 2012; Vörös et al. 2012).

In general, genetic manipulation of propionibacteria is difficult because of the low efficiency at which they are transformed with external DNA. Many factors contribute to the low transformation efficiency of propionibacteria including the presence of cell wall, the host restriction modification system, the presence of endogenous plasmid and the high GC content of the bacteria (Cheong et al. 2008; Genevieve 1988; Jore et al. 2001; Luijk et al. 2002). Generally speaking, little work has been done in this area and most published work aimed at improving vitamin B12 production using *P. freudenreichii*. p4 and p138 were recognized as strong promoter elements from *P. freudenreichii* (Piao et al. 2004a). Later, some promoters of a wide range of strengths, and allegedly conserved -10, -16 and -35 regions were identified (Faye et al. 2008; Piao et al. 2004a). So far, no similar study has been done for promoter elements from *P. acidipropionici*. 
Several plasmids were identified and characterized from different *Propionibacterium sp.* including pRGO1, 2, 3, 4, 5, 6 and 7 plasmids (Rehberger and Glatz 1990). In addition, several shuttle vectors that can shuttle between *E. coli* and propionibacteria were reported including pBRESP (Jore et al. 2001), pTD210 (Faye et al. 2008), pK705 (Kiatpapan et al. 2000), pKHEM01 and 04 (Kiatpapan and Murooka 2001) and pAMT1 (Brede et al. 2005). Of these vectors, we have interest in two vector systems, pKHEM and pTD210 (Faye et al. 2008; Kiatpapan et al. 2000; Kiatpapan and Murooka 2001; 2002).

An advantage of pKHEM01 and 04 vectors is the use of strong promoters, p138 and p4 respectively, to control the expression of the downstream gene. Alternatively, pTD210 vector has a promoterless *lacZ* gene marker to measure the strength of promoter sequences cloned into a multiple cloning site upstream of the *lacZ* gene. These vectors have the advantage of being derived from *propionibacteria sp.* which minimizes their rejection by the restriction modification (RM) system of the host, and makes it possible to extend their use in other *propionibacteria sp.* for controlling expression of different genes.

Not much work has been published on improving propionic acid and/or propanol production by genetic manipulation of propionibacteria-with the exception of knocking out the acetate kinase gene in *P. acidipropionici* ATCC 4875 (Suwannakham et al. 2006) and overexpressing *adhE* gene from *E. coli* in *P. freudenreichii* to produce n-propanol (Ammar et al. 2013). The knockout transformant in that study showed 14% decrease in acetate formation and a corresponding 13% increase in propionate, and unexpectedly, the
n-propanol producing mutants showed better propionic acid fermentation kinetics. In conclusion, many questions in this area still need answers.

In this research, a bifunctional aldehyde/alcohol dehydrogenase (adhE) cloned from \textit{E. coli} was successfully expressed in \textit{P. freudenreichii} and its effects on the transformants were studied (Ammar et al. 2013). The mutant expressing the \textit{adhE} gene not only produced n-propanol from glucose and glycerol, but also showed higher propionic acid productivity due to increased substrate uptake or consumption rate. This study is the first attempt to produce n-propanol from metabolically engineered propionibacteria, offering a novel route for n-propanol biosynthesis and probably for enhancing propionate biosynthesis as well. Similarly, this study demonstrated the feasibility of improving propionic acid production in \textit{P. freudenreichii} DSM 20271 by overexpressing a phosphoenolpyruvate carboxylase (PPC) from \textit{E. coli}. PPC should, in theory, direct more carbon to propionate at the expense of acetate increasing the propionate yield. Unexpectedly, the propionate yield did not go up, but the mutant showed better growth and production kinetics. Such kinetics enhancements were observed with glucose and more profound with glycerol. With further process engineering, such approach should contribute towards economic production of propionate from sugars and renewable feedstocks. Moreover, in this work eleven promoters from \textit{P. acidipropionici} were cloned into pTD210, a reporter vector with promoterless \textit{lacZ} gene, to analyze their strengths based on the corresponding activities of the reporter gene. The studied promoters enforced gene expression to different levels. No distinct promoter regions were observed in the given promoter sequences indicating that they are more complicated than those
from regular prokaryotes. These findings would contribute towards improving future metabolic engineering of *P. acidipropionici* for enhanced propionic acid/n-propanol fermentation.

In general, more investigations are still needed to better our understanding of the genetic elements and metabolic reactions in propionibacteria. Such investigations should help develop new economic processes for enhanced propionic acid and n-propanol biosynthesis from cheap renewable substrates.

**Objectives**

This work aimed at improving propionic acid fermentative production by using metabolic and bioprocess engineering approaches. Another main goal is to engineer propionibacteria to produce n-propanol which is hardly produced by wild type propionibacteria. The specific goals of this study are listed below:

1- **Metabolic engineering of propionibacteria for producing n-propanol by expressing a heterologous aldehyde/alcohol dehydrogenase:** By expressing an aldehyde/alcohol dehydrogenase in propionibacteria, propionyl-CoA should, in theory, be converted to n-propanol. For this purpose, two different aldehyde/alcohol dehydrogenases were cloned into two different vectors. Mutants producing n-propanol were obtained from *P. freudenreichii* DSM 4902 overexpressing heterologous *adhE* gene. Kinetics of n-propanol production from glucose in serum bottles was studied. Production
was limited by the availability of reducing equivalents which was alleviated by switching to glycerol as carbon source. Mutants demonstrated higher specific growth rates, carbon source consumption rates and propionate productivity compared to the wild type. Such enhancements were more manifest when using glycerol as carbon source. The effects of n-propanol on propionate fermentation kinetics and the metabolic flux distribution were investigated. This is the first report on successful n-propanol production from metabolically engineered *P. freudenreichii* and it offers a novel route to enhance propionic acid production as well.

2- **Enhancing propionic acid production in propionibacteria by expressing heterologous ppc gene:** PPC in theory should direct more carbon flux to propionate at the expense of acetate by converting PEP directly to OAA. The propionate yield and P/A ratio should therefore, in theory, go up. For this purpose two plasmids were constructed, and mutants overexpressing *ppc* were obtained from *P. freudenreichii* DSM 20271. Unexpectedly, the mutants did not show any significant increase in yields. However, the propionate productivity was enhanced especially with glycerol as carbon source. The kinetics of propionate production using glucose and glycerol as carbon sources were studied in serum bottles and bioreactor. The effects of *ppc* overexpression on growth and metabolic flux distribution were investigated. The observed results demonstrated distinct improvements in cell growth, consumption rate, productivity and P/A ratio with glycerol. This metabolic engineering approach together with further bioprocess engineering could facilitate the development of a fermentation process for the economic production of propionic acid from sugars and other renewable feedstocks.
3- Analyzing constitutive promoters from *P. acidipropionici* for enhancing the metabolic engineering of propionibacteria: Not much is known about the genetics of propionibacteria. Being key elements in controlling gene expression, some investigations have been published on promoters from *P. freudenreichii*, however nothing similar from *P. acidipropionici* has been reported. In this study, eleven promoters from *P. acidipropionici* ATCC 4875 were cloned into a reporter vector (pTD210) and transformed into propionibacteria. The promoter strengths in the mutants were analyzed as function of a reporter gene (β-galactosidase) activity. Results showed that these promoters were able to enforce expression of the reporter gene to different levels. In addition, sequences were analyzed and the promoters from *P. acidipropionici* seemed to be more complicated than those from regular prokaryotes. These findings will contribute towards improving future metabolic engineering of *P. acidipropionici* and other propionibacteria for enhanced propionic acid fermentation.
References


Hettinga DH and Reinbold GW (1972b) The propionic-acid bacteria II. Metabolism. J Milk Food Technol. 35:358-372

Hettinga DH and Reinbold GW (1972c) The propionic-acid bacteria III. Miscellaneous metabolic activities. J Milk Food Technol. 35:436-447


Neronova NM, Ibragimova SI and Ierusalimski ND (1967) Effect of propionate concentration on the specific growth rate of *Propionibacterium shermanii*. Mikrobiologiya. 36:404-409


CHAPTER 2

LITERATURE REVIEW

Propionic Acid

Propionic acid is also called propanoic acid and its salts and/or esters are called either propionates or propanoates. It is a natural carboxylic acid with three carbon atoms (CH₃CH₂COOH) and it is a clear pungent liquid. Physically, it is an intermediate between the smaller carboxylic acids, acetic and formic acids, and larger fatty acids. Chemically, it undergoes reactions typical to carboxylic acids such as amide and ester formation. Regarding solubility, it is miscible with water.

Propionic acid is commonly used as a chemical intermediate in various industrial processes such as manufacturing of polymers, pesticides, and pharmaceuticals. Propionates are widely used as food preservatives at concentrations ranging from 0.1 to 1% per weight due to their anti-mold activity (Boyaval and Corre 1995). In addition, they are used as solvents and as flavoring agents because of their fruity odors.
According to a report from ICIS Chemical Business on propionic acid market in the US, propionic acid consumption in the US in 2008 was estimated to 42 million pounds, and demand on propionic acid is generally connected to the food industry. The demand on propionic acid was 233, 240 and is expected to be 260 million lbs in 2007, 2008 and 2012, respectively. The market growth through (2003-2008) was 2.9% per year; the expected future growth through 2012 was 2.3% per year. Price used to be around 85 cents/lb through (2003-2008) and increased to 92-97 cents/lb in 2009 (web.ebscohost.com).

Currently, propionic acid is mainly produced in industry from petrochemicals (Bertleff et al. 2000). It is produced either by hydrocarboxylation of ethylene in presence of a catalyst, nickel carbonyl, according to the following reaction:

\[ \text{H}_2\text{C}=\text{CH}_2 + \text{H}_2\text{O} + \text{CO} \rightarrow \text{CH}_3\text{CH}_2\text{CO}_2\text{H} \]

Or, it is produced by oxidation of propionaldehyde at 40-50 °C in presence of catalysts, cobalt or magnesium ions, according to the following reaction (Boyaval and Corre 1995):

\[ \text{CH}_3\text{CH}_2\text{CHO} + \frac{1}{2} \text{O}_2 \rightarrow \text{CH}_3\text{CH}_2\text{COOH} \]

Propionic acid is a byproduct formed during industrial production of acetic acid. So far, BASF Chemicals is the largest global manufacturer of propionic acid with an annual capacity of 150,000 metric tons in 2009 (www.basf.com).

Recent increases in oil prices, environmental pollution associated with using petrochemicals, and rising demands for natural ingredients in different products by
consumers, have led to increasing interest in fermentative production of propionic acid from bio-renewable resource. Naturally, propionic acid is the main fermentation product of many microorganisms including *Clostridium propionicum*, Selenomonas and Veillonella (Boyaval et al. 1994; Playne 1985; Seshadri and Mukhopadhyay 1993). However, fermentative production is not yet satisfactory for industrial purposes and more enhancements are still required to make it competitive to petrochemicals processes.

**Propanol**

Propanol is also called normal propanol, n-propanol, n-propyl alcohol, 1-propyl alcohol or propan-1-ol (IUPAC name). It is a primary alcohol of three carbon atoms and its formula is (CH₃CH₂CH₂OH) or (C₃H₇O). Propanol is a colorless, volatile and highly inflammable liquid. It has a sharp musty odor like rubbing alcohol. Regarding solubility, it is highly miscible with water. Chemically, it undergoes reactions characteristic to primary alcohols such as forming alkyl halides upon reacting with halogens and forming esters upon reacting with organic acids. Depending on oxidation conditions applied, it can be oxidized to either propionaldehyde or it can be further oxidized to propionic acid.

Propanol is mainly used as a solvent and carrier for a variety of natural products and synthetic polymers. It is also used in pharmaceuticals and cosmetics, such as lotions and soaps, both as a solvent and as an anti-microbial agent. Moreover, it serves as an intermediate in the manufacturing of many industrially important chemicals such as propylene and propyl derivatives.
In earlier times, propanol was produced by fractional distillation of fusel oils or alcohols, a mixture of alcohols formed by fermentation. Currently, propanol production is achieved by hydroformylation (reaction with carbon monoxide and hydrogen) of ethylene leading to proionaldehyde which, in the presence of a catalyst, is subjected to hydrogenation and forms propanol. It is also a byproduct in some manufacturing processes such as in methanol formation using carbon monoxide and hydrogen, and in propane oxidation.

Nowadays, there has been increased interest in renewable energy sources due to increasing oil prices, and environmental problems associated with the use of petrochemicals. Ethanol, the most common gasoline substitute, has the downsides of being relatively of low energy density, high hygroscopicity and vapor pressure and incompatible with current combustion systems. This has motivated scientists to search for better gasoline substitutes. In general, alcohols with longer chains like n-propanol are better than ethanol as biofuel (Atsumi and Liao 2008; Atsumi et al. 2010). They have higher energy densities and are less hygroscopic. The latter feature makes them better in terms of storage and distribution. Moreover, they are more compatible with current combustion systems. When mixed with gasoline, n-propanol can serve as antiknock and an octane number increasing additive. Hence, its use in fuel blends has been suggested (Barannik et al. 2005). To date, no wild-type microorganism is known to produce n-propanol in sufficient quantities for industrial application purposes.
Propionibacteria

Propionibacteria are gram positive, rod shaped and facultative anaerobic bacteria widely used in industry for the production of Swiss cheese for eye formation and flavor development (Thierry et al. 2004). The characteristic flavor of Swiss cheese is caused by the produced acids (acetic and propionic), proline and metabolites generated during amino acids catabolism (Hettinga and Reinbold 1972c; Quelen et al. 1995). On the other hand, eye formation is brought by CO₂ generation (Langsrud and Reinbold 1973). Because of their GRAS (Generally Recognized as Safe) status granted by the FDA (United States Food and Drug Administration), propionibacteria are also the preferred and widely used organisms for propionic acid (food preservative) and vitamin B₁₂ production (Dishisha et al. 2013; Kagliwal et al. 2013; Kośmider et al. 2011; Liu et al. 2012; Piao et al. 2004b; Suwannakham and Yang 2005; Zhang and Yang 2009a; 2009b; Wang and Yang 2013).

There have been extensive studies on the growth and metabolic properties of propionibacteria (Hettinga and Reinbold 1972a; 1972b; 1972c; Playne 1985). Carbon and nitrogen sources, in addition to trace elements such as Mg²⁺ and Mn²⁺, are crucial for cell growth and products formation in propionibacteria fermentations (Balamurugan et al. 1999; Katagiri and Ichikawa 1953). Optimally, cells grow under anaerobic conditions in the presence of N₂ gas, at 30-32°C and pH 6-7 where below pH 4.5 cellular activities are halted (Hsu and Yang 1991; Jin and Yang 1998; Playne 1985). Metabolically, propionibacteria synthesize propionic acid through the dicarboxylic acid pathway together with other acids, mainly acetate and succinate.
**Metabolic Pathway**

In propionibacteria glucose is converted to propionate, with the formation of acetate and succinate as byproducts, through what is called the dicarboxylic acid pathway (see Figure 2.1). Like in many other bacteria, the phosphoenolpyruvate (PEP)–pyruvate–oxaloacetate node acts as a switch point for distributing the carbon flux towards catabolism, anabolism or energy generation for cellular activities (Sauer et al. 2005). From pyruvate the pathway diverges into two branches; one branch leading to propionate and the other leading to acetate.

In the propionate branch, oxaloacetate can be formed from pyruvate by pyruvate carboxylase and/or oxaloacetate transcarboxylase (Playne 1985). In most microorganisms, pyruvate carboxylase enzyme has $\alpha_4$ structure consisted of four 120-130 kD identical $\alpha$ subunits, however $\alpha_4\beta_4$ versions of the enzyme do exist (Jitrapakdee et al. 1999; Sauer et al. 2005).

Oxaloacetate can also be formed directly and irreversibly from phosphoenolpyruvate by phosphoenolpyruvate carboxylase. Most of phosphoenolpyruvate carboxylases consist of four 90-110kD subunits, and can coexist only with $\alpha_4\beta_4$ pyruvate carboxylases. Generally, phosphoenolpyruvate carboxylases are not present in bacteria with $\alpha_4$ pyruvate carboxylases (Dunn et al. 1996).

In the acetate branch, pyruvate is decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex, and then acetyl-CoA is converted to acetate by the actions of phosphotransacetylase and acetate kinase. ATP molecules generated in this branch serve
as energy source for cells. Meanwhile, energy can be provided from acetyl-CoA through the TCA cycle.

According to a recently published article on the whole genome sequence of *P. acidipropionici*, the phosphoenolpyruvate carboxylase did not exist in the studied bacteria, and CO$_2$ fixation capability was attributed to pyruvate carboxylase (Parizzi et al. 2012). Likewise, phosphotransacetylase and acetate kinase did not exist. An alternative one step mechanism for acetate formation using an ADP-forming acetyl-CoA synthetase, which converts acetyl-CoA, inorganic phosphate and ADP to acetate-CoA–SH and ATP, was suggested.

One of the major targets in propionic acid fermentation by propionibacteria is to direct the carbon flux more towards propionate at the expense of acetate raising the P/A ratio. And among studied approaches to raise the P/A ratio is the knocking out of the acetate kinase gene in the acetate branch (Suwannakham et al. 2006), and proper choice of carbon source (Wang and Yang 2013; Zhang and Yang 2009b)

**Transformation of Propionibacteria**

Electroporation is the main method by which propionibacteria is transformed with foreign DNA. However, the transformation efficiency is generally low. There are many factors that contribute to the low transformation efficiency, including but not limited to, 1- The wall thickness which is a common barrier in gram positive bacteria; 2- The presence of host restriction-modification system(s) that acts as a barrier against foreign
DNA (Jore et al. 2001); 3- The high GC content that stands as another barrier against transforming propionibacteria with exogenous DNA.

One way to improve the transformation efficiency of propionibacteria is to use plasmids originally derived from propionibacteria or extracted from propionibacteria transformants. This should lower the rejection of foreign DNA by the host restriction-modification system(s). For example, $10^3$-fold and up to $10^7$-fold improvement in transformation efficiency has been reported when *P. freudenreichii* was transformed with propionibacteria-derived DNA compared to *E. coli*-derived DNA materials (Jore et al. 2001; Kiatpapan et al. 2000).

In 2008, Cheong and co-workers investigated many conditions influencing the transformation efficiency of *P. acne* such as using cell wall weakening agents, growing cells at various temperatures, applying different electric field strengths and pulse durations, and extracting DNA from different *E. coli* strains. According to the former investigation, incubation temperature during competent cells preparation, pulse duration and using dam$^{-}$ *E.coli*-derived DNA were critical factors. It was believed that dam$^{-}$ *E.coli*-derived DNA would evade the host methyl-dependant restriction modification.

For selection of successfully transformed propionibacteria, many antibiotic resistance genes can serve as selective markers, including genes encoding chloramphenicol (*cat* and *cml*), erythromycin (*ery*), hygromycin B (*hyg*) and tetracycline (*tet*) antibiotics (Huang 1998; Jore et al. 2001; Suwannakham et al. 2006).
Metabolic Engineering of Propionibacteria

Metabolic engineering is a practice that aims at obtaining new phenotypes with increased or decreased production of specific metabolites, not necessarily produced naturally by cells, by altering or setting up new metabolic pathways in host cells using recombinant DNA technology. The debut of metabolic engineering is linked to the emergence of recombinant DNA technology (Stephanopoulos 1999), and it has been revolutionized with continuous advances in computational analysis and experimental tools used in genetics and biochemistry fields (Raab et al. 2005). Generally, metabolic engineering utilizes different tools for: 1- Cell genetic manipulation; 2- Metabolic flux analysis; 3- Cell status analysis (Raab et al. 2005).

With the help of metabolic engineering it became possible, for the first time, to introduce a gene or multiple genes from one organism to another, raising endless possibilities of achieving phenotypes with industrial significance (Raab et al. 2005). Another advantage of metabolic engineering is ability to target systematically large cellular metabolic networks, whereas genetic engineering strategies deal only with gene(s) directly connected to a target metabolite (Raab et al. 2005).

Engineering the metabolic pathway for propionate production in bacteria is an approach that can be used to improve propionic acid yield and/or switch the pathway towards propanol. Not much is known about the genomics of propionibacteria. Recently, the whole genome sequence of P. acidipropionici ATCC 4875 has been published (Parizzi et al. 2012).
In general, genetic manipulation of propionibacteria is difficult because of the low efficiency at which they are transformed with external DNA. The low efficiency results from the presence of a cell wall which is a common barrier in transforming gram positive bacteria. Another factor contributing to the low transformation rate is the presence of an endogenous plasmid in some propionibacteria which might compete with foreign counterparts (Genevieve 1988). In addition, the host restriction modification (RM) system plays a role in rejecting foreign DNA (Luijk et al. 2002), which is supported by the fact that more transformants are obtained using plasmids derived from propionibacteria compared to using plasmids derived from other sources (Jore et al. 2001). Using plasmids extracted from *E. coli* that have no methylation ability alleviates rejection of such plasmids by propionibacteria’s methyl-dependant RM systems (Cheong et al. 2008). Obviously, much work is still needed in this area to enhance the cells permeability and tolerability to foreign DNA. Another challenge is the high GC content of propionibacteria which makes expression of heterologous genes with a lower GC ratio difficult.

Generally, little work has been done in this area and most published work aimed at improving vitamin B12 production using *P. freudenreichii*. For the previous purpose, a reporter vector was constructed, pCVE1, which used cholesterol oxidase as a reporter gene to assess the strength of different promoter elements from *P. freudenreichii* (Piao et al. 2004). As a result of the previous work, p4 and p138 were recognized as strong promoter elements. p4 showed 100% homology with the upstream region of ORF4 of pRGO1 from *P. acidipropionici*, whereas no details were provided on p138. Also,
conserved -10 and -35 regions were identified, and a so-called -16 region was thought to be characteristic to propionibacteria. Later, another reporter vector was designed, pTD210, to overcome the laborious and time consuming assay associated with using pCVE1 (Faye et al. 2008). pTD210 used a lacZ gene that encodes β-galactosidase as a reporter gene which made possible using a simpler colorimetric assay. As a result, different promoters from *P. freudenreichii* with a wide range of strengths were identified which can be useful in controlling the level of gene expression. However, no characteristic promoter regions were identified as in the former study. So far, no study has been done for promoter elements from *P. acidipropionici*.

Several plasmids were identified and characterized from different *Propionibacterium sp.* including pRGO1, 2, 3, 4, 5, 6 and 7 plasmids (Rehberger and Glatz 1990). In addition, several shuttle vectors that can shuttle between *E. coli* and propionibacteria were reported including pBRESP (Jore et al. 2001), pTD210 (Faye et al. 2008), pK705 (Kiatpapan et al. 2000), pKHEM01 and 04 (Kiatpapan and Murooka 2001) and pAMT1 (Brede et al. 2005). Of these vectors, we have more interest in two vector systems, pKHEM and pTD210 (Faye et al. 2008; Kiatpapan and Murooka 2001; Kiatpapan and Murooka 2002; Kiatpapan et al. 2000). These vectors can shuttle between *E. coli* and propionibacteria; both have an ampicillin resistance marker to select for correct *E. coli* transformants while hygromycin B or chloramphenicol resistance markers are used to select for propionibacteria transformants with pKHEM or pTD210, respectively. An advantage of pKHEM01 and 04 vectors is the use of strong promoters, p138 and p4 respectively, to control the expression of the downstream gene. Alternatively, pTD210 vector has a
promoterless lacZ gene marker to measure the strength of promoter sequences cloned into a multiple cloning site upstream of the lacZ gene. These vectors have the advantage of being derived from propionibacteria sp. which minimizes their rejection by the RM system of the host, and makes it possible to extend their use in other propionibacteria sp. for controlling expression of different genes.

No work has been published on improving propionic acid and/or propanol production by genetic manipulation of propionibacteria with the exception of knocking out the acetate kinase gene in P. acidipropionici ATCC 4875 (Suwannakham et al. 2006), and overexpressing adhE gene from E. coli in P. freudenreichii (Ammar et al. 2013). The knockout transformant in that study showed 14% decrease in acetate (byproduct) formation and a corresponding 13% increase in propionate. Surprisingly, acetate was still detected, indicating the presence of alternative pathways through which it could be produced, and more work was still needed to completely eliminate production of acetate. On the other hand, mutants overexpressing adhE produced n-propanol, and exhibited better propionate production kinetics on glycerol as carbon source, unexpectedly. Not much work has been reported on improving propionic acid production by overexpressing genes in the dicarboxylic acid pathway of propionibacteria. In conclusion, many questions in this area still need answers.

**Metabolic Engineering of E. coli for Propanol Production**

In general, bio-alcohol production from sugars does not provide sufficient yields for industrial applications. An exception is the microbial production of ethanol from S.
and butanol from Clostridium sp. through ABE (acetone – butanol – ethanol) fermentation. Regarding n-propanol, there is no wild type microorganism known to produce it in significant amounts. Small quantities were produced from threonine catabolism in Clostridium sp., and from beer fermentation in yeast (Eden et al. 2001; Janssen 2004). Currently, researchers are trying to improve bio-propanol production by genetic engineering of well-studied and easily manipulated microbial systems such as E. coli or S. cervisiae. Basically, 2-ketoacid decarboxylases convert 2-ketoacid intermediates from amino acid biosynthetic pathways to aldehydes which are acted upon by alcohol dehydrogenases to produce alcohol (Atsumi and Liao 2008). In this context, JC Liao’s group has reported 2 g/L n-propanol production by genetic engineering of E. coli’s amino acid biosynthetic pathways (Shen and Liao 2008). 2-ketobutyrate, an intermediate produced from threonine and used for isoleucine biosynthesis, is converted to an aldehyde by 2-ketoacid decarboxylase (Kivd) which is then converted to n-propanol by alcohol dehydrogenase 2 (adh2) as shown in Figure 2.2.

Recently, an L-threonine-overproducing E. coli strain was metabolically engineered and produced 10 g/L n-propanol under aerobic conditions in fed-batch culture (Choi et al. 2012) as shown in Figure 2.3. However, further improvements in product titer, yield and productivity are necessary for industrial applications.

**Metabolic Flux Analysis**

Metabolic flux analysis (MFA) or flux balance analysis (FBA) is one of the tools widely used in metabolic engineering. In 1986, the earliest significant work in this area,
addressing the metabolic constraints in fat biosynthesis, was published (Fell and Small 1986). However, work in MFA started as of early 1980s.

MFA is a mathematical approach that calculates and analyzes fluxes through metabolic pathways providing better understanding of flux distribution. An important application of MFA is the calculation of the maximum theoretical yield for products based on stoichiometric equations for a specific pathway (Granström et al. 2002; Nielsen 1998; Stephanopoulos et al. 1998). For propionibacteria, the stoichiometric equations are available (Papoutsakis and Meyer 1985b). These equations can be used to determine the maximum theoretical yields of different products in the pathway, and how they compare to real data (Papoutsakis and Meyer 1985a). Also, using the same equations it was concluded that both EMP and HMP pathways are utilized in glycolysis (Papoutsakis and Meyer 1985a; 1985b).

**Fermentation Process**

Propionibacteria produce propionic acid through the dicarboxylic acid pathway with other acids, mainly acetate and succinate, and CO$_2$ as byproducts in the fermentation process. There have been continuous efforts from scientists to enhance propionic acid fermentation through improving the final concentration, yield and productivity. In theory, the maximum yield of propionic acid, acetic acid, CO$_2$ and total acids from glucose are 54.8%, 22%, 17% and 77% (w/w), respectively, based on Fitz equations (Boyaval and Corre 1995; Leaver et al. 1955):

\[
\text{Lactic acid} \rightarrow 2 \text{ propionic acid} + \text{ acetic acid} + \text{ CO}_2 + \text{ H}_2\text{O}
\]
1.5 Glucose → 2 propionic acid + acetic acid + CO₂ + H₂O

However, actual propionic acid yields are less than theoretical values because some of the carbon flux is directed to cell growth (Lewis and Yang 1992b; Obaya et al. 1994). Of the major downsides of propionic acid fermentation is the presence of byproducts, mainly acetic, which makes downstream processing more difficult and render the entire process costly. The lower the propionate/acetate (P/A) ratios, the higher the downstream processing cost and vice versa. Another factor that influences propionic acid fermentation negatively is the inhibitory effect of propionic acid on growth and product formation (Blanc and Goma 1987; Gu et al. 1998; Herrero 1983; Ibragimova et al. 1969; Lewis and Yang 1992a; Nanba et al. 1983; Neronova et al. 1967; Ozadali et al. 1996; Paik and Glatz 1994; Yang et al. 1994). It has been shown that propionic acid has a stronger inhibitory effect on both cell growth and its own formation compared to acetic acid (Neronova et al. 1967; Obaya et al. 1994).

According to Gu et al. 1998 and Pérez Chaia et al. 1994, it is believed that propionic acid inhibitory effects are attributed to perturbation of the pH gradient across the cell membrane. Normally, the pH gradient is crucial for the transfer of molecules across the membrane from and into the cell. With the accumulation of propionic acid in the medium, the pH goes down and acids become in the unionized form which is diffusible through the cell membrane. Inside the cell, the pH is higher than in the outer acidic fermentation broth, leading acids to dissociate into anions and protons. The accumulation of excessive protons inside the cell constitutes a metabolic burden. And, in order to
maintain a normal pH gradient, cell has to pump the excessive protons out by enforcing higher H\(^+\)ATPase activity. Consequently, more ATPs will be consumed and less ATPs are available for regular metabolic functions. Subsequently, both cell growth and product formation will slow down.

Because of the previously mentioned inhibition effects of propionic acid, conventional propionic acid fermentation exhibits low product concentration, yield and productivity (Jin and Yang 1998). The lower kinetic parameters, in addition to the costly downstream processing, make fermentative production of propionic acid generally not economically competitive to chemical processes.

**Carbon Source**

An important factor in the economics of a fermentation process is the cost of production medium. Of all components, the carbon source contributes significantly to the total cost, and hence the final price of a fermentation product.

From the literature, we know that propionibacteria can utilize many carbon sources for propionic acid production such as glucose (Chen et al. 2012b; Liu et al. 2011; Suwannakham and Yang 2005), glycerol (Ammar et al. 2013; Barbirato et al. 1997; Chen et al. 2012a; Dishisha et al. 2012; Himmi et al. 2000; Liu et al. 2011; Wang and Yang 2013; Zhang and Yang 2009b), lactate (Coral et al. 2008; Emde et al. 1990), lactose (Goswami and Srivastava 2001; Jin and Yang 1998; Lewis et al. 1992a; Yang et al. 1995) sorbitol (Suwannakham 2005), and xylose (Liu et al. 2012.).
Industrial byproducts can serve as inexpensive carbon sources, which both lower the cost and help get rid of environmental waste materials. Examples of industrial byproducts that proved useful in propionic acid fermentation are whey and glycerol.

Whey is a byproduct in cheese manufacturing and it can serve as cheap source for lactose. Using whey permeate as a carbon source, up to 65 g/L were reported (Boyaval and Corre 1987; Yang et al. 1995). Like whey, corn steep liquor is a cheap byproduct from corn milling processes; however it has relatively less carbon sources. The ability of propionibacteria to utilize corn steep liquor for propionic acid production was reported (Paik et al. 1994).

Glycerol is a byproduct in biodiesel industry. Compared to glucose, with glycerol the carbon flux goes to propionate at the expense of acetate and higher P/A ratios are achieved. This can be explained as glycerol has a higher reducing power than glucose, and thus favors the product with a higher redox status. Consequently, in the presence of glycerol, the more reduced propionate is favored over the less reduced acetate (Ammar et al. 2013; Chen et al. 2012a; Coral et al. 2008; Dishisha et al. 2012; Himmi et al. 2000; Liu et al. 2011; Wang and Yang 2013; Zhang and Yang 2009b). For these reasons, the low cost of glycerol and its selectivity for propionate, glycerol is becoming an increasingly attractive carbon source in propionic acid fermentation. Using glycerol as a carbon source, up to ~ 106 g/L propionic acid were reported (Zhang and Yang 2009b).

In a recent study (Liu et al. 2012), hemicellulose which is a source of glucose, xylose and arabinose, was suggested as a cheap renewable carbon source in propionic acid
fermentation. In batch fermentation using corncob molasses, a byproduct from xylitol manufacturing similar to hemicellulose hydrolysate, 72 g/L of propionic acid was reported.

No doubt, the ability of propionibacteria to use cheap carbon sources would increase the competitiveness of fermentation production to chemical synthesis. However, more studies seem necessary to enhance the fermentation process and get it to a point where it is economically feasible.

**Propionate Yield and P/A Ratio**

As mentioned earlier, the maximum theoretical yields of propionic acid, acetic acid, CO₂ and total acids from glucose are 54.8%, 22%, 17% and 77% (w/w), respectively, based on Fitz equations (Boyaval and Corre 1995; Leaver et al. 1955). However, actual propionic acid yields are less than theoretical values because some of the carbon flux is directed to cell growth and byproducts (Lewis and Yang 1992b; Lewis and Yang 1992c; Obaya et al. 1994; Suwannakham and Yang 2005).

In order to increase the yield and raise the P/A ratio, different strategies have been adopted to enforce more flux to propionate rather than acetate. One strategy is lower the fermentation pH to an acidic range (4.5-5) at which cell growth is minimal and more flux can be directed to propionic acid biosynthesis. Following this approach, the propionic acid yield reached 0.63 g/g from glucose. However, the yield improvement was countered by the low productivity levels (Hus et al. 1991).
It has been shown that using carbon sources with high reducing properties would favor the more reduced product in the pathway which is propionate. With glycerol, higher yields of propionate were reported compared to with glucose (Ammar et al. 2013; Coral et al. 2008; Himmi et al. 2000; Wang and Yang 2013; Zhang and Yang 2009b). In 2000, Himmi and co-workers reported a propionate yield of 0.64 g/g from glycerol. Similar results were obtained by pressurizing the production medium with H₂ gas, which led to less acetate production and subsequently higher propionate yields of (Thompson et al. 1984). Further yield enhancements were reported using electrodes to apply reducing conditions (Emde and Schink 1990). In the latter investigation, the ratio of propionate to propionate plus acetate reached 97% compared to 94% reported from the earlier mentioned H₂ approach (Thompson et al. 1984).

**Propionate Productivity**

Because of the inhibitory effect of propionic acid on cell growth, the fermentation is typically slow and takes several days to complete. The higher the propionate concentration in the fermentation broth, the lower the specific growth rate of cells (Balamurugan et al. 1999; Neronova et al. 1967). The slow growth rate results in low propionate productivity below 1 g/L·h (Blanc and Goma 1989; Carrondo et al. 1988; Paik and Glatz 1994).

A commonly used approach to enhance propionic acid productivity is high cell density fermentation. Using a high concentration of cells shortens the lag phase for cell growth
and makes cells more tolerable to propionic acid inhibitory effects (Boyaval and Corre 1987; Suwannakham and Yang 2005).

In the literature, many attempts to enhance productivity through high cell density approach are available. With glycerol as the carbon source and a high cell density maintained, a continuous fermentation was run in a membrane bioreactor reaching productivity of 1 g/L·h (Boyaval et al. 1994). Similarly, in sequential batch fermentation with high cell density (50 g/L) productivity of 1.2 g/L·h was achieved (Colomban et al. 1993). Higher productivity of 2.2 g/L·h was attained in a continuous stirred tank bioreactor with xylose as the carbon source (Carrondo et al. 1988). In different continuous batch fermentation, productivity jumped up to 5 g/L·h where a high cell density of 130 g/L was maintained by recycling cells using ultra filtration (Blanc and Goma 1989). Likewise, Boyaval and Corre (1987) reported 14.3 g/L·h productivity from whey permeat in continuous batch fermentation with high cell density maintained by ultra filtration.

**Propionate Final Concentration**

As mentioned before, the inhibitory effect of propionic acid on growth and product formation is an important factor that influences propionic acid fermentation negatively (Blanc and Goma 1987; Gu et al. 1998; Herrero 1983; Ibragimova et al. 1969; Lewis and Yang 1992a; Nanba et al. 1983; Neronova et al. 1967; Ozadali et al. 1996; Paik and Glatz 1994; Yang et al. 1994). Also, it was shown that propionic acid has a stronger inhibitory effect than acetate, on both cell growth and its own biosynthesis (Neronova et al. 1967;
At 1% propionic acid concentration, the specific growth rate goes down by 50% (Blanc and Goma 1987). In another study, the propionic acid inhibitory effect was considered the cause for the reduced yield (less than 0.5 g/g), productivity (less than 1 g/L·h) and final concentration of propionate (Jin and Yang 1998).

There have been continuous attempts to alleviate the inhibitory effect of propionate on the process. Jin and Yang (1995) reported 75 g/L propionate using extractive fermentation. Propionate was selectively removed from the fermentation broth using an amine extractant and a hollow-fiber membrane extractor. The process was stable over the period of study, in addition acetate and succinate levels were significantly reduced. Suwannakham and Yang (2005) reported 70 g/L propionate using cells immobilized in a fibrous bed bioreactor (FBB). The cells adapted in FBB demonstrated a higher density of viable cells and higher tolerance to propionic acid, as opposed to free-cell culture fermentation. Similarly, when \textit{P. acidipropionici} mutant with its acetate kinase gene knocked out was adapted in FBB, cells produced 70 g/L propionate (Suwannakham and Yang 2005). More recently, 106 g/L propionate was reported using the same mutant from fed-batch fermentation on glycerol (Zhang and Yang 2009b).

**Cell Immobilization**

Cell immobilization is very useful in maintaining high cell density of viable cells which are protected from shear stress, with subsequent enhanced tolerance to propionic acid and improved productivity. Many methods have been applied for cell immobilization; of which entrapment and binding are the two major (Chun et al. 1991; Hilge-Rotmann et al. 1991).
Physical cell entrapment in porous matrices is one of the most widely used techniques for cell immobilization (Shuler and Kargi 1992). Binding is mainly covalent binding or physical adsorption. Covalent binding is mainly for enzymes and has limited applications in cells, whilst physical adsorption has been extensively applied in cell immobilization (Shuler and Kargi 1992).

So far, some of the highest propionic acid concentrations reported are from cells immobilized in fibrous bed bioreactor (Suwannakham and Yang 2005; Zhang and Yang 2009b). The FBB is a bioreactor that increases cells tolerability to inhibitory metabolites in the medium. It has a highly porous fibrous matrix enabling efficient mass transfer, cell immobilization, and continued cell growth and regeneration (Yang et al. 2004). Thus, it supports a high density of viable and productive cell populations. Using a FBB, Yang’s lab enhanced propionic acid production from a transformant strain to the highest reported propionic acid concentration (~106 g/L) (Suwannakham and Yang 2005; Suwannakham et al. 2006; Zhang and Yang 2009a; Zhang and Yang 2009b). Interestingly, it has been reported that cell morphological changes existed in the highly productive transformants. With a threefold increase in its length and ~ 24% decrease in its diameter, the transformant cells had ~ 10% higher specific surface area making them more efficient in transporting substrates and metabolites across the cell membrane (Suwannakham and Yang 2005; Zhang and Yang 2009a).

Generally, not many dedicated investigations were done on the physiological influence of immobilization on immobilized cells. Morphological changes in *P. acidipropionici* have been reported when cells were immobilized in a fibrous bed bioreactor (Suwannakham 1991 and Junter et al. 2002).
and Yang 2005; Zhang and Yang 2009a). Changes in cell membrane structure were found in cells used in immobilized cell fermentation. It was reported that in immobilized cells, cell membranes had more saturated fatty acids compared to their counterparts in free cells (Cotter and Hill 2003; Hilge-Rotmann et al. 1991; Suwannakham and Yang 2005). These modifications are seen as a contributing factor to the higher tolerance to end product’s inhibitory effects which is generally observed in immobilized cells systems (Zhang and Yang 2009a). It was also noticed that metabolites expression levels might change in immobilized cells because immobilized cells are usually used under conditions that are not the best for normal cell growth. In order to adapt to unfavorable conditions, immobilized cells overexpress or limit the expression of relevant metabolites (Cotter and Hill 2003; O'Sullivan and Condon 1999). In the literature, there are no consistent reports on the effect of immobilization on the growth rate. Some groups reported no change in growth rate, while others reported higher or lower rates (Chun and Agathos 1991; Kiy and Tiedtke 1993).
References


Chen F, Feng XH, Liang JF, Xu H and Ouyang PK (2012a) An oxidoreduction potential shift control strategy for high purity propionic acid production by Propionibacterium freudenreichii CCTCC M207015 with glycerol as sole carbon source. Bioprocess Biosyst Eng. Published online ahead of print


Hettinga DH and Reinbold GW (1972b) The propionic-acid bacteria II. Metabolism. J Milk Food Technol. 35:358-372

Hettinga DH and Reinbold GW (1972c) The propionic-acid bacteria III. Miscellaneous metabolic activities. J Milk Food Technol. 35:436-447


Himmi EH, Bories A, Boussaid A and Hassani L (2000) Propionic acid fermentation of glycerol and glucose by Propionibacterium acidipropionici and


Langsrud T and Reinbold GW (1973) Flavour development and microbiology of Swiss cheese. II. Starters, manufacturing process and procedure. J Milk Food Technol. 36:531-542


Neronova NM, Ibragimova SI and Ierusalimski ND (1967) Effect of propionate concentration on the specific growth rate of *Propionibacterium shermanii*. Mikrobiologiya. 36:404-409


(1) Pyruvate kinase; (2) Pyruvate dehydrogenase; (3) Phosphate acetyltransferase; (4) Acetate kinase; (5) Phosphoenolpyruvate carboxylase; (6) Methylmalonyl-CoA carboxyltransferase; (7) Malate dehydrogenase; (8) Fumarate hydratase; (9) Succinate dehydrogenase; (10) Propionyl-CoA: succinate CoA-transferase; (11) Methylmalonyl-CoA mutase; (12) Methylmalonyl-CoA epimerase; (13) Acetyl-CoA synthetase

Figure 2.1 Dicarboxylic acid pathway in propionibacteria.
Figure 2.2 Conversion of 2-ketoacid to propanol in *E. coli* (Shen and Liao 2008).
Figure 2.3 Engineering *E. coli* to produce n-propanol (Choi et al. 2012).
CHAPTER 3

METABOLIC ENGINEERING OF *PROPIONIBACTERIUM FREUDENREICHII* FOR N-PROPANOL PRODUCTION

Summary

Propionibacteria are widely used in industry for manufacturing of Swiss cheese, vitamin B\textsubscript{12}, and propionic acid. However, little is known about their genetics and only a few reports are available on the metabolic engineering of propionibacteria aiming at enhancing fermentative production of vitamin B\textsubscript{12} and propionic acid. n-Propanol is a common solvent, an intermediate in many industrial applications, and a promising biofuel. To date, no wild-type microorganism is known to produce n-propanol in sufficient quantities for industrial application purposes. In this study, a bifunctional aldehyde/alcohol dehydrogenase (*adhE*) was cloned from *Escherichia coli* and expressed in *Propionibacterium freudenreichii*. The mutants expressing the *adhE* gene converted propionyl-coenzyme A, which is the precursor for propionic acid biosynthesis, to n-propanol. The production of n-propanol was limited by NADH availability, which was improved significantly by using glycerol as the carbon source. Interestingly, the
improved propanol production was accompanied by a significant increase in propionic acid productivity, indicating a positive effect of n-propanol biosynthesis on propionic acid fermentative production.

To our best knowledge, this is the first report on producing n-propanol by metabolically engineered propionibacteria, which offers a novel route to produce n-propanol from renewable feedstock, and possibly a new way to boost propionic acid fermentation.
Introduction

1-Propanol (also known as n-propanol or propyl alcohol) is a primary alcohol mainly used as a solvent and carrier for a variety of natural products and synthetic polymers. It is also used in pharmaceuticals and cosmetics, both as a solvent and as an antimicrobial agent. Moreover, it serves as an intermediate in the manufacturing of many industrially important chemicals such as propylene and propyl derivatives. Currently, propanol is mainly produced via petrochemical processes, which are environmentally unfriendly and costly. With the rising oil prices, there has been increasing interest in producing biobased propanol from renewable biomass. However, no known wild-type microorganism can produce propanol in significant amounts for industrial applications, although propanol production was detected in some *Clostridium* spp. from threonine catabolism (Janssen 2004), in beer fermentation by yeast (Eden et al. 2001), and in propionic acid fermentation by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp. *shermanii* (Himmi et al. 2000; Liu et al. 2011). Current research efforts to improve biopropanol production focus on genetic engineering of *Escherichia coli* and *Saccharomyces cerevisiae* (Atsumi and Liao 2008; Choi et al. 2012; Matsuda et al. 2011; Shen and Liao 2008). By engineering the amino acid biosynthetic pathways in *E. coli*, up to 4 and 10 g/L of n-propanol was produced from glucose and glycerol, respectively (Atsumi and Liao 2008; Choi et al. 2012; Shen and Liao 2008). However, further improvements in product titer, yield, and productivity are necessary for industrial applications.
Propionibacteria are Gram-positive, rod-shaped, and facultative anaerobic bacteria widely used in industry for the production of Swiss cheese for eye formation and flavor development (Thierry et al. 2004). Because of their Generally Recognized as Safe status granted by the United States Food and Drug Administration, propionibacteria are also the preferred and widely used organisms for propionic acid (mainly as food/feed preservative) and vitamin B$_{12}$ production (Kośmider et al. 2011; Piao et al. 2004b; Suwannakham and Yang 2005; Zhang and Yang 2009a; b). Metabolically, propionibacteria synthesize propionic acid through the dicarboxylic acid pathway with acetate and succinate as two coproducts (see Figure 3.1). This study explored the feasibility of producing n-propanol from propionyl-coenzyme A (CoA) by overexpressing a bifunctional aldehyde/alcohol dehydrogenase encoded by, such as, aldehyde/alcohol dehydrogenase (adhE) in propionic acid bacteria. Bifunctional aldehyde/alcohol dehydrogenases are capable of converting acetyl-CoA and butyryl-CoA to ethanol and butanol, respectively (Nair et al. 1994; Yu et al. 2011). Our hypothesis is that such enzymes should be able to utilize propionyl-CoA as a substrate to make n-propanol. It can be inferred that considerable quantities of propionyl-CoA are produced in propionibacteria as evident by the high propionate levels reported (Suwannakham and Yang 2005; Suwannakham et al. 2006; Zhang and Yang 2009a; b). Thus, a significant amount of n-propanol should arise upon transforming propionibacteria with adhE. Another advantage of using the dicarboxylic acid pathway for producing n-propanol is the possibility of minimizing byproducts formation through proper control of carbon source and fermentation conditions, and through the deletion of the pathway leading to
acetate (Suwannakham et al. 2006). Furthermore, only one gene needs to be introduced, reducing the difficulty in cloning and complexity of byproducts formation as compared to introducing multiple genes in other hosts such as *E. coli* and yeasts.

In this research, a bifunctional *adhE* cloned from *E. coli* was successfully expressed in *P. freudenreichii* and its effects on the transformants were studied. The mutant expressing the *adhE* gene not only produced n-propanol from glucose and glycerol, but also showed higher propionic acid productivity due to increased substrate uptake or consumption rate. To our best knowledge, this study is the first attempt to produce n-propanol through metabolically engineered propionibacteria, offering a novel route for n-propanol biosynthesis.

**Materials and Methods**

**Bacterial Strains, Plasmids, and Media**

All bacterial strains and plasmids used in this study are listed in Table 3.1. *E. coli* DH5α were grown aerobically at 37 °C in Luria–Bertani (LB) medium, supplemented with 100 μg/mL ampicillin for its transformants. *Clostridium acetobutylicum* ATCC 824 was cultured anaerobically at 37 °C in the Reinforced Clostridial Medium (RCM, Difco). Propionibacteria were grown anaerobically at 32 °C in sodium lactate broth (NLB) medium containing 10 g/L sodium lactate, 10 g/L yeast extract, and 10 g/L trypticase soy broth (Kiatpapan and Murooka 2001) to prepare competent cells and for cells to recover after electroporation. For fermentation kinetics studies, cells were grown anaerobically in the medium containing 10 g/L yeast extract, 5 g/L trypticase, 0.25 g/L K₂HPO₄, 0.05 g/L
MnSO₄, 25 g/L glucose or glycerol as carbon source, and 2 % CaCO₃ to buffer the medium pH (Zhang and Yang 2009a). These media were sterilized by autoclaving at 121 °C, 103.4 kPa for 30 min. Unless otherwise noted, filter-sterilized hygromycin B was aseptically added to the sterile media to a final concentration of 250 μg/ml for culturing and maintaining propionibacteria transformants in serum bottles. All stock cultures were kept at 4 °C for short-term usage and at -80 °C for long-term storage.

**Construction of aad and adhE Expression Vectors**

Cells were lysed after incubation in 10 mg/mL lysozyme at 37 °C for 20–30 min (Jore et al. 2001). Chromosomal DNA was then isolated using the QIAGEN genomic DNA kit (Qiagen, Valencia, CA, USA). The *E. coli adhE* and *C. acetobutylicum aad* genes, each encoding a different aldehyde/alcohol dehydrogenase, were PCR-amplified using the respective genomic DNA as template and primers with introduced *Bsp*HI and *Nde*I restriction sites (see Table 3.1) in a DNA engine (MJ Research, Reno, NV, USA). Briefly, the reaction mixture (50 μl) containing 5 μl of 10× PCR buffer (Invitrogen, Grand Island, NY, USA), 1 μl of 25 mM MgCl₂, 1 μl of 10 mM dNTPs (each), 1 μl of 10 mM forward primer, 1 μl of 10 mM reverse primer, 1 μl of genomic DNA, and 2.5 U Taq DNA polymerase (Invitrogen) was subject to an initial denaturation step at 94 °C for 2 min, 34 cycles of repeated denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 3 min, and a final extension of PCR products at 72 °C for 10 min. The PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA) to construct the corresponding pGEM-aad and pGEM-adhE vectors, which were isolated and purified using the QIAprep MiniPrep plasmid purification kit. To construct
the expression vectors pKHEM04-aad and pKHEM04-adhE, pGEM-aad and pGEM-adhE were double digested with BspHI and Ndel, and the corresponding aad and adhE DNA fragments, after being purified from agarose gels using QIAquick gel extraction kit, were ligated with pKHEM04 digested with NcoI and Ndel (see Figure 3.2). Both BspHI and NcoI have compatible cohesive ends. To verify the cloned genes, their DNA sequences were sequenced by the Plant–Microbe Genomic Facility at the Ohio State University.

**Transformation of Propionibacteria**

The transformation of propionibacteria with pKHEM04-aad and pKHEM04-adhE was carried out by electroporation following the procedures described elsewhere (Xue et al. 1999). Briefly, cells grown in NLB medium to the absorbance $A_{600}$ of 0.6–0.8 were cooled on ice water for 10 min, harvested by centrifugation at 4 °C and 4,000 rpm for 10 min, washed with ice-cold electroporation medium (10 % glycerol) four times, and resuspended in the electroporation medium (1/40 of the original culture volume). Then, 100 μl of competent cells mixed with 1–2 μg of plasmid DNA in 0.1 cm cuvette were incubated on ice for 2–5 min and electroporated with a Gene-Pulser (Bio-Rad Laboratories, Richmond, CA, USA) at 25 μF, 200 Ω, 20 kV/cm, and time constants between 4.5 and 5.0 ms. The electroporated cells were transferred into 1 ml of the recovery medium for 3-h cultivation before plating on the NLB agar medium with 250 μg/ml hygromycin B. After ~10 days of cultivation, colonies on the plates were picked and the transformants were confirmed by PCR cloning using the corresponding adhE or aad gene primers shown in Table 3.1.
**Fermentation Kinetics**

Batch fermentations of glucose and glycerol with selected transformants and parental strains were studied in serum bottles under anaerobic conditions at 32 °C and ~pH 6.8 with CaCO₃. Samples were taken periodically to monitor substrate consumption and production of n-propanol and organic acids. To study the effects of n-propanol on cell growth and fermentation, various amounts of n-propanol were added in the media in serum bottles. No CaCO₃ was added in the media for the growth tests. Unless otherwise noted, at least duplicate bottles were used for each condition studied.

**Stoichiometric Analysis of Carbon Flux Distribution**

To evaluate the effects of *adhE* expression and n-propanol biosynthesis on propionic acid fermentation, stoichiometric analysis of the carbon flux distributions in the wild-type and mutant strains was performed using the stoichiometric equations listed in Table 3.2 and the following assumptions: (1) No net accumulation or consumption of intermediate metabolites including pyruvate and phosphoenolpyruvate (PEP); (2) redox is balanced such that no net change in NADH or NAD⁺; (3) sufficient amounts of ATP are produced for cell growth and maintenance or there is a net ATP generation. Carbon flux distributions were estimated based on the amounts of the end products (propionate, succinate, acetate, and n-propanol) and the substrate (glucose or glycerol) consumed in the fermentation. The molar carbon flux distributions to the fermentation end products and cell biomass were normalized with the substrate consumed.
Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm in a 1.5-mL cuvette (light path length = 1 cm) with a spectrophotometer (Shimadzu, Columbia, MD, UV-16-1). n-propanol was analyzed with a gas chromatograph (GC; GC-2014 Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector and a 30.0-m fused silica column (Stabilwax-DA, 0.25 mm film thickness and 0.25 mm ID, Restek, Bellefonte, PA, USA). The injection temperature was 200 °C, and 1 μl of sample was injected using an auto-injector (AOC-20i, Shimadzu). Initially, the column temperature was kept at 60 °C for 3 min. Then, column temperature was increased to 150 °C at a constant rate of 30 °C per min, and remained at 150 °C for 4 min for a total of 10 min per sample. Glucose, glycerol, and organic acids (acetic, succinic, and propionic) were analyzed using a high-performance liquid chromatography equipped with an organic acid column (Bio-Rad, HPX-87H) operated at 45 °C with 0.005 M H₂SO₄ as the eluant at 0.6 mL/min following the procedures described elsewhere (Suwannakham and Yang 2005).

Statistical Analysis

Unless otherwise noted, all experiments were conducted in duplicate and the means and standard errors are reported. Student’s t test analysis of data was performed with statistically significant difference at $p < 0.05$. 
Results

Cloning and Expressing *adhE* and *aad* in Propionibacteria

The *aad* and *adhE* genes encoding bifunctional aldehyde/alcohol dehydrogenases from *C. acetobutylicum* ATCC 824 and *E. coli* str. K-12 substr. DH10B, respectively, were successfully cloned into pKHEM04, which contained the p4 promoter from *P. freudenreichii* subsp. *shermanii* IFO12424 for gene expression (Piao et al. 2004a). The resulting plasmids pKHEM04-aad and pKHEM04-adhE were transformed into three different propionibacteria (*P. acidipropionici* ATCC 4875, *P. freudenreichii* DSM 20271, and *P. freudenreichii* DSM 4902) by electroporation. No colonies were obtained from *P. acidipropionici*, indicating the incompatibility of pKHEM04 with the host cells. Stable colonies were obtained for both *P. freudenreichii* strains transformed with pKHEM04-aad and pKHEM04-adhE. To eliminate false positive from contamination, colonies on the NLB agar plates were picked and examined for their ability to produce propionic acid in liquid media. Positive transformation was verified for colonies giving positive culture PCR with primers for *aad* or *adhE*, and giving positive PCR with *aad* or *adhE* primers using the plasmids extracted from transformants as templates. Finally, plasmids extracted from the transformants were transformed into *E. coli* and re-extracted, and positive PCR was detected with *aad* or *adhE* primers from *E. coli* cultures and from re-extracted plasmids (see Figure 3.3).

Mutants proven to be positive transformants were then tested for n-propanol production from glucose. n-Propanol production was detected in the fermentation broth of only two
transformants with *adhE* gene expressed in *P. freudenreichii* DSM 4902, namely Pf(adhE)-1 and Pf(adhE)-2, which were further characterized for their fermentation kinetics with glucose and glycerol as carbon source. No detectable levels of n-propanol were produced from any other positive transformants, suggesting that the expression level of the enzyme was too low or the enzyme was not functional in the host cell. The wild-type (WT) strains of these propionibacteria did not produce any detectable amount of n-propanol under the same testing conditions (see Figures 3.5 and 3.6).

**Effect of *adhE* Expression on Cell Growth**

The effect of *adhE* expression on cell growth on glucose and glycerol as substrate, respectively, was studied and is shown in Figure 3.4. In general, the WT and *adhE*-expressing mutant Pf(adhE) had similar growth rates, although the mutant showed a slightly lower specific growth rate on glucose (0.087 ± 0.003 vs. 0.094 ± 0.008 h⁻¹) but higher on glycerol (0.082 ± 0.001 vs. 0.078 ± 0.001 h⁻¹) compared to the wild type. Compared to glucose, cell growth on glycerol stopped and entered the stationary phase earlier at a much lower cell density. However, the mutant was able to grow on glycerol for a longer period to reach a higher final cell density. The beneficial effect of *adhE* expression on propionibacteria cell growth on glycerol can be attributed to its effect on improving redox balance, which will be further discussed later in this paper.

**Batch Fermentation Kinetics with Glucose as Carbon Source**

Figure 3.5 shows the fermentation kinetics with *P. freudenreichii* DSM 4902 WT and two mutants expressing *adhE* gene, Pf(adhE)-1 and Pf(adhE)-2, grown on glucose as
carbon source. As expected, only the two mutants were able to produce n-propanol but not the WT. n-Propanol was detected in the fermentation broth of mutants after 72 h and reached a maximum concentration of 342 mg/L from Pf(adhE)-1 and 291 mg/L from Pf(adhE)-2 at ~270 h. Thereafter, propanol production leveled off due to limited availability of glucose in the medium. Interestingly, both glucose consumption and acids production were significantly faster with the mutants than with the WT, especially during the first 150 h. Compared to the WT, the mutants consumed more glucose and produced more propionic and acetic acids to reach higher final concentrations in these fermentations. About 9.9–11.1 g/L of propionic acid and 4–4.5 g/L of acetic acid were produced from 20–23.5 g/L of glucose in the mutants, while only 8.7 g/L of propionic acid and 2.8 g/L of acetic acid were produced from 20 g/L of glucose by the WT. Table 3.3 summarizes and compares the substrate consumption rates and acid productivities and yields from these fermentations. In general, comparable propionic acid yields were obtained in all strains although the mutants had slightly higher productivities. Clearly, the expression of adhE gene in the mutants not only resulted in n-propanol biosynthesis, but also affected organic acids production in the mutants. The effects will be discussed in more details later in this chapter.

**Batch Fermentation Kinetics with Glycerol as Carbon Source**

Batch fermentation kinetics with various strains was also studied with glycerol as carbon source and the results are shown in Figure 3.6 with key kinetic parameters also summarized in Table 3.3. Again, the mutants produced significant amounts of n-propanol while the wild type did not produce any detectable quantity. Compared to glucose
fermentation, about 50–65 % more n-propanol was produced from glycerol at a faster rate, reaching a maximum propanol titer of ~500 mg/L at the end of the fermentation. More interestingly, the mutants were able to use glycerol much faster and more effectively with all 20 g/L of glycerol consumed in 200 h (consumption rate, ~0.10 g/(L·h)), whereas the wild type only used ~10 g/L of glycerol in 300 h (consumption rate, ~0.032 g/(L·h)). Consequently, more propionic acid was produced by the mutants at a higher productivity of 0.047 g/(L·h), which was more than double of that with the wild type (0.021 g/(L·h)) and even higher than that with glucose as carbon source (~0.031 g/(L·h)). However, propionic acid yields were similar in both the WT and mutants, ~0.64 g/g, which was higher than that from glucose (~0.49 g/g). Similar to glucose fermentation, more acetate was also produced from glycerol by the mutants than the WT (~1.35 vs. 0.6 g/L). The P/A ratio from glycerol fermentation was three to four times higher than that with glucose (~10 vs. ~3), indicating more glycerol carbon was directed towards propionic acid biosynthesis over acetic acid.

When the same fermentation batch was repeated in the bioreactor under controlled pH of 6.5 using Pf(adhE)-2 strain, the n-propanol titer went up to 1300 mg/L as shown in Figure 3.7.

Effects of adhE Expression and n-Propanol Biosynthesis on Flux Distribution

The expression of adhE and n-propanol biosynthesis could change the NADH balance and thus affect the carbon flux distribution in propionic acid fermentation. Based on the assumptions of no net change in the fermentation intermediates (pyruvate and PEP) and a redox balance, the molar flux distributions under various fermentation conditions were
calculated from the experimental data on the substrate (glucose or glycerol) consumption and products formation, and the results are summarized in Table 3.4. With glucose as the substrate, most of the pyruvate was produced through the Embden–Meyerhof–Parnas (EMP) pathway. Compared to the wild type, the mutant had less substrate carbon going into cell biomass (5.9 vs. 15.8 %) but more into acetate (28.0 vs. 21.1 %), probably because cells needed more NADH for n-propanol and increased propionic acid biosynthesis. With the more reduced substrate glycerol, all pyruvate was produced through the EMP pathway, and the mutant had more substrate carbon going into cell biomass (11.9 vs. 5.3 %) and less into succinate (4.8 vs. 11.6 %) as compared to the wild type. On the other hand, the total carbon flux into propionate and n-propanol was almost the same at ~75 % for the mutant and wild type. The carbon flux into acetate was also similar for both mutant (8.6 %) and wild type (8.2 %). Slightly more acetate was produced in the mutant to compensate for the additional NADH consumption by adhE in the biosynthesis of n-propanol.

In propionic acid fermentation, CO₂ is produced from glucose via the HMP pathway (reaction b) and coproduced with acetic acid (reaction c). Meanwhile, it is consumed in the carboxylation of pyruvate (reaction d). So the net change of CO₂ in the fermentation is 3b + c − d when glucose is the substrate and c − d with glycerol as the substrate (see Table 3.2). Based on the stoichiometric analysis, much less CO₂ was produced from glycerol than from glucose (see Table 3.4), which was consistent with the experimental observation. It is interesting to see that the mutant increased CO₂ (and acetate) production, which also improved cell growth (and fermentation productivity), especially
from glycerol. In fact, the wild type did not produce any CO$_2$, instead it had to uptake some CO$_2$ in order to maintain redox balance. These model predictions are consistent with experimental observations (Zhang and Yang 2009b).

**Effects of Propanol on Propionic Acid Fermentation**

*P. freudenreichii* DSM 4902 wild-type cells were cultured in the medium with glycerol as carbon source and various amounts of n-propanol (0, 0.5, 1, 5, and 10 g/L) to study possible effects of n-propanol on cell growth and the fermentation. As can be seen in Figure 3.8, propanol did not significantly affect cell growth or the fermentation at a low concentration of 0.1–1 g/L but inhibited cell growth with a significantly longer lag phase at the higher concentrations of 10 g/L. However, in the presence of 5 g/L propanol, cells were able to consume glycerol and produce propionic acid at significantly higher rates after a short adaptation in the lag phase. The specific growth rate, glycerol consumption rate, and propionic acid productivity and yield were estimated from the time–course data and summarized in Table 3.5. The results showed that propionic acid productivity and titer increased ~20% in the presence of 5 g/L of n-propanol ($p < 0.05$). Meanwhile, acetate production was significantly reduced in the presence of 5–10 g/L of propanol, although the effect on P/A ratio did not seem to be significant due to the large variation in acetate production at the low concentration level. Succinic acid production was not affected by n-propanol in the concentration range studied.
Discussion

Propionibacteria produce propionic acid through the dicarboxylic acid pathway, with acetate and succinate coproduced as two byproducts. Except for a few cases, n-propanol was usually not detected in propionic acid fermentation (Himmi et al. 2000; Liu et al. 2011). Several propionibacteria have been fully sequenced for their genomes (Falentin et al. 2010; Horváth et al. 2012; Meuricea et al. 2004; Parizzi et al. 2012; Vörös et al. 2012). However, not much has been done on the genetic engineering of these bacteria because of the high GC content in their genomes, difficulty to transform the Gram-positive bacteria, and the lack of cloning tools. So far, only two expression vectors using two different promoters, pKHEM01 and pKHEM04 (Kiatpapan et al. 2000; Kiatpapan and Murooka 2001; 2002), and two reporter vectors without a promoter, pTD210 (Faye et al. 2008) and pCVE1 (Piao et al. 2004a), have been developed based on a plasmid originally isolated from *P. acidipropionici*. To date, most published work in this area aimed at improving vitamin B\(_{12}\) production (Piao et al. 2004b); only one paper is focused on improving propionic acid production by knocking out the acetate kinase (*ack*) gene in *P. acidipropionici* ATCC 4875 (Suwannakham et al. 2006). To the best of our knowledge, there has been no report on manipulating the metabolic pathway of propionibacteria to produce n-propanol.

With the help of alcohol and aldehyde dehydrogenases encoded by genes present in their genomes, propionibacteria should, in theory, be capable of converting propionyl-CoA to n-propanol (Falentin et al. 2010; Parizzi et al. 2012). However, n-propanol was rarely detected in propionic acid fermentation, with only a few exceptions showing 0.5–1 g/L
of n-propanol produced from glucose or glycerol by *P. freudenreichii* ssp. *shermanii* ATCC 9614, *P. acidipropionici* ATCC 25562, and *P. acidipropionici* ATCC 4965 (Himmi et al. 2000; Liu et al. 2011). Apparently, the native aldehyde and alcohol dehydrogenases present in most of the propionibacteria have low specificities to propionyl-CoA and propionaldehyde, respectively, or such enzymes are not expressed in sufficient quantities to drive the reactions. In this study, we successfully demonstrated the feasibility of producing n-propanol in *P. freudenreichii* DSM 4902 by overexpressing a bifunctional *adhE* using pKHEM04. About 300 and 500 mg/L of propanol were produced from glucose and glycerol, respectively, by the mutants in serum bottles. With pH controlled at 6.5 in the bioreactor, mutant produced 1300 mg/L of propanol from glycerol. More propanol was produced from glycerol than from glucose, which can be attributed to the fact that glycerol has a higher reductance degree than glucose and thus favors the product with a higher reductance degree (Coral et al. 2008; Himmi et al. 2000; Zhang and Yang 2009b). It is noted that n-propanol production by the mutants was relatively low compared to acids, which could be due to low gene expression or enzyme activity of the heterologous *adhE* (from *E. coli*) controlled by the heterologous p4 promoter (from *P. freudenreichii* ssp. *shermanii* IFO12424). n-Propanol production also could be limited by the availability of NADH needed for the reduction of propionyl-CoA. Based on stoichiometric analysis and redox balance, the maximum theoretical yield of n-propanol from glucose is 0.267 g/g when EMP is used in glycolysis and 0.356 g/g when HMP is used, whereas the maximum theoretical yield of n-propanol from glycerol is 0.391 g/g. The theoretical yield of n-propanol from glucose and glycerol could be
higher than 0.5 g/g if all propionyl-CoA generated in the dicarboxylic acid pathway could be converted to n-propanol, instead of propionic acid, with sufficient hydrogen and reducing power provided to the system. In contrast, the theoretical yields for n-propanol production via the keto-acid pathway in engineered E. coli are 0.444 g/g for glucose and 0.496 g/g for glycerol. However, so far, the highest n-propanol yields were 0.107 g/g glucose and 0.259 g/g glycerol obtained in fermentations with E. coli using the engineered keto-acid pathway (Choi et al. 2012). Although the n-propanol titer and yield obtained in the present study were low due to redox limitation, the propionibacteria mutants, after further engineering, have potential to produce n-propanol at a high titer and yield that would be competitive to the engineered E. coli strains.

Interestingly, adhE expression also increased cell growth (see Figure 3.3b) and acetate and propionate production from glycerol in the mutants (see Table 3.3). In general, cell growth on glycerol, which has a high reductance degree of 4.67, will generate excess NADH; and thus, propionic acid, also with a reductance degree of 4.67, becomes the favorable end product, instead of acetic acid, which has a reductance degree of 4.0 (Liu et al. 2011). The more NADH required for the biosynthesis of n-propanol could create a physiological state favoring consuming more glycerol to maintain the NADH balance and hence increase glycerol consumption, cell growth, and propionic acid production in mutants as observed in this study. Compared to the wild type, the adhE-expressing mutants had a threefold glycerol consumption rate and twofold propionic acid productivity in the batch fermentations that could not be accounted totally by the increased cell biomass in the fermentation, suggesting much higher specific cell
productivity for the mutants with glycerol as the substrate. Such enhancements in acids production could also be due to increased cell membrane permeability in the presence of propanol, resulting in more acids excreted from cells to the fermentation broth. In agreement with the increased acids production, the consumption of substrate, especially glycerol, by cells also increased significantly. These effects were also observed for the wild type growing in the presence of n-propanol at 1–5 g/L (see Figure 3.6). However, no significant effect was observed at 0.5 g/L n-propanol, which is comparable to the amount of n-propanol produced by the mutants, suggesting that the extracellular propanol added to the fermentation medium might not be as effective on membrane permeability as the intracellular propanol produced by the cell. It is well known that alcohol could increase membrane permeability and adding a small amount of alcohol such as methanol during citric acid fermentation can increase citric acid production (El-Samragy et al. 1996; Haq et al. 2003; Rivas et al. 2008). The reasons why mutants had higher propionic acid production and glycerol consumption rates thus might be related to NADH balance and the altered membrane permeability caused by propanol, which showed a more profound effect when produced intracellularly than when added extracellularly. Nevertheless, the finding that a low concentration of n-propanol can increase propionic acid production by propionibacteria has never been reported before and worth further investigation.

A stoichiometric model was used to further evaluate the effects of adhE expression and n-propanol biosynthesis on carbon flux distributions in the dicarboxylic acid pathway. For cell growth on glucose, more carbon flowed towards acetate and less towards biomass in the mutants as compared to the wild type. This flux shift could
provide additional NADH for n-propanol biosynthesis and also increased propionic acid production. Unexpectedly, flux analysis showed that the percentage of pyruvate derived from HMP, which could provide more NADH, in mutants was less than that in the wild type (see Table 3.4). Normally, one would expect mutants to use HMP more to provide higher levels of NADH to support increasing demand on it for propanol and propionate biosynthesis. Instead, mutants increased acetate production to generate additional NADH and decreased biomass to balance NADH. For cell growth on the more reduced substrate glycerol, carbon fluxes to acetate and biomass decreased as expected in the wild type (Zhang and Yang 2009b). However, the flux to biomass went up in mutants unexpectedly at the expense of succinate. The increase in biomass probably was a result of the increased glycerol consumption, which was approximately three times of that in the wild type. In such case, cells produced NADH at a rate higher than the rate it could be consumed in propanol and propionic acid biosynthesis, which also showed an approximately twofold increase in its rate (see Table 3.3). Consequently, to maintain NADH balance, more NADH was directed to biomass production.

In conclusion, overexpressing adhE in P. freudenreichii DSM 4902 can convert propionyl-CoA to n-propanol, which is not produced by most of known propionibacteria. With proper control of the carbon source, the limitation of reducing equivalents can be partially overcome leading to higher n-propanol production by the mutants. To the best of our knowledge, this is the first report on metabolic engineering of Propionibacterium for n-propanol production. However, further research is needed to overcome the limited availability of reducing equivalents to enhance the performance of adhE. Similarly, more
investigations are needed to better our understanding of the kinetic changes taking place in mutants, which might be helpful towards improving propionic acid fermentation as well.
References


Table 3.1 Bacterial strains, plasmids and primers used in this study.

<table>
<thead>
<tr>
<th>Strain/Plasmid/Primer</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. acidipropionici</strong> ATCC 4875</td>
<td>Wild type (WT)</td>
<td>ATCC</td>
</tr>
<tr>
<td><strong>P. freudenreichii</strong> DSM 20271</td>
<td>Wild type (WT)</td>
<td>DSM</td>
</tr>
<tr>
<td>DSM 4902</td>
<td>Wild type (WT)</td>
<td>DSM</td>
</tr>
<tr>
<td>Pf(adhE)-1</td>
<td>DSM 4902 transformant of pKHEM04-adhE</td>
<td>This study</td>
</tr>
<tr>
<td>Pf(adhE)-2</td>
<td>DSM 4902 transformant of pKHEM04-adhE</td>
<td>This study</td>
</tr>
<tr>
<td><strong>C. acetobutylicum</strong> ATCC 824</td>
<td>Wild type (WT)</td>
<td>ATCC</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>Host cells for plasmids amplification</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DH5α</td>
<td>DNA template for cloning adhE</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>K-12 substr. DH10B</td>
<td>E. coli with plasmid pGEM-aad</td>
<td>This study</td>
</tr>
<tr>
<td>EC-pGEM-adhE</td>
<td>E. coli with plasmid pGEM-adhE</td>
<td>This study</td>
</tr>
<tr>
<td>EC-4A</td>
<td>E. coli with plasmid pKHEM04-aad</td>
<td>This study</td>
</tr>
<tr>
<td>EC-4E</td>
<td>E. coli with plasmid pKHEM04-adhE</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T</td>
<td>Cloning vector, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEM-aad</td>
<td>pGEM-T with aad</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-adhE</td>
<td>pGEM-T with adhE</td>
<td>This study</td>
</tr>
<tr>
<td>pKHEM04</td>
<td>Expression vector with p4 promoter, Ap&lt;sup&gt;+&lt;/sup&gt; and Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Kiatpapan &amp; Murooka 2001</td>
</tr>
<tr>
<td>pKHEM04-aad</td>
<td>pKHEM04 with aad</td>
<td>This study</td>
</tr>
<tr>
<td>pKHEM04-adhE</td>
<td>pKHEM04 with adhE</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>aad-F (forward primer)</td>
<td>aacgtcatgacgagggaaatttagatcggcagggataaatgacgcataggttcgcagc</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>aad-R (reverse primer)</td>
<td>gtcgcatacatgctattttttttttaaatattatacgcggaggccccgaaaatgagtcgcagc</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>adhE-F (forward primer)</td>
<td>aacgtcatgacgagggaaatttagatcggcagggataaatgacgcataggttcgcagc</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>adhE-R (reverse primer)</td>
<td>gtcgcatacatgctattttttttttaaatattatacgcggaggccccgaaaatgagtcgcagc</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

Ap<sup>+</sup>, ampicillin resistance gene; Hyg<sup>+</sup>, hygromycin B resistance gene
Table 3.2 Stoichiometric equations and constraints used in the metabolic carbon flux distribution analysis.

<table>
<thead>
<tr>
<th>Coeff.</th>
<th>Stoichiometric equations (Papoutsakis and Meyer 1985)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Embden-Meyerhof-Parnas Pathway (EMP)</td>
</tr>
<tr>
<td></td>
<td>Glucose + 2 NAD(^+) + 2 ADP → 2 Pyruvate + 2 NADH + 2 ATP</td>
</tr>
<tr>
<td></td>
<td>Glycerol + 2 NAD(^+) + ADP → Pyruvate + 2 NADH + ATP</td>
</tr>
<tr>
<td>b</td>
<td>Hexose Monophosphate Pathway (HMP)</td>
</tr>
<tr>
<td></td>
<td>3 Glucose + 11 NAD(^+) + 5 ADP → 5 Pyruvate + 3 CO(_2) + 11 NADH + 5 ATP</td>
</tr>
<tr>
<td>c</td>
<td>Pyruvate + NAD(^+) + ADP → Acetate + NADH + ATP + CO(_2)</td>
</tr>
<tr>
<td>d</td>
<td>Pyruvate + CO(_2) + 2 NADH + ADP → Succinate + 2 NAD(^+) + ATP</td>
</tr>
<tr>
<td>e</td>
<td>Pyruvate + 2 NADH + ADP → Propionate + 2 NAD(^+) + ATP</td>
</tr>
<tr>
<td>f</td>
<td>Pyruvate + 4 NADH + ADP → n-Propanol + 4 NAD(^+) + ATP</td>
</tr>
<tr>
<td>g</td>
<td>4 Pyruvate + 5.75 NADH + 33.7 ATP → 3 C(_4)H(_4)O(_4)N(_4) + 5.75 NAD(^+) + 33.7 ADP</td>
</tr>
</tbody>
</table>

Conditions or constraints used in the metabolic carbon flux distribution calculations:

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate consumption:</td>
<td>a + 3b</td>
<td>A</td>
</tr>
<tr>
<td>Pyruvate balance:</td>
<td>2a+5b = c+d+e+f+4g</td>
<td>a = c+d+e+f+4g</td>
</tr>
<tr>
<td>NADH(_2) balance:</td>
<td>2a+11b+c = 2d+2e+4f+5.75g</td>
<td>2a+c = 2d+2e+4f+5.75g</td>
</tr>
<tr>
<td>ATP generation:</td>
<td>2a+5b+c+d+e+f+33.7g ≥ 0</td>
<td>a+c+d+e+f+33.7g ≥ 0</td>
</tr>
<tr>
<td>Net CO(_2) (produced-fixed):</td>
<td>3b+c-d</td>
<td>c-d</td>
</tr>
</tbody>
</table>

Cell biomass is represented by the elemental formula C\(_4\)H\(_4\)O\(_4\)N\(_4\). Moles of pyruvate used for various products: Acetate: c; Succinate: d; Propionate: e; n-Propanol: f; Biomass: 4g
Table 3.3 Kinetic parameters of WT, Pf(adhE)-1 and Pf(adhE)-2 on different carbon sources.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate consumption rate (g/(L-h))</td>
<td>Wild type</td>
</tr>
<tr>
<td></td>
<td>0.057 ± 0.006</td>
<td>0.063 ± 0.005</td>
</tr>
<tr>
<td>Final product concentration (g/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propanol</td>
<td>0</td>
<td>0.32 ± 0.04*</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>8.7 ± 0.4</td>
<td>9.9 ± 1.3</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>2.8 ± 0.2</td>
<td>4 ± 0.6</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>1.7 ± 0.2</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Total acids</td>
<td>13.2 ± 0.9</td>
<td>15.5 ± 2</td>
</tr>
<tr>
<td>P/A ratio (g/g)</td>
<td>3.4 ± 0.2</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>Product yield (g/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.49 ± 0.04</td>
<td>0.5 ± 0.05</td>
</tr>
<tr>
<td>Total acids</td>
<td>0.72 ± 0.05</td>
<td>0.76 ± 0.07</td>
</tr>
<tr>
<td>Productivity (g/(L-h))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.029 ± 0.001</td>
<td>0.031 ± 0.001</td>
</tr>
<tr>
<td>Total acids</td>
<td>0.043 ± 0.002</td>
<td>0.048 ± 0.002</td>
</tr>
</tbody>
</table>

P/A ratio: propionate/acetate ratio

* indicates the value is significantly higher than that of the control (wild type) with $p < 0.05$

** indicates the value is significantly lower than that of the control (wild type) with $p < 0.05$
Table 3.4 Stoichiometric analysis of carbon flux distributions in wild type and adhE-mutant grown on glucose and glycerol in batch fermentations.

<table>
<thead>
<tr>
<th>Carbon Flux distr. (mol %)</th>
<th>Glucose</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>adhE-mutant</td>
</tr>
<tr>
<td>EMP</td>
<td>73.2 ± 0.3</td>
<td>83.6 ± 4.0</td>
</tr>
<tr>
<td>HMP</td>
<td>26.8 ± 0.3</td>
<td>16.4 ± 4.0</td>
</tr>
<tr>
<td>Pyruvate to cell biomass</td>
<td>15.8 ± 3.7</td>
<td>5.9 ± 1.6</td>
</tr>
<tr>
<td>Pyruvate to acetate and CO₂</td>
<td>21.1 ± 0.5</td>
<td>28.0 ± 0.6</td>
</tr>
<tr>
<td>Pyruvate to succinate</td>
<td>7.1 ± 0.1</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>Pyruvate to propionate</td>
<td>56.0 ± 3.2</td>
<td>57.8 ± 1.2</td>
</tr>
<tr>
<td>Pyruvate to propanol</td>
<td>0</td>
<td>2.1 ± 0.8</td>
</tr>
<tr>
<td>Succinate+Propionate+Propanol</td>
<td>63.1 ± 3.3</td>
<td>66.1 ± 2.3</td>
</tr>
<tr>
<td>Net CO₂ (mol)*</td>
<td>0.061 ± 0.005</td>
<td>0.072 ± 0.006</td>
</tr>
</tbody>
</table>

*A positive value indicates net CO₂ production while a negative one indicates net CO₂ fixation or uptake.*
Table 3.5 Effects of n-propanol on propionic acid fermentation by *P. freudenreichii* WT grown on glycerol in serum bottles.

<table>
<thead>
<tr>
<th></th>
<th>Initial propanol concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Specific growth rate (h⁻¹)</td>
<td>0.092 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>Glycerol consumption rate (g/(L·h))</td>
<td>0.021 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Final product concentration. (g/L)</td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>6.2 ± 0.0</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Total acids</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>P/A ratio (g/g)</td>
<td>10.5 ± 2.4</td>
</tr>
<tr>
<td>Product yield (g/g)</td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.67 ± 0.10</td>
</tr>
<tr>
<td>Total acids</td>
<td>0.97 ± 0.06</td>
</tr>
<tr>
<td>Productivity (g/(L·h))</td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.019 ±</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Total acids</td>
<td>0.026 ±</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
</tr>
</tbody>
</table>

P/A ratio: propionate/acetate ratio

* indicates the value is significantly higher than that of the control (0 g/L propanol) with *p* < 0.05

** indicates the value is significantly lower than that of the control (0 g/L propanol) with *p* < 0.05
Figure 3.1 Dicarboxylic acid pathway and the expression of heterologous *adhE* for n-propanol biosynthesis in propionibacteria.
Figure 3.2 Construction of pKHEM04-adhE.
Figure 3.3 PCR confirmation of adhE-mutants (a) Culture PCR using prospective mutants (b) Culture PCR using *E. coli* (c) PCR using pKHEM04-adhE.
Figure 3.4 Cell growth kinetics on glucose (a) and glycerol (b) for the wild type (WT) and mutant Pf(adhE). (c) Comparison of the specific growth rates of the WT and mutant.
Figure 3.5 Kinetics of batch fermentation of glucose by *P. freudenreichii* wild type and mutant strains Pf(adhE)-1 and Pf(adhE)-2 expressing *adhE*.
Figure 3.6 Kinetics of batch fermentation of glycerol by *P. freudenreichii* wild type and mutant strains Pf(adhE)-1 and Pf(adhE)-2 expressing *adhE*.
Figure 3.7 Kinetics of batch fermentation of glycerol by mutant strain Pf(adhE)-2 expressing adhE in the bioreactor at pH 6.5.
Figure 3.8 Effects of propanol on cell growth (a), glycerol consumption (b) and propionic acid production (c) in batch fermentations of *P. freudenreichii* wild type grown on glycerol in the presence of various concentrations of propanol.
CHAPTER 4

METABOLIC ENGINEERING OF *PROPIONIBACTERIUM FREUDENREICHII*: EFFECT OF EXPRESSING *PPC* FROM *E. COLI* ON PROPIONIC ACID PRODUCTION

Summary

Propionic acid is widely used as a chemical intermediate in various manufacturing processes, and its salts are common food preservatives. It is currently produced mainly via petrochemicals, but there is increasing interest in its fermentative production from renewable biomass. However, current propionic acid fermentation process suffers from low product yield and productivity. In this work, the gene encoding phosphoenolpyruvate carboxylase (PPC) was cloned from *E. coli* and expressed in *Propionibacterium freudenreichii*. PPC catalyzes the conversion of phosphoenolpyruvate to oxaloacetate with the fixation of one CO$_2$. Its expression in *P. freudenreichii* thus showed profound effects on propionic acid fermentation. Compared to the wild type, the mutant expressing the *ppc* gene grew significantly faster on glycerol, consumed more glycerol and produced
propionate to a higher final titer at a faster rate. These effects could be attributed to changes in the flux distributions in the dicarboxylic acid pathway.
Introduction

Propionic acid is commonly used as a food preservative and as chemical intermediate in various industrial processes for the manufacturing of polymers, pesticides, and pharmaceuticals (Boyaval and Corre 1995). Currently, propionic acid is mainly produced from petrochemicals which are non-renewable and increasingly costly due to the rising oil prices. These, in addition to the rising demands for natural ingredients in consumer products, have led to increasing interests in fermentative production of propionic acid from bio-renewable resources (Dishisha et al. 2013; Kagliwal et al. 2013; Liu et al. 2012; Wang and Yang 2013). However, much work is still required to make fermentative propionic acid production competitive to petrochemical processes. Propionibacteria are gram-positive bacteria that can synthesize propionic acid through the dicarboxylic acid pathway with acetate and succinate as two main byproducts. Byproducts formation and inhibition effect of propionic acid on cells are two major concerns that have ignited continuous research efforts aiming to enhance propionic acid fermentation with higher final product concentration, yield and productivity (Coral et al. 2008; Himmi et al. 2000; Suwannakham and Yang 2005; Zhang and Yang 2009b; Zhu et al. 2012). However, most of the previous studies focused on process optimization and strain development through conventional mutagenesis and adaptation. To date, little has been done on the metabolic engineering of propionibacteria for enhanced propionic acid production (Ammar et al. 2013; Suwannakham et al. 2006).

At the branch point of the dicarboxylic acid pathway of propionibacteria, pyruvate is partitioned to acetyl-CoA to form acetate, and oxaloacetate (OAA) to form propionate
Theoretically, the flux to OAA can be increased by overexpressing pyruvate carboxylase (PYC), which converts pyruvate to OAA with CO$_2$ fixation and the consumption of one ATP, or phosphoenolpyruvate carboxylase (PPC), which converts phosphoenolpyruvate (PEP) to OAA by fixing CO$_2$ with the co-generation of one GTP. According to the published whole genome sequences (Falentin et al. 2010; Horváth et al. 2012; Meuricea et al. 2004; Parizzi et al. 2012; Vörös et al. 2012), PYC should be present in some propionibacteria such as *P. acidipropionici* but not in *P. freudenreichii*, while PPC is not present in any known propionibacteria. In general, the PPC pathway is more energetically favorable than the PYC pathway as it would provide more energy equivalents for cell growth. Moreover, directing carbon from PEP to OAA by PPC occurs at an earlier point that bypasses the pyruvate branch point. In addition, overexpressing *ppc* has been proven to be effective for increasing succinic and fumaric acids production in *E. coli* and *Rhizopus oryzae*, respectively (Millard et al. 1996; Song et al. 2013; Zhang et al. 2012). A mutant of *Corynebacterium glutamicum* with increased PPC activity and higher glutamic acid productivity has also been reported (Sawada et al. 2010).

Therefore, we postulated that overexpressing *ppc* can increase carbon flux towards OAA and thus propionate production in propionibacteria. The goal of this study was thus to overexpress a heterologous *ppc* in propionibacteria and evaluate its effects on propionic acid production. In this work, *ppc* from *E. coli* was successfully expressed in *P. freudenreichii* using the expression vector pKHEM04 (Kiatpapan et al. 2000; Kiatpapan and Murooka 2001; 2002) and the mutant showed increased cell growth and propionate
productivity. To the best of our knowledge, this is the first report on enhancing propionic acid production by overexpressing *ppc* in propionibacteria.

**Materials and Methods**

**Bacterial Strains, Plasmids, and Media**

Table 4.1 lists all bacterial strains and plasmids used in this work. Both *E. coli* DH5α and str. K12 substr. DH10B were grown aerobically in Luria-Bertani (LB) medium at 37 °C. For *E. coli* DH5α transformants, LB was supplemented with 100 μg/mL ampicillin. To prepare competent cells and for cells recovery after electroporation, propionibacteria were grown anaerobically in sodium lactate broth (NLB) containing 10 g/L sodium lactate, 10 g/L yeast extract, and 10 g/L trypticase soy broth at 32 °C. For fermentation kinetics studies, cells were grown anaerobically at 32 °C in the fermentation medium (10 g/L yeast extract, 5 g/L trypticase, 0.25 g/L K$_2$HPO$_4$, 0.05 g/L MnSO$_4$, 25-50 g/L carbon source, pH 6.5) supplemented with 2-4% CaCO$_3$ to buffer the pH during fermentation, unless otherwise noted. To study the effect of propionic acid on cell growth, media with different propionate concentrations (0, 5, 10 and 20 g/L) but without CaCO$_3$ were used. All media were autoclaved at 121 °C, 103.4 kPa for 30 min. To culture and maintain mutants, sterile hygromycin B was added to the autoclaved media to a final concentration of 250 μg/ml. Working cultures were kept at 4 °C for short-term storage, while the stock cultures were kept at -80 °C for long term storage.
Construction of *ppc* Expression Vector and Transformation

The expression vector (pKHEM04-ppc) was constructed from the shuttle vector pKHEM04 and *ppc* gene was amplified, using the primers shown in Table 4.1, from *E. coli* genome (see Figure 4.2) following similar procedures described by Ammar et al. (2013). For verification, the cloned gene in the constructed vector was sequenced at the Plant-Microbe Genomic Facility at the Ohio State University. The transformation of propionibacteria with pKHEM04-ppc was done by electroporation following the procedures described elsewhere (Ammar et al. 2013). After electroporation, cells were grown on the NLB agar supplemented with 250 µg/ml hygromycin B for 7 to 10 days before colonies were picked from the plates. The transformants were confirmed by PCR cloning using the *ppc* primers shown in Table 4.1.

**PPC Enzyme Activity Assay**

To prepare the cell extract for activity assay, exponential-phase cells grown on NLB were harvested by centrifugation (7,000 rpm for 10 min), washed with ice-cold water three times, resuspended in 3 mL of ice-cold Tris-HCl buffer (25 mM, pH 7.4), and then treated with ultra-sonication using a sonic dismembrator (Fisher Scientific, Model 100) to disrupt cells, which included 20 cycles of 5 seconds ultra-sonication followed by 25 seconds standing on ice to avoid protein damage by heat. Protein concentration in cell extracts was determined by Bradford protein assay (Bio-Rad) in duplicate using bovine serum albumin as standard.

The PPC activity was measured following the procedures described by Zhang et al. (2012). Aliquots of cell extracts were added to the reaction mixture (0.1 M Tris-HCl
buffer at pH 8.0, 0.01 M MgCl₂, 2.5 mM phosphoenolpyruvic acid, 0.2 mM NADH, 0.01 M NaHCO₃ and 5 units of malate dehydrogenase) and the decrease in absorbance of NADH at 340 nm was monitored to determine the change in NADH with the extinction coefficient of 6.22 mM⁻¹ cm⁻¹ and the amount of PPC. One activity unit is defined as the amount of PPC capable of oxidizing 1 μmol NADH per minute at 25 °C and pH 8.0.

**Fermentation Kinetics**

Batch fermentations of glucose and glycerol were first studied in serum bottles under anaerobic conditions by purging fermentation media with nitrogen gas prior to autoclaving. Unless otherwise mentioned, freshly prepared cells grown on NLB to an OD₆₀₀ ~2.0 were used to inoculate the bottles at 5% (v/v). Samples were collected at suitable time intervals to analyze for carbon source consumption and organic acids production.

Batch fermentations in bioreactor were also studied in 1-L fermentors, each containing 0.5 L medium, at 32 °C, with pH controlled at 6.5 using 6 M NaOH, and agitation at 100 rpm. The inoculum used was 25 ml freshly prepared cells in NLB to an OD₆₀₀ ~2.0. After inoculation, N₂ gas was sparged into the medium for ~30 min to purge oxygen from the bioreactor.

**Stoichiometric Analysis of Carbon Flux Distribution**

Stoichiometric analysis of the carbon flux distributions in the wild-type and mutant strains was performed to evaluate the effects of ppc expression on propionic acid fermentation. The stoichiometric equations used in the analysis are listed in Table 4.2,
and calculations were based on the assumptions of no net accumulation of intermediate metabolites and balanced redox (NAD$^+/\text{NADH}$).

**Analytical Methods**

Optical density as a measure of cell density was determined in a 1.5-mL cuvette (light path length = 1 cm) with a spectrophotometer (Shimazu, Columbia, MD, UV-16-1) at a wavelength of 600 nm. One $\text{OD}_{600}$ unit was found to be equivalent to ~0.2 g/L cell dry weight. The concentrations of substrate (glucose or glycerol) and products (organic acids) in the fermentation broths were determined using high performance liquid chromatography (HPLC) with an organic acid column (Bio-Rad, HPX-87H) following the procedures described by Suwannakham and Yang (2005).

**Statistical Analysis**

All experiments were carried out in duplicate or triplicate, and the mean and standard error values are reported. To assess the significance of different kinetic parameters, data was subject to Student’s t-test analysis with $p < 0.05$ being significantly different.

**Results**

**Cloning and Expressing ppc in Propionibacteria**

pKHEM04-ppc with ppc genes encoding phosphoenolpyruvate carboxylase from *E. coli* str. K-12 substr. DH10B under the control of the p4 promoter from *P. freudenreichii* subsp. *shermanii* IFO12424 (Piao et al. 2004a) was constructed and used to transform several propionibacteria (*P. acidipropionici* ATCC 4875, *P. freudenreichii* DSM 20271
and *P. freudenreichii* DSM 4902). After electroporation, no colony from *P. acidipropionici* was obtained on the NLB agar plates with antibiotic (hygromycin B), whereas some colonies were obtained from the two *P. freudenreichii* strains (DSM 4902 and 20271). These colonies were picked and subcultured in NLB liquid media with hygromycin B to check their ability to grow and produce propionic acid. Positive transformants were confirmed with: 1) positive culture PCR with *ppc* primers; 2) positive PCR with *ppc* primers and the plasmids isolated and purified from transformed colonies; 3) positive PCR with *ppc* primers using plasmids extracted from *E. coli* which had been transformed with plasmids extracted from transformants earlier (data not shown). Finally, all positive transformants were screened against their corresponding wild type (WT) cells for produced propionate and acetate titers in serum-bottle fermentations, which showed that the transformants from DSM 4902 produced less organic acids while those from DSM 20271 generally gave better performance. The *P. freudenreichii* DSM 20271 mutant, namely Pf(*ppc*), producing the highest level of propionate and best P/A ratio was further investigated to study effects of expressing *ppc* on the PPC enzyme activity and the fermentation kinetics.

**PPC Enzyme Activity**

Based on the NADH consumption rate, the PPC activities in the cell extracts of Pf(*ppc*) and WT were estimated and compared in Figure 4.3. Compared to the WT, the mutant showed a 50% higher specific enzyme activity, confirming that *ppc* from *E. coli* was expressed and functional in *P. freudenreichii* DSM 20271. It should be noted that the WT has no endogenous PPC and the observed activity based on the NADH consumption was
attributed to other reactions that might also consume NADH and thus should be regarded as background noise.

**Effect of Propionic Acid on Growth**

To study the effect of *ppc* expression on cell growth, the WT and Pf(*ppc*) mutant cells were grown on glycerol in the presence of various amounts of propionic acid (0, 5, 10 and 20 g/L) and the results are shown in Figure 4.4. Without propionic acid, Pf(*ppc*) showed a significantly higher specific growth rate (0.089 ± 0.003 h⁻¹ vs. 0.078 ± 0.002 h⁻¹) and grew to a higher final cell density (OD 4.7 vs. 3.7). However, the effect was not as pronounced in the presence of 5 g/L propionic acid (final OD: 2.5 for mutant vs. 1.9 for WT) and diminished at the higher propionic acid concentrations of 10 and 20 g/L, which strongly inhibited cell growth in both cultures.

**Batch Fermentation Kinetics with Glucose as Carbon Source in Serum Bottles**

Figure 4.5 shows the fermentation kinetics with *P. freudenreichii* DSM 20271 WT and the mutant expressing *ppc* gene, Pf(*ppc*), grown on glucose as the carbon source. Without CaCO₃ for buffering the pH, the mutant grew faster (0.046 ± 0.001 h⁻¹ vs. 0.038 ± 0.001 h⁻¹ for the WT) and reached a higher OD₆₀₀ of 6.8 (vs. 5.2 for the WT) in 100 h (see Figure 4.5). Thereafter both WT and mutant cell growth leveled off due to the drop in pH to below 4.5 because of the accumulating acids. Compared to the WT, the mutant Pf(*ppc*) also gave a slightly higher glucose consumption rate (0.046 ± 0.003 vs. 0.040 ± 0.001 g/L·h) and a significantly higher propionate productivity (0.029 ± 0.002 vs. 0.020 ± 0.000 g/L·h), although the P/A ratio and acid yields were not significantly different (see Table
The fermentation kinetics was also studied with CaCO$_3$ to buffer the pH and neutralize the produced organic acids. Compared to the WT, the mutant Pf(ppc) had a much higher propionate productivity (0.066 ± 0.006 vs. 0.046 ± 0.000 g/L·h) and glucose consumption rate (0.14 ± 0.13 vs. 0.09 ± 0.02 g/L·h). Interestingly, the fermentation with the mutant reached significantly higher final concentrations of propionate (14.9 vs. 10.7 g/L) and acetate (7.5 vs. 4.6 g/L). Consequently, significantly higher P/S and lower P/A ratios were obtained in the fermentation with the mutant as compared to the WT (see Table 4.3). The PA yield from the mutant fermentation was slightly lower probably because more carbon source was used for cell growth. However, the difference was statistically insignificant.

**Batch Fermentation Kinetics with Glycerol as Carbon Source in Serum Bottles**

Figure 4.6 shows the fermentation kinetics of the WT and Pf(ppc) grown on glycerol as carbon source in serum bottles. In the absence of CaCO$_3$ for pH buffering, the mutant grew faster (0.089 h$^{-1}$ ± 0.004 vs. 0.078 ± 0.003 h$^{-1}$), consumed glycerol much faster (0.053 ± 0.004 vs. 0.038 ± 0.002 g/L·h) and produced more propionate (6.3 vs. 4.7 g/L) at a higher rate (0.039 ± 0.003 vs. 0.030 ± 0.003 g/L·h) as compared to the WT (see Figure 4.6). The mutant also gave a higher propionate yield (0.084 ± 0.08 vs. 0.071 ± 0.08 g/g), but the difference was not statistically significant (see Table 4.3). To increase propionic acid production, CaCO$_3$ was added to the medium to buffer the pH and neutralize produced organic acids in the fermentation. Again, the Pf(ppc) showed a 50% higher glycerol consumption rate (0.104 ± 0.008 vs. 0.073 ± 0.005 g/L·h) and 40% increase in propionate productivity (0.073 ± 0.007 vs. 0.054 ± 0.000 g/L·h) as compared to WT.
Also, Pf(ppc) produced 36% more propionate (13.1 vs. 9.6 g/L for WT). On the other hand, the acetate titer was the same (0.3 g/L) and it was less than in glucose fermentation, as glycerol is a more reduced carbon source. Succinate concentrations were 1.3 and 1.7 g/L in the WT and mutant, respectively. As expected, the propionate yields from glycerol were higher than those from glucose, but there was no significant difference between the WT and mutant. The mutant also showed a significantly higher P/A ratio in the fermentation with CaCO₃.

Pf(ppc) also produced propionate faster and accumulated more propionate in the fermentation broth when 50 g/L glycerol was used as the carbon source (data not shown). However, only a small amount of glycerol was consumed in the fermentation and all kinetic parameters measured were lower than those with 25 g/L of glycerol, indicating a carbon catabolite repression effect.

**Batch Fermentation Kinetics in Bioreactor with pH Controlled at 6.5**

When Pf(ppc) and WT were grown on glucose in the bioreactor controlled at pH 6.5, higher glucose consumption rate (0.227 g/L·h) and organic acid titers (up to 5, 7 and 20 g/L of acetate, succinate and propionate, respectively) were observed with the mutant, although the increase in titers was not statistically significant (see Figure 4.7). In general, the mutant performed similarly to the WT with regards to propionate production over three independent batches (data not shown), suggesting no reproducible superiority of the mutant compared to the WT on glucose fermentation with pH controlled at 6.5. However, when glycerol was used as the carbon source, Pf(ppc) had a significantly higher specific growth rate (0.060 ± 0.003 h⁻¹ vs. 0.052 ± 0.004 h⁻¹), and produced more
propionate (13.2 vs. ~9 g/L) in 240 h (see Figure 4.7) with a 68% higher propionate productivity (0.064 ± 0.006 vs. 0.038 ± 0.009 g/L·h) and 173% higher glycerol consumption rate (0.120 ± 0.014 vs. 0.044 ± 0.005 g/L·h) as compared to the wild type (see Table 4.3). It is clear that Pf(ppc) consumed and grew faster on glycerol, and produced propionate at a higher rate than did the WT.

**Effects of ppc Expression on Flux Distribution**

The expression of *ppc* could change the carbon flux distribution in propionic acid fermentation. Based on the assumptions of no net change in the fermentation intermediates (pyruvate and PEP) and a redox balance, the molar flux distributions under various fermentation conditions were calculated from the experimental data on the substrate (glucose or glycerol) consumption and products formation, and the results are summarized in Table 4.4. With glucose, most of the glycolysis was through the Embden-Meyerhof-Parnas (EMP) pathway in both the WT and mutant, and the *ppc* expression in the mutant did not cause substantial changes in the flux distributions towards different metabolic products (acetate, succinate and propionate) although a substantial amount (26.3%) of PEP was converted directly to OAA, bypassing pyruvate. The increased flux through OAA in the mutant (2.41 ± 0.13 vs. 1.56 ± 0.20 mol/mol glucose for the wild type) did not increase propionate and succinate yields because more pyruvate was used in cell growth. There was a net CO$_2$ fixation (0.487 ± 0.004 mol/mol glucose) with the mutant, whereas there was a net CO$_2$ formation (0.847 ± 0.147 mol/mol glucose) with the WT, which did not have the enzyme PPC needed for CO$_2$ fixation. Similar but more profound effects were observed with glycerol. A large fraction (41%) of PEP was
converted directly to OAA in the mutant, which also fixed a substantial amount of CO$_2$ (2.2 mol/mol glycerol consumed). Compared to glucose, the carbon flux towards propionate increased significantly at the expense of acetate for both the WT and mutant with the more reduced substrate glycerol. However, $ppc$ expression did not significantly change the flux to propionate and succinate, although the flux to acetate was reduced compared to the wild type.

**Discussion**

The genetic manipulation of propionibacteria is relatively difficult because of the limited availability of cloning vectors and low transformation efficiency (Cheong et al. 2008; Genevieve 1988; Jore et al. 2001; Luijk et al. 2002). To date, a few expression vectors such as pKHEM01 and pKHEM04 with promoters p138 and p4, respectively (Kiatpapan et al. 2000; Kiatpapan and Murooka 2001; 2002) and two promoterless reporter vectors (pTD210 and pCVE1) (Faye et al. 2008; Piao et al. 2004a) have been developed to overexpress genes in propionibacteria. However, most of the prior studies focused on vitamin B$_{12}$ production by *P. freudenreichii* (Piao et al. 2004b). To date, only a few studies were on the metabolic engineering of propionibacteria for enhanced propionic acid production. In our recent study, *P. freudenreichii* DSM 4902 was engineered to produce n-propanol by expressing an exogenous aldehyde/alcohol dehydrogenase ($adhE$), which also increased propionate productivity especially when glycerol was the carbon source (Ammar et al. 2013). In the present study, we aimed at increasing the carbon flux towards OAA in the dicarboxylic acid pathway to enhance propionic acid biosynthesis by overexpressing $ppc$ in *P. freudenreichii* DSM 20271. We chose to overexpress $ppc$
instead of *pyc* based on its potential energetic advantages and the proven outcomes from similar studies on other microbes (Millard et al. 1996; Song et al. 2013; Zhang et al. 2012). In theory, expressing *ppc* for the direct conversion of PEP to OAA to bypass pyruvate should increase the carbon flux towards propionate and decrease acetate production. Unexpectedly, the *ppc*-expressing mutant did not show any significant increase in propionate yield or decrease in acetate production (see Figure 4.8). Nevertheless, the mutant had a significantly higher specific growth rate and fermentation productivity, which could be attributed to the additional GTP generation through the PPC-catalyzed reaction (see Figure 4.1) and the higher cell density reached in the fermentation (see Figures 4.5-7). However, the higher cell density itself cannot fully account for the increased substrate consumption rate and productivity, suggesting that the mutant had a higher specific productivity. Thus, the *ppc*-expressing mutant could be used to effectively increase propionic acid production rate.

The higher propionate production rate and titer obtained with the mutant in serum-bottle fermentations, where the pH was maintained at ~5 or lower (see Figures 4.5-6), also suggested that the ppc-mutant had better tolerance to propionic acid, which is a strong inhibitor to cells. This finding was consistent with the results that the mutant grew to a higher final cell density in the presence of 5-10 g/L propionic acid (see Figure 4.4). However, the mutant’s propionic acid tolerance advantage diminished when the propionic acid concentration increased to 20 g/L or became less noticeable in the bioreactor fermentation with pH controlled at 6.5 (see Figure 4.7). The latter is understandable as only the undissociated propionic acid is toxic to cells while at pH 6.5
most of the propionic acid is present as salt or in the dissociated form, which is not as toxic to cells (Gu et al. 1998; Hsu and Yang 1991).

It is noteworthy that the effects of ppc expression were more profound in fermentations with glycerol than with glucose as the carbon source. Glucose being a less reduced carbon source stimulates acetate formation to provide additional NADH to support propionate biosynthesis and maintain the intracellular NADH balance. Consequently, the effects of PPC on flux distribution and fermentation kinetics (i.e., substrate consumption rate, cell growth rate, and propionate production rate) were not as profound with glucose compared to glycerol. On the other hand, more propionate can be produced from glycerol than from glucose as glycerol being the more reduced substrate would favor the more reduced product, propionic acid (Coral et al. 2008; Himmi et al. 2000; Liu et al. 2011; Zhang and Yang 2009b). Compared to the WT, the ppc-mutant consistently gave a higher P/A ratio in glycerol fermentations under all conditions studied (see Table 4.3), confirming our hypothesis that overexpressing ppc would increase carbon flux towards propionate instead of acetate. However, the propionate yield from glycerol was not increased, which probably was because more carbon source was used for cell growth due to the increased GTP supply. Besides being an energy carrier, GTP is known to be critical for DNA and protein biosynthesis and its level is up-regulated in proliferating cells because protein translation is GTP-dependent (Yalowitz and Jayaram 2000). Thus, PPC activity produces GTP, which stimulates cell growth. Consequently, more glycerol was consumed to generate extra energy needed for cell growth and maintenance, resulting in fast glycerol consumption, more biomass formation, and faster propionate production in
the fermentation. Overexpressing ppc did not significantly change the P/S ratio since both propionate and succinate are in the downstream of OAA in the dicarboxylic acid pathway.

The effects of ppc expression on carbon flux distributions were also manifested with a stoichiometric analysis, which confirmed that there were significant amounts of OAA formed directly from PEP with the fixation of CO₂ in the ppc-mutant. The increased flux from PEP to OAA should produce more GTP that in turn favored cell growth and specific activity, resulting in increased substrate consumption rate and propionate production rate in the fermentation with the mutant.

In conclusion, overexpressing ppc in P. freudenreichii DSM 20271 improves propionate productivity. By controlling the carbon source, the limitation of required cell maintenance energy and reducing equivalents is mitigated, and the mutant expressing ppc gene becomes distinctly more productive and gives a higher P/A ratio compared to the wild type. To the best of our knowledge, this is the first report on metabolic engineering of propionibacteria for propionic acid production by up-regulating the propionate branch of the dicarboxylic acid pathway using exogenous ppc gene.
References


Hettinga DH and Reinbold GW (1972b) The propionic-acid bacteria II. Metabolism. J Milk Food Technol. 35:358-372
Hettinga DH and Reinbold GW (1972c) The propionic-acid bacteria III. Miscellaneous metabolic activities. J Milk Food Technol. 35:436-447


105


Xue GP, Johnson JS and Dalrymple BP (1999) High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J Microbiol Methods. 34:183-191


Zhang A and Yang ST (2009b) Propionic acid production from glycerol by metabolically engineered *Propionibacterium acidipropionici*. Process Biochem. 44:1346-1351


Table 4.1 Bacterial strains, plasmids and primers used in this study.

<table>
<thead>
<tr>
<th>Strain/Plasmid/Primer</th>
<th>Characteristics</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. acidipropionici</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 4875</td>
<td>Wild type (WT)</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>P. freudenreichii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSM 20271</td>
<td>Wild type (WT)</td>
<td>DSM</td>
</tr>
<tr>
<td>DSM 4902</td>
<td>Wild type (WT)</td>
<td>DSM</td>
</tr>
<tr>
<td>Pf(ppc)</td>
<td>DSM 20271 transformant of pKHEM04-ppc</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Host cells for plasmids amplification</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>K-12 substr. DH10B</td>
<td>DNA template for cloning <em>ppc</em></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>EC-pGEM-ppc</td>
<td><em>E. coli</em> with plasmid pGEM-ppc</td>
<td>This study</td>
</tr>
<tr>
<td>EC-4P</td>
<td><em>E. coli</em> with plasmid pKHEM04-ppc</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Cloning vector, <em>Ap</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEM-ppc</td>
<td>pGEM-T with <em>ppc</em></td>
<td>This study</td>
</tr>
<tr>
<td>pKHEM04</td>
<td>Expression vector with p4 promoter, <em>Ap</em>&lt;sup&gt;+&lt;/sup&gt; and <em>Hyg</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Kiatpapan &amp; Murooka 2001</td>
</tr>
<tr>
<td>pKHEM04-ppc</td>
<td>pKHEM04 with <em>ppc</em></td>
<td>This study</td>
</tr>
<tr>
<td><strong>Primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ppc-F (forward primer)</td>
<td>gctagtcagggcgatgaacgaataatccgattgc</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>ppc-R (reverse primer)</td>
<td>atcgcaatatgtattagccggttacgcactagtgc</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

*Ap*<sup>+</sup>, ampicillin resistance gene; *Hyg*<sup>+</sup>, hygromycin B resistance gene
Table 4.2 Stoichiometric equations and constraints used in the metabolic carbon flux distribution analysis.

<table>
<thead>
<tr>
<th>Stoichiometric equations</th>
<th>Glucose + 2 NAD(^+) + 2 ADP → 2 PEP + 2 NADH + 2 ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Substrate uptake</td>
<td>Glycerol + 2 NAD(^+) → PEP + 2 NADH</td>
</tr>
<tr>
<td>b. Substrate uptake</td>
<td>Hexose Monophosphate Pathway (HMP):</td>
</tr>
<tr>
<td></td>
<td>3 Glucose + 11 NAD(^+) + 5 ADP → 5 PEP + 3 CO(_2) + 11 NADH(_2) + 5 ATP</td>
</tr>
<tr>
<td>c. Pyruvate synthesis</td>
<td>PEP + ADP → Pyruvate + ATP</td>
</tr>
<tr>
<td>d. CO(_2) Fixation</td>
<td>PEP + CO(_2) + GDP → Oxaloacetate + GTP</td>
</tr>
<tr>
<td>e. Biomass synthesis</td>
<td>4 Pyruvate + 5.75 NADH + 33.7 ATP → 3 C(_4)H(_4)O(_4)N(_4) + 5.75 NAD(^+) + 33.7 ADP</td>
</tr>
<tr>
<td>f. Oxaloacetate synthesis</td>
<td>Pyruvate + Succinyl CoA → Propionyl CoA + Oxaloacetate</td>
</tr>
<tr>
<td>g. Succinate synthesis</td>
<td>Oxaloacetate + 2 NADH + ADP → Succinate + ATP + 2 NAD(^+)</td>
</tr>
<tr>
<td>h. Propionate synthesis</td>
<td>Succinate + Propionyl CoA → Succinyl CoA + Propionate</td>
</tr>
<tr>
<td>i. Acetate synthesis</td>
<td>Pyruvate + NAD(^+) + ADP → Acetate + NADH + ATP + CO(_2)</td>
</tr>
</tbody>
</table>

Constraints on the intermediate metabolites used in the calculation of coefficients in the stoichiometric equations.

<table>
<thead>
<tr>
<th>Constraint</th>
<th>Glucose</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ[PEP] = 0</td>
<td>2a + 5b = c + d</td>
<td>a = c + d</td>
</tr>
<tr>
<td>Δ[Pyruvate] = 0</td>
<td>c = 4e + f + i</td>
<td>c = 4e + f + i</td>
</tr>
<tr>
<td>Δ[Oxaloacetate] = 0</td>
<td>d + f = g</td>
<td>d + f = g</td>
</tr>
<tr>
<td>Δ[Succinyl CoA] = 0</td>
<td>f = h</td>
<td>f = h</td>
</tr>
<tr>
<td>Δ[Propionyl CoA] = 0</td>
<td>f = h</td>
<td>f = h</td>
</tr>
<tr>
<td>Δ[NADH] = 0</td>
<td>2a + 11b + i = 5.75e + 2g</td>
<td>2a + i = 5.75e + 2g</td>
</tr>
<tr>
<td>Δ[ATP] ≥ 0</td>
<td>2a + 5b + c + d + g + i + 33.7e ≥ 0</td>
<td>c + d + g + i + 33.7e ≥ 0</td>
</tr>
<tr>
<td>Δ[CO(_2)]</td>
<td>3b + i - d</td>
<td>i - d</td>
</tr>
</tbody>
</table>

Cell biomass is represented by the elemental formula C\(_4\)H\(_4\)O\(_4\)N\(_4\). Moles of pyruvate used for various products: Acetate: i; Succinate: g; Propionate: h; Biomass: e
Table 4.3 Fermentation kinetics parameters from glucose and glycerol batches.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No CaCO₃</th>
<th>With CaCO₃</th>
<th>Bioreactor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Mutant</td>
<td>WT</td>
</tr>
<tr>
<td>μ (h⁻¹)</td>
<td>0.095 ± 0.001</td>
<td>0.102 ± 0.001</td>
<td>NA</td>
</tr>
<tr>
<td>Acetate (g/L)</td>
<td>1.8 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>Succinate (g/L)</td>
<td>NA</td>
<td>NA</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>Propionate (g/L)</td>
<td>3.5 ± 0.1</td>
<td>4.0 ± 0.2*</td>
<td>10.7 ± 0.1</td>
</tr>
<tr>
<td>Total acids (g/L)</td>
<td>5.4 ± 0.1</td>
<td>6.3 ± 0.2*</td>
<td>17.6 ± 0.1</td>
</tr>
<tr>
<td>P/A (g/g)</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>P/S (g/g)</td>
<td>NA</td>
<td>NA</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>PA yield (g/g)</td>
<td>0.52 ± 0.07</td>
<td>0.53 ± 0.04</td>
<td>0.51 ± 0.06</td>
</tr>
<tr>
<td>Total acid yield (g/g)</td>
<td>0.80 ± 0.10</td>
<td>0.82 ± 0.07</td>
<td>0.82 ± 0.09</td>
</tr>
<tr>
<td>PA productivity (g/L·h)</td>
<td>0.021 ± 0.001</td>
<td>0.029 ± 0.002*</td>
<td>0.046 ± 0.001</td>
</tr>
<tr>
<td>Total acid productivity (g/L·h)</td>
<td>0.031 ± 0.001</td>
<td>0.036 ± 0.004*</td>
<td>0.074 ± 0.001</td>
</tr>
<tr>
<td>Glucose consumption rate (g/L·h)</td>
<td>0.040 ± 0.001</td>
<td>0.046 ± 0.003*</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parameter</td>
<td>No CaCO₃</td>
<td>With CaCO₃</td>
<td>Bioreactor</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>Mutant</td>
<td>WT</td>
</tr>
<tr>
<td>μ (h⁻¹)</td>
<td>0.078 ± 0.003</td>
<td>0.089 ± 0.002*</td>
<td>NA</td>
</tr>
<tr>
<td>Acetate (g/L)</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>Succinate (g/L)</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>Propionate (g/L)</td>
<td>4.7 ± 0.3</td>
<td>6.3 ± 0.4*</td>
<td>9.6 ± 0.1</td>
</tr>
<tr>
<td>Total acids (g/L)</td>
<td>5.7 ± 0.3</td>
<td>7.4 ± 0.4*</td>
<td>11.4 ± 0.1</td>
</tr>
<tr>
<td>P/A (g/g)</td>
<td>13.0 ± 0.2</td>
<td>16.7 ± 1.9*</td>
<td>29.3 ± 0.3</td>
</tr>
<tr>
<td>P/S (g/g)</td>
<td>10.4 ± 1.2</td>
<td>11.3 ± 2.7</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>PA yield (g/g)</td>
<td>0.78 ± 0.08</td>
<td>0.86 ± 0.08</td>
<td>0.85 ± 0.05</td>
</tr>
<tr>
<td>Total acid yield (g/g)</td>
<td>0.85 ± 0.09</td>
<td>0.94 ± 0.08</td>
<td>0.98 ± 0.06</td>
</tr>
<tr>
<td>PA productivity (g/L·h)</td>
<td>0.030 ± 0.003</td>
<td>0.039 ± 0.003*</td>
<td>0.054 ± 0.000</td>
</tr>
<tr>
<td>Total acid productivity (g/L·h)</td>
<td>0.033 ± 0.003</td>
<td>0.042 ± 0.003*</td>
<td>0.071 ± 0.000</td>
</tr>
<tr>
<td>Glycerol consumption (g/L·h)</td>
<td>0.038 ± 0.002</td>
<td>0.053 ± 0.004*</td>
<td>0.073 ± 0.005</td>
</tr>
</tbody>
</table>

* indicates \( p < 0.05 \)
Table 4.4 Stoichiometric analysis of carbon flux distributions in wild type and ppc-mutant grown on glucose and glycerol in batch fermentations in serum bottles with CaCO<sub>3</sub> (~pH 5.0).

| Carbon Flux distr. (mol %) | Glucose  | Glycerol  |  |  |
|---------------------------|----------|-----------|  |  |
|                           | Wild type | ppc-mutant| Wild type | ppc-mutant |
| EMP                       | 94.1 ± 1.4 | 96.1 ± 0.7 | 100 | 100 |
| HMP                       | 5.9 ± 1.5  | 3.9 ± 0.6  | 0   | 0   |
| PEP to OAA                | 0         | 26.3 ± 0.56 | 0   | 41 ± 0.1 |
| PEP to Pyruvate           | 100       | 73.7 ± 0.55 | 100 | 59 ± 0.1 |
| Pyruvate to cell biomass  | 12.0 ± 2.0 | 15.4 ± 1.2 | 11.5 ± 0.8 | 12.1 ± 0.1 |
| Pyruvate to acetate       | 22.8 ± 0.3 | 22.3 ± 0.2 | 3 ± 0.1 | 2.4 ± 0.4 |
| Pyruvate to succinate     | 7.4 ± 0.3  | 6.2 ± 0.6  | 6.5 ± 0.1 | 6.2 ± 0.1 |
| Pyruvate to propionate    | 57.8 ± 1.4 | 56.1 ± 0.4 | 79 ± 0.7 | 79.3 ± 0.3 |
| Succinate+Propionate      | 65.2 ± 1.7 | 62.3 ± 1.0 | 85.5 ± 0.8 | 85.5 ± 0.4 |
| Net CO₂ (mol/mol carbon source)* | 0.847 ± 0.147 | -0.487 ± 0.004 | 0.077 ± 0.004 | -2.207 ± 0.017 |
| Total OAA (mol/mol carbon source) | 1.56 ± 0.20  | 2.41 ± 0.13 | 2.05 ± 0.1 | 4.26 ± 0.03 |

*The negative value indicates net CO₂ fixation or uptake in the fermentation.
Figure 4.1 Dicarboxylic acid pathway for propionic acid biosynthesis in propionibacteria. Some propionibacteria such as *P. acidipropionici* ATCC 4875 has pyruvate carboxylase (PYC) that can convert pyruvate to oxaloacetate with CO$_2$ fixation.
Figure 4.2 Construction of pKHEM04-ppc.
Figure 4.3 Comparison of PPC enzyme activity in *P. freudenreichii* DSM 20271 (wild type, WT) and mutant Pf(ppc) expressing *ppc*. 
Figure 4.4 Effect of propionic acid on cell growth on glycerol without CaCO$_3$ for pH buffering. The initial medium pH was ~6.5. WT: wild type; Pf(ppc): mutant expressing ppc.
Figure 4.5 Batch fermentation kinetics with glucose in serum bottles with and without CaCO$_3$ for pH buffering.
Figure 4.6 Batch fermentation kinetics with glycerol in serum bottles with and without CaCO\textsubscript{3} for pH buffering.
Figure 4.7 Batch fermentation kinetics in bioreactor with pH controlled at ~6.5.
Figure 4.8 Propionate yields from glucose and glycerol in serum bottles with CaCO$_3$ (~pH 5) and in bioreactor (pH 6.5).
CHAPTER 5

CLONING AND FUNCTIONAL ANALYSIS OF CONSTITUTIVE PROMOTERS FROM *PROPIONIBACTERIUM ACIDIPROPIONICI* ATCC 4875

Summary

Propionibacteria can be used to produce propionic acid, which currently is mainly produced via petrochemicals. The fermentation production of propionic acid can be improved by metabolic engineering of propionibacteria. However, little is known about the genetics of these bacteria. Promoters are key elements in metabolic engineering as they control the level of gene expression. Eleven promoters from *P. acidipropionici* ATCC 4875 were cloned into a shuttle vector with a promoterless *LacZ* reporter gene and transformed into the host *P. freudenreichii*. Promoter strengths were analyzed as function of the β-galactosidase activity. Results showed that these promoters were able to enforce the expression of the reporter gene to different levels in propionibacteria. In addition, sequence analysis showed that these promoters from *P. acidipropionici* were more complicated than those from other known prokaryotes, and hence more investigations are
needed in this area. These findings will contribute towards improving future metabolic engineering of propionibacteria for enhanced propionic acid fermentation.
Introduction

Propionibacteria are commercially important for their use in the production of Swiss-type cheese for eye formation and flavor development (Thierry et al. 2004). Propionibacteria are also known for their high production of propionic acid and vitamin B12 and this has led to the development of commercially interesting industrial processes. Since some Propionibacterium spp. have been granted GRAS (Generally Recognized As Safe) status by the United States Food and Drug Administration (FDA) and are known to produce neither endo- nor exotoxins (Salminen et al. 1998), they are preferred for the production of vitamin B12 and other food additives.

In order to improve the characteristics of propionibacteria, some genetic manipulation systems have been developed, including shuttle vectors between E. coli and propionibacteria such as pK705 (Kiatpapan et al. 2000) and a series of vectors based on plasmid p545 (Jore et al. 2001). In 2001, Kiatpapan and co-workers succeeded in overexpressing heterologous genes in P. freudenreichii ssp. shermanii (choA encoding cholesterol oxidase from streptomyces and hemA encoding 5-aminolevulinic acid (ALA) synthase from Rhodobacter sphaeroides based on pK705), and screened some endogenous promoters as well. Similarly, some strong promoters (p4, p138 and others) were isolated from P. freudenreichii (Faye et al. 2008; Piao et al. 2004). When some genes for vitamin B12 biosynthesis were overexpressed driven by such strong promoters, the production of vitamin B12 in mutants increased 2.2 times (Piao et al. 2004). More recently, P. freudenreichii was successfully engineered to produce n-propanol (Ammar et al. 2013).
Promoter is an important DNA element that influences gene expression. Promoter activity determines gene expression efficiency and is important in the metabolic engineering of microorganisms. Strong promoters could be very useful for overexpressing some genes and improving their corresponding metabolite levels. When 18 promoters from *P. freudenreichii* were analyzed, -10 and -35 motifs were not consistent with their counterparts in *E. coli* (Piao et al. 2004). The -16 region ACGCGCA, allegedly characteristic of propionibacteria (Piao et al. 2004), was not found in other promoters from *P. freudenreichii* (Faye et al. 2008). These findings suggest that more investigations are needed in this area.

Although there were some improvements on metabolic engineering of propionibacteria, most reports focused mainly on *P. freudenreichii*. The propionic acid production was enhanced in a *P. acidipropionici* mutant by knocking out *ack* gene using an integration vector (Suwannakham et al. 2006). More recently, some whole genome sequences of propionibacteria have been published indicating a growing interest in exploring their genetics (Falentin et al. 2010; Horváth et al. 2012; Meuricea et al. 2004; Parizzi et al. 2012; Vörös et al. 2012). However, there is no report on the analysis of *P. acidipropionici* promoters. In this work, eleven promoters were cloned into pTD210, a vector with promoterless *lacZ* gene previously used with *P. freudenreichii* (Faye et al. 2008) to evaluate the strength of promoters based on the corresponding β-galactosidase activities of the reporter gene.
Materials and Methods

Bacterial Strains and Plasmids

Table 5.1 lists all bacterial strains and plasmids used and created in this work. *P. acidipropionici* ATCC 4875 and *P. freudenreichii* stock cultures were kept in serum bottles under anaerobic conditions at 4 °C for short-term storage and at -80 °C for long-term storage. *E. coli* DH5α was stored at -80 °C. The promoterless vector pTD210, kindly provided by Dr. Brede (Faye et al. 2008), was used to construct and analyze the strength of cloned promoters in *P. freudenreichii*.

Culture Media

*P. acidipropionici* and *P. freudenreichii* were grown anaerobically at 32 °C in sodium lactate broth (NLB) medium containing 1% sodium lactate, 1% yeast extract, and 1% trypticase soy broth (Kiatpapan and Murooka 2001). NLB medium with 0.5 M sorbitol and 0.5 M mannitol was used as growth medium for preparing propionibacteria competent cells. The recovery medium for selecting the propionibacteria transformants was NLB with 0.5 M sorbitol and 0.38 M mannitol. The electroporation medium was a solution of 0.5 M sorbitol, 0.5 M mannitol and 10% glycerol. All media were sterilized by autoclaving at 121 °C, 103.4 kPa for 30 min. Propionibacteria transformants were grown in NLB medium supplemented with 10 µg/ml of chloramphenicol. *E. coli* DH5α transformants were grown aerobically at 37 °C in Luria-Bertani (LB) medium supplemented with 100 µg/mL ampicillin.
DNA Manipulations

Chromosomal DNA from *P. acidipropionici* was isolated using the QIAGEN genomic DNA kit (Qiagen, Valencia, CA). Plasmid DNA from *E. coli* was isolated using the QIAprep MiniPrep plasmid purification kit. QIAquick gel extraction kit was used to purify DNA fragments from agarose gel. Restriction enzymes and T4 DNA ligase from either Invitrogen (USA) or Promega (USA) were used according to the supplier's instructions.

Cloning Promoters by PCR Amplification

Eleven constitutive promoters for genes encoding triose-phosphate isomerase (tpiA), chaperone protein DnaJ (DnaJ), ribosomal protein S10 (rpsJ), ribosomal protein L28 (rpmB), ribosomal protein S15 (rpsO), acetyl-CoA transferase (CoAT), pyruvate dehydrogenase operon (pdh), pyruvate carboxylase (pyc), outer membrane lipoprotein (omL), protein recA (recA), DNA-directed RNA polymerase beta subunit (rpoB) were cloned from the genome of *P. acidipropionici* ATCC 4875 using primers shown in Table 5.2. Two restriction enzyme sites, *Bgl*II and *EcoRV*, were introduced at the forward and reverse primers, respectively. These promoters were amplified using *P. acidipropionici* genomic DNA as template in a DNA engine (MJ Research, Reno, NV). The reaction mixture (50 μl) contained 5 μl of 10X PCR buffer (Invitrogen, US), 1 μl of 25 mM MgCl₂, 1 μl of 10 mM dNTPs (each), 1 μl of 10 mM forward primer, 1 μl of 10 mM reverse primer, 1 μl of genomic DNA, and 2.5 U Taq DNA polymerase (Invitrogen). The cycle program consisted of an initial denaturation step at 94 °C for 5 min followed by 35 cycles of repeated denaturation at 94 °C for 50 s, annealing for 50 s, and extension at 72
°C for 50 s. The annealing temperature was 60 °C for cloning pdh and 55 °C for cloning the other ten promoters. Final extension of incomplete PCR products was conducted at 72 °C for 10 min. The size of PCR products ranged from 300 to 500 bp and the PCR product of each promoter was cloned into pGEM-T vector (Promega, USA) to construct the corresponding cloning vector. The cloned 11 promoters were verified for their sequences at the Plant-Microbe Genomic Facility of the Ohio State University.

Construction of the Expression Vectors
The promoterless reporter vector pTD210 (Faye et al. 2008) was used to construct expression vectors with cloned promoters. The cloned 11 promoters and pTD210 plasmid were double digested with BglII and EcoRV. Expression vectors were constructed by ligating the purified promoters and vector fragments (see Figure 5.1). After ligation, the constructed plasmids were isolated and checked by running an agarose gel for both the newly constructed plasmids and their double digestion products with BglII and EcoRV (see Figure 5.2).

Transformation of Propionibacteria
The promoterless vector pTD210 and the 11 constructed expression vectors were transformed into P. freudenreichii by electroporation following the procedures described by Xue et al. (1999). Propionibacteria were grown in the growth medium until the culture OD$_{600}$ reached 0.6 ~ 0.8. Cells were cooled on ice for 10 min, harvested by centrifugation at 4 °C, 4000 rpm for 10 min, and washed 4 times with ice-cold electroporation medium. For electroporation, cells were resuspended in the electroporation medium equal to 1/40
of the original culture volume. 70 μl of competent cells were mixed with 0.5-1 μg DNA in 0.1 cm cuvette and incubated on ice for 2-5 min. Electroporation was conducted with Gene-Pulser (Bio–Rad Laboratories, Richmond, CA) at 25 μF, 200 Ω, 20 kV/cm, and time constants were between 4.0 and 5.0 ms. The electroporated cells were transferred into 1 ml recovery medium for 9 h cultivation before screening with NLB agar medium supplemented with 10 μg/ml chloramphenicol. After 10 days of cultivation, transformants were analyzed by PCR using partial cmxA gene primers (see Table 5.2).

Checking Prospective Mutants

After expression vectors were electroporated into propionibacteria, prospective transformants with pTD210, pTD-tpiA, pTD-DnaJ, pTD-rpsJ, pTD-rpmB, pTD-rpsO, pTD-CoAT, pTD-pdh, pTD-pyc, pTD-omL, pTD-recA, and pTD-rpoB were cultivated and corresponding plasmids were isolated. Then, plasmids were double checked by PCR using the partial cmxA gene primers and the specific primer set for each promoter fragment, in addition to double digestion with restriction enzymes as mentioned before.

β-Galactosidase Assay

Promoter strengths were tested by measuring the β-galactosidase activity in propionibacteria in 96-well plate (Griffith and Wolf 2002). Propionibacteria mutants were grown anaerobically at 32 °C for 48 h in NLB medium with 5 μg/ml chloramphenicol. Cells were collected by centrifugation at 4 °C and resuspended in Z-buffer (Na-phosphate (pH 7.0), 100 mM; KCl, 10 mM; MgSO₄, 1 mM; β-mercaptoethanol, 50 mM). 100 μl cells diluted with Z-buffer (OD₆₀₀ = 0.25) were
pippeted into 96-well plate and mixed with 20 μl of ONPG (4 mg/ml) and 80 μl Z-buffer. The microplate was then incubated at 30 °C for an appropriate time until the yellow color was developed; at this point the reaction was terminated by adding 50 μl of 1 M Na₂CO₃. Then, the microplate was introduced into the plate reader, and absorption values at 420 (A₄₂₀) and 550 nm (A₅₅₀) were determined. The β-galactosidase activity was determined as Miller units (MU) (Miller 1972). MU = 1000 x [(A₄₂₀ - 1.75 × A₅₅₀)] / (Time × Vol × OD₆₀₀); Time = reaction time (min); Vol = cell volume (mL). One MU describes the change in A₄₂₀/min/mL of cells/OD₆₀₀. For each mutant, four reactions were prepared simultaneously and final calculations were based on the average of the four readings obtained at A₄₂₀ and A₅₅₀.

**SDS-PAGE Analysis**

50 ml cells grown to the exponential phase were harvested by centrifugation at 7,000 rpm for 10 min, washed three times, and resuspended in 3 mL of ice cold Tris/HCl buffer (25 mM, pH 7.4). Resuspended cells were ultra-sonicated through 20 cycles using a sonic dismembrator (Fisher Scientific, Model 100); each cycle consisted of 5 seconds ultrasonication then 25 seconds resting on ice to prevent protein damage by generated heat. During the disruption process, cells were kept on ice all time. Later, protein concentration was determined in duplicate using Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard. For analysis, a 10 % SDS–PAGE gel was prepared.
Results

Confirmation of Propionibacteria Mutants

The mutants were able to grow on NLB medium with 5 μg/mL chloramphenicol. Also, Figure 5.3 shows that they gave similar PCR product (lane 1-12) to the positive control (lane +) using the corresponding plasmids extracted from mutants as templates, however there was no PCR band in the negative control (lane -). Both antibiotic resistance and PCR results confirmed that mutants were true.

Promoter Activity

Promoter activities analyzed by measuring the β-galactosidase activity in P. freudenreichii are compared in Figure 5.4. Six mutants (PAB-pTD-tpiA, PAB-pTD-rpmB, PAB-pTD-rpsO, PAB-pTD-CoAT, PAB-pTD-pdh and PAB-pTD-rpoB) showed low β-galactosidase activity of less than 15 MU; 2 mutants (PAB-pTD-rpsJ and PAB-pTD-omL) showed moderate activity (32 and 34 MU, respectively); 3 mutants (PAB-pTD-DnaJ, PAB-pTD-pyc and PAB-pTD-recA) showed high activity (123, 135 and 79 MU, respectively). Both controls, propionibacteria with promoterless pTD210 and the WT, showed almost zero β-galactosidase activity.

SDS-PAGE Analysis

To test the correlation between enzyme activity and protein expression level, cell extracts from mutants with the highest promoter activity (DnaJ and pyc) were run on SDS-PAGE against the mutant with promoterless plasmid pTD210 as control. Results showed a band for pyc mutant between 100 and 120 KDa which is where one would expect a β-
galactosidase band of 116 KDa to appear (see Figure 5.5). Similarly, a band at the same location but of weaker intensity was found for DnaJ. However, such band was not found for the control.

**Promoter Sequence Analysis**

To analyze the promoter region for each gene, the cloned 300-500 bp upstream of the corresponding ATG start codon was analyzed using the multiple sequence alignment tool T-COFFEE Version 8.93 (http://www.ebi.ac.uk/Tools/msa/tcoffee/). There was no obvious -10 (CCGTTG) or -35 (CGCACA) regions characteristic of prokaryotic promoters (see Figure 5.6). The -16 (ACGCGCA) region, reportedly characteristic of propionibacteria by Piao et al. (2004) was not found in these promoters either. On the other hand, a region equivalent to the Shine Dalgarno (SD) sequence, which is a ribosome bind site rich in A and G bases (AGGAGG, for example) and generally located 8 bases upstream of the start codon, existed in some promoters (CoAT, oml, dnaJ, recA, rpmB, rpsJ, rpsO).

**Discussion**

Although there were some improvements in the metabolic engineering of propionibacteria, most reports focused on *P. freudenreichii* rather than *P. acidipropionici*. There is no report on analyzing the strength and sequence of promoters from *P. acidipropionici*. In this work, eleven promoters from *P. acidipropionici* were cloned into pTD210, a vector with promoterless lacZ gene, to analyze their strengths based on the corresponding β-galactosidase activity (Faye et al. 2008). To ensure that each promoter
sequence covers all probable promoter elements, 300-500 bp upstream of the ATG start codon of the gene it controls was cloned by PCR. All selected promoters control genes which perform important physiological functions and are expected to be constitutively expressed in propionibacteria, such as genes encoding glycolytic enzymes (tpiA), chaperons (DnaJ), ribosomal proteins (rpsJ, rpmB and rpsO), dicarboxylic acid pathway enzymes (CoAT, pdh, and pyc) and others (oml, outer membrane lipoprotein; recA, DNA repair and maintenance; rpoB, RNA polymerase beta subunit). Focusing on such genes was meant to raise the chances of finding strong promoter(s).

The constructed pTD210-promoter vectors were transformed into propionibacteria and mutants were confirmed by PCR (see Figure 5.3), in addition to their ability to grow in the presence of 5-10 µg/ml of the selection marker (chloramphenicol antibiotic).

Based on the β-galactosidase assay results shown in Figure 5.4, DnaJ, pyc and recA promoters are relatively stronger than the rest of studied promoters. Also, there was a good correlation between enzyme activity and protein expression level. Such good correlation confirms that improved enzyme activity in mutants with potential strong promoters is a result of enhanced gene expression driven by these promoters. Therefore, DnaJ, pyc and recA promoters are potential candidates in future metabolic engineering work to overexpress genes of interest in propionibacteria. Nonetheless, it is recommended to conduct a follow-up study to compare the strengths of these three promoters against the two strong promoters reported by other groups, namely p4 and p138 (Kiatpapan and Murooka 2001; Faye et al. 2008). This would help improve
propionic acid fermentation production by better metabolic engineering of the host propionibacteria.

On the other hand, variation in promoter strengths, as manifested by the wide range of enzyme activities obtained, should be useful in future work towards optimizing the expression of a given gene to a desired level. As for mutants where the activity was very low or null, this could be explained by being weak promoters or by the absence of promoter elements due to functioning as part of an operon. Therefore more analysis is required to find these elements. Another possibility is that these promoters are not constitutively expressed as expected and they need to be induced to function.

The absence of obvious -10 (CCGTTG) and -35 (CGCACA) regions in all cloned promoters in this study, is consistent with promoters analysis by Piao et al. (2004). The -16 region, ACGCGCA motif consensus sequence, which was proposed as the characteristic of some propionibacteria promoters by Piao et al. (2004), cannot be generalized as evident by the promoters investigated in this work. In addition, promoters with -16 motifs like CoAT promoter showed a similar activity to many of the promoters lacking them. In the same context, the strong promoter $P_{pcfAS}$ (Faye et al. 2008) isolated from $P. freudenreichii$ also showed no significant similarity to the consensus sequences identified by Piao et al. (2004).

In conclusion, DnaJ, pyc and recA promoters are potential strong promoters for future metabolic engineering work to overexpress genes of interest in propionibacteria. The variation in promoter strengths can be useful in modulating gene expression to a desired
level. No strong evidence was found to support the existence of characteristic -10, -16 or -35 regions for propionibacteria promoters based on sequences investigated in this and other studies. Finally, promoter structure and gene regulation in propionibacteria are more complicated than in simple prokaryotes like *E. coli*, and more investigations in this area are needed to better understand it. This is the first report on the analysis of promoters from *P. acidipropionici*. 
References


Table 5.1 Bacterial strains and plasmids.*

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Characteristics</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. acidipropionici</em> ATCC 4875</td>
<td>WT propionibacteria used for cloning the promoter sequences</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>P. freudenreichii</em></td>
<td>WT propionibacteria used for obtaining transformants with pTD-promoter plasmids</td>
<td>DSM</td>
</tr>
<tr>
<td>PA-pTD210</td>
<td><em>P. freudenreichii</em> mutant with plasmid pTD210</td>
<td>This study</td>
</tr>
<tr>
<td>PA-pTD-tpiA, -DnaJ, -rpsJ, -rpmB, -rpsO, -CoAT, -pdh, -pyc, -omL, -recA, -rpoB</td>
<td><em>P. freudenreichii</em> mutants with the corresponding pTD-promoter plasmids</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong> DH5α</td>
<td>Commercial competent cells for cloning</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>EC-pTD210</td>
<td><em>E. coli</em> mutant with pTD210 plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>EC-pTD-tpiA, -DnaJ, -rpsJ, -rpmB, -rpsO, -CoAT, -pdh, -pyc, -omL, -recA, -rpoB</td>
<td><em>E. coli</em> mutants with the corresponding pTD-promoter plasmids</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Commercial cloning vector, Ap'</td>
<td>Promega</td>
</tr>
<tr>
<td>pTD-tpiA, DnaJ, -rpsJ, -rpmB, -rpsO, -CoAT, -pdh, -pyc, -omL, -recA, -rpoB</td>
<td>Expression vectors composed of pTD210 plasmid and the corresponding promoters</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Note:** Ap', ampiciline resistance; Cl', chloramphenicol resistance
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>tpiA-F</td>
<td>5'-TGAAGATCTTGCCGTCCCTTGACCACCAC-3'</td>
</tr>
<tr>
<td>tpiA-R</td>
<td>5'-GCAGTATCGAGTTTCACTTGATGCAAG-3'</td>
</tr>
<tr>
<td>DnaJ-F</td>
<td>5'-GTCGAAGATCTGCAGCAGATCCCTCGAGATC-3'</td>
</tr>
<tr>
<td>DnaJ-R</td>
<td>5'-CTTCTGATATCGACAGGAAACCGGCCACGTACC-3'</td>
</tr>
<tr>
<td>rpsJ-F</td>
<td>5'-CGAGCAAGATCTGCTTTGCGAGCGAAGGGGATC-3'</td>
</tr>
<tr>
<td>rpsJ-R</td>
<td>5'-CTTCTGATATCGACTCCCTGGCCCGGCGATTTTTC-3'</td>
</tr>
<tr>
<td>rpmB-F</td>
<td>5'-TGAAGATCTTTGACGCCTTACATCTTTC-3'</td>
</tr>
<tr>
<td>rpmB-R</td>
<td>5'-CGCGATATCGTCACTGGAAGCG-3'</td>
</tr>
<tr>
<td>rpsO-F</td>
<td>5'-CAGGTAGATCTCGTGTTGATGTTGATCACGCG-3'</td>
</tr>
<tr>
<td>rpsO-R</td>
<td>5'-TCCCTGATATCCTCGTGCTTGCGCGCGCTCCCCGATGC-3'</td>
</tr>
<tr>
<td>CoAT-F</td>
<td>5'-TGAAGATCTACTGACACCGTGCTCC-3'</td>
</tr>
<tr>
<td>CoAT-R</td>
<td>5'-CGCGATATCGCAACCGCTTTGAAAATG-3'</td>
</tr>
<tr>
<td>pdh-F</td>
<td>5'-TGAAGATCTTCTGACGCCATCCTACTCT-3'</td>
</tr>
<tr>
<td>pdh-R</td>
<td>5'-CGCGATATCGGTAAGGTCCCACAAC-3'</td>
</tr>
<tr>
<td>pyc-F</td>
<td>5'-GTTCCAGATCTCTCGGCTTCCAGATCCGCAC-3'</td>
</tr>
<tr>
<td>pyc-R</td>
<td>5'-TCCCTGATATCGCCGAACGAGTCCATGTAAGTC-3'</td>
</tr>
<tr>
<td>omL-F</td>
<td>5'-GCAGTACTGCTGCCGACCAGCCCTCAAGCG-3'</td>
</tr>
<tr>
<td>omL-R</td>
<td>5'-CTCCTGATATCGACATGCGTGATGCCGCGGGCGCCGCTGC-3'</td>
</tr>
<tr>
<td>recA-F</td>
<td>5'-TCATGGAGATCTCCGCGCTTTTCAATGAAACCCTGC-3'</td>
</tr>
<tr>
<td>recA-R</td>
<td>5'-TCCTCTGATATCGAAGTGCTGGTCATGGGAGAGCGCGCTGC-3'</td>
</tr>
<tr>
<td>rpoB-F</td>
<td>5'-TGAAGATCTGCAACCCTCAACTCAAGTG-3'</td>
</tr>
<tr>
<td>rpoB-R</td>
<td>5'-CGAGTATCGACCGGCCTTTCGCTGTTC-3'</td>
</tr>
<tr>
<td>chl-F</td>
<td>5'-GATGGGTGCGAGAAGGT-3'</td>
</tr>
<tr>
<td>chl-R</td>
<td>5'-GCCGTGTGCAAGAAGAAC-3'</td>
</tr>
</tbody>
</table>
Figure 5.1 Construction of expression vectors.
Figure 5.2 Some constructed promoter plasmids and their corresponding double digestion pattern with Bg/II and EcoRV.

Note: Lane M, DNA marker; Lane rpsJ, pTD-rpsJ; Lane DnaJ, pTD-DnaJ; Lane rpsO, pTD-rpsO. Each plasmid lane is followed by a lane showing the products of its double digestion products.
Figure 5.3 PCR of chl gene using plasmids extracted from propionibacteria mutants.

Note: Lane 1, PAB-pTD-tpiA; Lane 2, PAB-pTD-DnaJ; Lane 3, PAB-pTD-rpsJ; Lane 4, PAB-pTD-rpmB; Lane 5, PAB-pTD-rpsO; Lane 6, PAB-pTD-CoAT; Lane 7, PAB-pTD-pdh; Lane 8, PAB-pTD-pyc; Lane 9 PAB-pTD-omL; Lane 10, PAB-pTD-recA; Lane 11, PAB-pTD-rpo; Lane 12, PAB-pTD210; Lane M, DNA marker; Lane +, pTD210 from *E.coli*; and Lane -, Genomic DNA of propionibacteria
Figure 5.4 β-Galactosidase activity in different propionibacteria mutants.
Figure 5.5 SDS-PAGE analysis of the β-galactosidase expression.

Note: Lane M, protein marker; Arrows refer to β-galactosidase protein band (116 KDa) between 100 and 120 KDa.
Figure 5.6 Alignment of 13 propionibacteria promoter sequences showing lack of consensus or similarity at -35, -16, and -10 regions.

The cloned 300-500 bp upstream of the ATG start codon of each corresponding gene were analyzed for -10 (CCGTTG), -16 (ACGCGCA) or -35 (CGCACA) consensus elements. Also p4 (~ 600 bp showing 100% homology with the upstream region of ORF4 of pRGO1 from *P. acidipropionici*) and p138 (no details are available on which upstream region it constitutes) were included in the analysis. The figure shows lack of these elements at the corresponding regions in the analyzed sequences. On the other hand, A and G rich region corresponding to the SD sequence, ribosome binding site, existed in some promoters (CoAT, oml, dnaJ, recA, rpmB, rpsJ, rpsO) prior to the -10 region.
CHAPTER 6

CONCLUSIONS AND RECOMMENDATION

This research demonstrated the feasibility of producing n-propanol and enhancing propionic acid productivity by metabolic engineering of *P. freudenreichii* and improving gene expression in propionibacteria with potential strong promoters by analyzing the strengths of different promoters from *P. acidipropionici*. The main results and conclusions obtained from this study are summarized below.

6.1 Metabolic Engineering for n-Propanol Production

This study demonstrated the feasibility of producing n-propanol from *P. freudenreichii* DSM 4902 by overexpressing an aldehyde/alcohol dehydrogenase (*adhE*) from *E. coli* that converts propionyl-CoA to n-propanol. Reducing equivalents (NADH) are a limiting factor in n-propanol production from metabolically engineered propionibacteria. With glucose as the carbon source, n-propanol production was limited by NADH availability and only 0.3 g/L n-propanol was reached in serum bottle fermentation. With the more reduced carbon source, glycerol, the limitation of NADH was alleviated and n-propanol
titer reached 0.5 g/L. Under controlled pH in the bioreactor, the n-propanol titer increased up to 1.3 g/L with glycerol as carbon source.

The adhE-overexpressing mutant, on glycerol, showed higher final OD$_{600}$ (8 vs 5 for the WT), glycerol consumption rate (3-fold higher than WT), and propionate titer (2-fold higher than WT) and productivity (2-fold higher than WT). The enhanced propionate fermentation kinetics with glycerol can be attributed to increased membrane permeability caused by n-propanol. In addition, n-propanol biosynthesis may have disrupted the metabolic flux balance in a way that favored consuming more glycerol and producing more propionate at a higher rate than in the WT. Similar effects were observed when the WT was grown on glycerol in the presence of 5 g/L externally added n-propanol in the fermentation medium. This approach can provide a novel route for n-propanol fermentative production and for enhancing propionic acid fermentation as well.

**Further research in the following areas is recommended.**

- Transforming *P. acidipropionici* ATCC 4875 with adhE in an attempt to boost the n-propanol yield and titer. High propionate titers over 100 g/L have been reported from *P. acidipropionici* ATCC 4875 which makes it an affluent source for propionyl-CoA, the substrate for adhE’s enzyme product.

- Overexpressing formate dehydrogenase gene to overcome the NADH limitation; therefore, providing more NADH needed for n-propanol biosynthesis.

- Searching for other aldehyde/alcohol dehydrogenases with a higher GC content and better selectivity and affinity towards propionyl-CoA to mitigate the competition
from the native propionyl-CoA:succinyl-CoA transferase which converts propionyl-CoA to propionic acid.

- Investigating more deeply the possible causes of the enhanced propionic acid production in adhE-expressing mutants.

- Increasing propionyl-CoA availability for adhE’s enzyme product by overexpressing a gene which converts propionate to propionyl-CoA such as acetyl-CoA:acetoacetyl-CoA synthase.

- Engineering the fermentation process using fed-batch mode, high cell density and fibrous bed bioreactor approaches to boost the n-propanol productivity, titer and yield.

- Carrying out proteomic investigations to see if other genes are up or down regulated in the mutant. The outcomes of such investigations should be useful in subsequent rational metabolic engineering of propionibacteria to enhance propionic acid production.

6.2 Metabolic Engineering for Propionic Acid Production

This is the first study on metabolic engineering of propionibacteria for propionic acid production by up-regulating the propionate branch of the dicarboxylic acid pathway using exogenous ppc gene. This study demonstrated the feasibility of improving propionic acid production in P. freudenreichii DSM 20271 by overexpressing phosphoenol pyruvate carboxylase (ppc) gene from E. coli which converts phosphoenolpyruvate (PEP) directly
to oxaloacetate (OAA). Overexpressing ppc gene should, in theory, direct more carbon flux to propionate at the expense of acetate. Thus, propionate yield is expected to increase in the mutant cells. Unexpectedly, propionate yield was not significantly different from the WT.

The ppc-overexpressing mutants grew faster than the WT in serum bottles with no pH control, indicating possible higher tolerance to propionic acid. This effect diminished in batch fermentation under controlled pH of 6.5 in the bioreactor. In the presence of different concentrations of propionic acid (0, 5, 10 and 20 g/L) in the fermentation medium with glycerol as carbon source, the mutant grew faster at 0 g/L, and to a higher OD$_{600}$ at 5 and 10 g/L. Growth was inhibited at 20 g/L for the WT and mutant. The mutant consumed carbon source faster and produced more propionic acid at a higher rate than the WT. The effect was more pronounced with glycerol as carbon source.

Metabolic flux distribution analysis revealed 26 and 41% of OAA were formed directly from PEP by the action of PPC in the mutant. It also revealed the ability of mutants rather than WT to fix CO$_2$. The enhanced growth rate of the mutant on glycerol could be attributed to the generation of GTP molecules in the PPC-catalyzed reaction; GTP is essential for DNA and protein synthesis. A reasonable explanation for the increased biomass formation, glycerol consumption and propionate productivity is that PPC activity produces GTP, which stimulates cell growth. Consequently, more glycerol was consumed to generate additional energy needed for cell growth and maintenance, resulting in fast glycerol consumption, more biomass formation, and faster propionate production.
Further research in the following areas is recommended.

- Transforming *P. acidipropionici* ATCC 4875 with *ppc* in an attempt to develop an economic process for propionic acid production from renewable feedstocks. High propionate titers over 100 g/L have been reported from *P. acidipropionici* ATCC 4875 which makes it a potential target for metabolic engineering; however it is very difficult to transform *P. acidipropionici* with foreign DNA.

- Using more reduced carbon sources similar to glycerol to study their influences on the fermentation kinetics.

- Running fermentation batches under anaerobic conditions where anaerobiosis is maintained by purging CO₂ to take advantage of the fact that the mutant can fix CO₂. Alternatively, the pH in the bioreactor can be controlled by adding NaHCO₃ to neutralize the accumulating organic acids with the release of CO₂ into the fermentation broth.

- Investigating why the mutant is more tolerant to propionic acid.

- Operating the fermentation process in a fed-batch mode, with a high cell density and cell immobilization in a fibrous bed bioreactor to boost the propionate productivity, titer and yield.

- Developing a dynamic model that takes time into consideration to better our understanding of the metabolic changes taking place in the mutant versus the WT.
6.3 Analysis of Promoters from \textit{P. acidipropionici} ATCC 4875

In this study eleven promoters from \textit{P. acidipropionici} were cloned into a promoterless reporter vector (pTD210) to analyze their relative strengths. These promoters were cloned from the upstream sequences of genes known to perform vital functions to the cell in order to increase the chances of finding strong constitutive promoters. Strengths of promoters were tested by measuring the corresponding $\beta$-galactosidase activity encoded by the reporter gene ($\text{lacZ}$). These 11 promoters enforced the reporter gene expression to different levels. Among them, pyc, DnaJ and recA were distinctly stronger than the rest. Mutants with strong promoters also showed distinctly stronger bands on SDS-PAGE when compared to the WT.

When all promoters were aligned against each other for sequence similarity analysis, no promoter regions characteristic of prokaryotes, such as -10 or -35 regions, were observed, indicating a more complicated nature of propionibacteria promoters. This is the first promoter analysis work done using promoters from the difficult-to-manipulate \textit{P. acidipropionici} ATCC 4875, and this study would contribute towards enhancing future metabolic engineering of propionibacteria.

**Further research in the following areas is recommended.**

- Analyzing more promoters from \textit{P. acidipropionici} to increase the chance of finding stronger promoters.
- Conducting a comparison study involving strong promoters reported from *P. freudenreichii* such as p4 and p138 against the strong promoters (pyc, DnaJ and recA) found in this study.

- Carrying out real-time PCR to get better insight into the relative strengths of promoters by making the comparison at the transcription level.

- Using the potential strong promoters reported in this study for overexpressing genes of interest in propionibacteria.
BIBLIOGRAPHY


Chen F, Feng XH, Liang JF, Xu H and Ouyang PK (2012a) An oxidoreduction potential shift control strategy for high purity propionic acid production by Propionibacterium freudenreichii CCTCC M207015 with glycerol as sole carbon source. Bioprocess Biosyst Eng. Published online ahead of print


151


Hettinga DH and Reinbold GW (1972b) The propionic-acid bacteria II. Metabolism. J Milk Food Technol. 35:358-372

Hettinga DH and Reinbold GW (1972c) The propionic-acid bacteria III. Miscellaneous metabolic activities. J Milk Food Technol. 35:436-447


Langsrud T and Reinbold GW (1973) Flavour development and microbiology of Swiss cheese. II. Starters, manufacturing process and procedure. J Milk Food Technol. 36:531-542


155


Neronova NM, Ibragimova SI and Ierusalimski ND (1967) Effect of propionate concentration on the specific growth rate of Propionibacterium shermanii. Mikrobiologiya. 36:404-409


156


APPENDIX: SUPPLEMENTARY FIGURES

Figure A.1 Serum bottles used in anaerobic fermentation.
Figure A.2 Custom made bioreactor.
Figure A.3 Examples for calculation of some fermentation kinetics parameters.

Glucose consumption rate

$y = -0.0807x + 34.853$

$R^2 = 0.8631$

Propionate yield

$y = -0.4445x + 18.327$

$R^2 = 0.9142$

Propionate productivity

$y = 0.0456x + 2.32$

$R^2 = 0.9238$
Figure A.4 GC chromatogram showing n-propanol peak.
Figure A.5 HPLC chromatogram showing peaks of carbon source and products.