

Synthetic Biology & Metabolic Engineering of *Clostridium pasteurianum*

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degree requirements for a Doctor of Philosophy

By

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Author's Declaration

I hereby declare that I am the sole author of this thesis and that the work contained within is my own. No work contained within this thesis has been submitted for any other degree at the University of Nottingham nor any other institute of higher learning.

David Ortega

December 2019

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Abstract

Clostridia are a class of anaerobic bacteria with exquisite metabolisms and which have the potential to be exploited for production of industrial chemicals and biofuels. The advancements in genetic tools available are allowing for applying modern synthetic biology and metabolic engineering techniques to be applied for the development of industrially relevant recombinant strains of non-model organisms. One such organism is *Clostridium pasteurianum*, which can naturally ferment glycerol – a waste by product of the biodiesel industry – and produce the biofuel butanol and the industrial chemical 1,3-propanediol (1,3-PDO). In this thesis, a strain of *C. pasteurianum* with increased transformation efficiency (TE) is developed through successive deletion of host restriction modification (RM) systems. *C. pasteurianum* was also engineered to produce a non-natural product for the first time. Co-production of 1,3-PDO and the platform chemical 3-hydroxypropionic acid (3-HP) is achieved by introduction of two heterologous 3-HP pathways, a CoA-dependent and CoA-independent 3-HP pathway. A native glycerol inducible promoter is characterised and shown to have strong level of expression and induction and is utilised for overexpression of key enzymes in the 3-HP pathways. Using metabolic engineering, host metabolism is engineered to redirect carbon flux towards product pathways. We report the deletion of glycerol dehydrogenase (*dhaD*), dihydroxyacetone kinase (*dhaK*) and the active thiolase (*thl*) enzymes, confirming their roles in host metabolism and resulting fermentation phenotypes. Both 3-HP pathways are introduced into resulting mutants for improved product yield of 3-HP and 1,3-PDO. Final strains are then subject to process optimisation to evaluate the effects of pH on product formation. The best performing strains produced 66.6 moles of 3-HP and 1,3-PDO per 100 moles of glycerol compared to 11.4 moles of product in the wildtype. Lastly, a strain of *C. pasteurianum* was developed for efficient whole cell biocatalysis of chiral alcohols. By introducing a (R)-dependent alcohol dehydrogenase (Adh), we demonstrate high conversion of acetophenone to (R)-1-phenylethanol and high enantiomeric excess (*ee*). Expression of the Adh in $\Delta thlA2$ resulted in increased substrate concentrations and conversion percentages. This thesis furthers our understanding of production of non-natural products within anaerobic systems and promotes the industrial relevance of *C. pasteurianum*.

Keywords: *Clostridium pasteurianum*, glycerol fermentation, metabolic engineering, synthetic biology, 3-hydroxypropionic acid, 1,3-propanediol

Structure of thesis

A quick word on the organisational structure of this thesis. Chapter 1 introduces the importance of the environment and the relation between greenhouse gas (GHG) emissions and climate change. A summary of the main applications of biotechnology is provided covering the last 100 or so years, including the importance of fermentation-based products. The difference between fermentation, biomanufacturing and biocatalysis is explained from the author's perspective. The class of bacteria *Clostridia* is introduced with its relevance and potential for industrial biotechnology with a focus on *Clostridium pasteurianum*, the host strain used throughout this thesis. A summary of the tools, techniques and applications of synthetic biology and metabolic engineering towards industrial biotechnology is described. The relevant literature for each chapter topic is thoroughly summarised, describing the previous work, strategies and findings. The introduction ends with the aims and objectives of the work completed during the thesis.

Chapter 2 contains the materials and methods used for the work completed in this thesis. Molecular biology techniques, fermentation conditions and analytical methods are described. Oligonucleotide design and assembly methods used for construction of plasmids are described with some plasmid maps provided. All plasmids constructed and strains generated during this thesis are listed.

Chapters 3 – 5 are the results chapters, which begin with the aims and objectives followed by the results. Chapter 3 is focused on improving the genetic tractability of the host by improving the transformation efficiency through targeted deletion of the host restriction modification systems. Chapter 4 is focused on engineering *C. pasteurianum* to produce a non-native product for the first time through introduction of heterologous pathways. Chapter 5 is focused on the exploitation of tools and strains generated in Chapter 4 towards whole-cell biocatalysis.

Chapter 6 is a general discussion for the results presented in the previous three chapters, including significance, impact and future directions.

Chapter 7 briefly summarises two collaborative projects undertaken during the time of this PhD. The first was the supervision of a visiting graduate student whose work resulted in a publication. The second involved the generation of mutants for a group in Switzerland interested in the bioremediation properties of *C. pasteurianum*.

Abbreviations

%	Percentage
µg	Microgram
1,3-PDO	1,3-Propanediol
3-HP	3-Hydroxypropionic Acid
3-HPA	3-Hydroxypropionaldehyde
AAV	Adeno-associated Virus
ABE	Acetone-Butanol-Ethanol
ACE	Allele Coupled Exchange
Adh	Alcohol dehydrogenase
ADP	Adenosine Diphosphate
AE	Allelic Exchange
ALDH	Aldehyde dehydrogenase
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
C2-8	molecules containing between two and eight carbons
Cas	CRISPR Associated
CatP	Chloramphenicol Acetyltransferase
CBM	Clostridial Basal Media
CFU	Colony Forming Units
CoA	Co-enzyme A
CO ₂	Carbon Dioxide Gas
CRG	Clostridia Research Group
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
ee	Enantiomeric Excess
EMP	Embden-Meyerhof-Parnas
Erm	Erythromycin
FOA	5-fluoroorotic acid
gDNA	Genomic DNA
g/L	gram per litre
GHG	Greenhouse Gas
GM	Genetically Modified
h	Hours
H ₂	Hydrogen Gas
H4	H0D0R4 hypertransformable variant of <i>C. pasteurianum</i>
kbp	Kilo Base Pairs
KI	Knock-in
KO	Knock-out
LCA	Life Cycle Analysis
MCS	Multiple Cloning Site
M	Molar
mg	Milligram

mM	Millimolar
NHEJ	Non-homologous End Joining
N ₂	Nitrogen Gas
OECD	Organisation for Economic Co-operation and Development
PCR	Polymerase Chain Reaction
ppm	Parts Per Million
RBS	Ribosome Binding Site
RM	Restriction Modification
RPM	Rotations Per Minute
SDM	Site-directed Mutagenesis
SLP	Substrate Level Phosphorylation
SNP	Single Nucleotide Polymorphism
SOE	Splicing by Overlap Extension
t	Time
TE	Transformation Efficiency
T _m	Annealing Temperature
US	United States
UTR	Untranslated Region
w/v	Weight / Volume

Table of Contents

Author's Declaration	i
Acknowledgements	ii
Abstract	iii
Structure of thesis	iv
Abbreviations	v
List of Figures	x
List of Tables	xi
Chapter 1 Introduction, Literature Review & Research Objectives	1
1.1.0 Introduction	2
1.1.1 <i>Society is becoming more conscience about the environment</i>	2
1.1.2 <i>What are the main sources of GHG emissions?</i>	2
1.1.3 <i>Bioeconomy and biotechnology</i>	5
1.1.4 <i>A brief introduction into fermentation</i>	5
1.2.0 Literature Review	6
1.2.1 <i>Modern biomanufacturing is centred on medicinal and food products</i>	6
1.2.2 <i>Biocatalysis vs. biomanufacturing</i>	7
1.2.3 <i>Biomanufacturing for the fuel and bulk chemical industries</i>	8
1.2.4 <i>The circular economy and using waste as a feedstock for bioprocesses</i>	9
1.2.5 <i>Overview of Clostridia and Clostridium pasteurianum</i>	11
1.2.6 <i>Microbial cell factories and metabolic engineering</i>	12
1.2.7 <i>The metabolism of Clostridium pasteurianum</i>	13
1.2.8 <i>Transformation efficiency and the restriction modification systems in Clostridium pasteurianum</i>	17
1.2.9 <i>Microbial production of the platform chemical 3-hydroxypropionic acid and 1,3-propanediol</i>	19
1.2.10. <i>Production of high value compounds through whole-cell biocatalysis</i>	24
1.2.10.1 <i>A short summary of enzyme biocatalysis</i>	24
1.2.10.2 <i>C. pasteurianum as a host for whole cell biocatalysis</i>	26
1.2.10.3 <i>Choosing an alcohol dehydrogenase and substrate for whole cell biocatalysis</i>	27
1.3 <i>Research objectives of this thesis</i>	28
Chapter 2 Materials and Methods	29
2.1 Materials and Methods	30
2.1.1 <i>Reagents, growth and maintenance conditions of bacterial strains</i>	30
2.1.2 <i>Standard Molecular Biology Techniques</i>	33
2.1.3 <i>Construction of expression plasmids</i>	35

2.1.4 Construction of genome modification plasmids and associated methods .	40
2.1.5 In vivo plasmid methylation, DNA transfer into <i>C. pasteurianum</i> and screening transformants.....	42
2.1.6 Calculating transformation efficiency.....	43
2.1.7 Analysis of fermentation products.....	43
2.1.8 Measuring activity of ALDHs	44
2.1.9 Promoter identification, cloning and characterization by chloramphenicol acetyltransferase assay	45
2.1.10 Batch fermentations	46
2.1.11 pH controlled fermentations.....	46
2.1.12 Whole cell biocatalysis fermentations.....	47
2.1.13 Measuring conversion efficiency of biotransformations	47
2.1.14 Calculations for mass balance	47
2.2.1 Tables of strains, plasmids and oligonucleotides used in this thesis.....	49
Chapter 3 Improving Transformation Efficiency in <i>Clostridium pasteurianum</i>	59
3.1 Chapter Introduction	60
3.2 Results.....	61
3.2.1 Transformation Efficiency (TE) in <i>C. pasteurianum</i>	61
3.2.2 Deletion of the Type II CpaAIP RM system in <i>C. pasteurianum</i>	65
3.2.3 Successive deletion of the Type I CpaAII RM system in <i>C. pasteurianum</i> ...	69
Chapter 4 Co-production of 3-Hydroxypropionic Acid and 1,3-Propanediol From Glycerol	73
4.1 Chapter Introduction	75
4.2 Results.....	78
4.2.1 3-HP is not consumed by nor is it toxic to <i>C. pasteurianum</i>	78
4.2.2 Expression plasmids do not cause significant effects on fermentation	79
4.2.2 The CoA-dependent (Pdu) 3-HP pathway	81
4.2.3 The CoA-independent (ALDH) 3-HP pathway	84
4.2.4 Increasing carbon flux towards reductive branch of metabolism increases 3-HP and 1,3-PDO titres.....	87
4.2.5 Expression of <i>dhaB₁B₂</i> and 3-HP pathways from a single plasmid increases biomass formation but does not increase 3-HP titres	91
4.2.6 Metabolic engineering – deletion of <i>dhaD</i> and <i>dhaK</i> prevents growth on glycerol.....	94
4.2.7 Fermentation of glucose-glycerol mixtures with the mutant Δ <i>dhaD_K</i> expressing the 3-HP pathways.....	97
4.2.8 Conversion of glycerol to 3-HP and 1,3-PDO using resting cells of Δ <i>dhaDK</i> expressing the Pdu pathway.....	101

4.2.9 Metabolic engineering – deletion of active thiolase abolishes production of C4 products butyrate and butanol	104
4.2.10 Plasmid based expression of Pdu 3-HP pathway in $\Delta thlA2$	109
4.2.11 Integration of 3-HP constructs in $\Delta thlA2$ – batch fermentations	111
4.2.12 Process optimisation – using static pH fermentations to control product formation	113
Chapter 5 Whole-cell Biocatalytic Reduction of Ketones to Chiral Alcohols	117
5.1 Chapter Introduction	119
5.2.0 Results.....	120
5.2.1 Establishing a <i>Clostridium</i> based whole cell system: toxicity, background activity and comparison to the <i>E. coli</i> system.....	120
5.2.2 Optimising the <i>Clostridium</i> system: premixing substrate with ethanol....	123
5.2.3 Time of addition affects substrate conversion.....	125
5.2.4 Comparing the mutant $\Delta thlA2$ to wildtype <i>C. pasteurianum</i> for reduction of acetophenone to (R)-1-phenylethanol.....	127
5.2.5 Expanding the substrate scope to aromatic ketones.....	129
Chapter 6 General Discussion	131
6.1 Overview of thesis	132
6.2 Discussion on improving TE through successive deletion of RM systems in <i>C. pasteurianum</i>	132
6.3 Discussion on co-production of 3-HP and 1,3-PDO from glycerol.....	134
6.3.1 Probing potential causes for the low production of 3-HP	134
6.3.2 Production of 3-HP is influenced by the availability of NAD^+	135
6.3.3 Using glycerol as a growth substrate can cause shifts in product formation by influencing the intracellular NAD^+ / $NADH$ ratio	136
6.3.4 Microbial production of 3-HP works best under microaerobic conditions	137
6.3.5 The Pdu 3-HP pathway has not been as explored or successful for production of 3-HP.....	138
6.3.6 An inducible glycerol promoter is an important synthetic biology tool for the fermentation of glycerol	139
6.3.7 The interesting phenotypes of mutants $\Delta thlA2$ and $\Delta dhaDK$	140
6.4. Discussion on the use of <i>C. pasteurianum</i> as a platform for whole cell biocatalytic reduction of ketones to chiral alcohols.....	142
Chapter 7 Collaborative Projects & Industrial Placement	145
7.1 Description of non-core PhD activities	146
8.1 References	148

List of Figures

Figure 1.1 World energy consumption and EU GHG emissions.	4
Figure 1.2 Overview of central carbon metabolism of <i>C. pasteurianum</i>	16
Figure 2.1 Circular and linear maps of the expression plasmid Q5_82251.	38
Figure 2.2 Illustration of cloning procedure for insertion of <i>dhaB₁B₂</i> downstream of inducible glycerol promoter P _{dhaBL}	39
Figure 3.1 Probing the TE of commonly used plasmids in <i>C. pasteurianum</i>	64
Figure 3.2 Confirming and characterizing the TE of the mutant H4 Δ <i>pyrE</i> Δ <i>cpaAIP</i>	67
Figure 3.3 Fermentation phenotype of mutant H4 Δ <i>pyrE</i> Δ <i>cpaAIP</i>	68
Figure 3.4 Confirming the isolation of the mutant Δ <i>pyrE</i> Δ <i>cpaAIP</i> Δ <i>cpaAII</i>	70
Figure 3.5 Transformation efficiency of H4 Δ <i>pyrE</i> Δ <i>cpaAIP</i> Δ <i>cpaAII</i>	71
Figure 4.1 Glycerol metabolism in <i>C. pasteurianum</i> , key metabolites and the two 3-HP formation pathways.	76
Figure 4.2 Evaluating toxicity and consumption of 3-HP on growing cultures of <i>C. pasteurianum</i>	78
Figure 4.3 Control fermentations with <i>C. pasteurianum</i> harbouring empty expression plasmids.	80
Figure 4.4 Characterising the CoA-dependent 3-HP pathway in <i>C. pasteurianum</i> ..	83
Figure 4.5 Characterising the CoA-independent 3-HP pathway in <i>C. pasteurianum</i>	85
Figure 4.6 Characterisation of the native inducible glycerol promoter and overexpression of heterologous <i>dhaB₁B₂</i>	88
Figure 4.7 Evaluating co-production of 3-HP and 1,3-PDO by overproducing <i>DhaB₁B₂</i> and <i>AIDHs</i> KGSADH or <i>GabD4</i>	90
Figure 4.8 Reducing metabolic burden by expressing both 3-HP pathways from a single plasmid and testing <i>GabD4</i> variants.	92
Figure 4.9 Characterising enzyme activity of <i>GabD4</i> variants.	93
Figure 4.10 Confirming in-frame deletion of <i>dhaD</i> and <i>dhak</i> in H4 Δ <i>pyrE</i> (H4P5). ..	95
Figure 4.11 Characterisation of Δ <i>dhaDK</i> and its complement Δ <i>dhaDK</i> <i>pyrE::dhaDK</i>	95
Figure 4.12 Characterising the growth phenotype of <i>C. pasteurianum</i> with varied concentrations of glucose.	96
Figure 4.13 Introducing the CoA-dependent and CoA-independent pathways into Δ <i>dhaDK</i>	98
Figure 4.14 Fermentation of 0.25% glucose and 5.75% glycerol using Δ <i>dhaDK</i> expressing solely the CoA-independent pathway or in combination with <i>dhaB₁B₂</i> . ..	99
Figure 4.15 Co-production of 1,3-PDO and 3-HP using resting cells of Δ <i>dhaDK</i> expressing the <i>Pdu</i> pathway.	102
Figure 4.16 Gels confirming deletion of <i>thlA2</i> , chromosomal complementation of <i>thlA2</i> and repairing <i>pyrE</i> in Δ <i>thlA2</i> Δ <i>pyrE</i>	105
Figure 4.17 Confirming the role of <i>Thl</i> in host metabolism.	107
Figure 4.18 Comparing the glucose and glycerol fermentation product profile of Δ <i>thlA2</i>	108
Figure 4.19 Expression of <i>dhaB₁B₂</i> and the <i>pdu</i> 3-HP pathway in Δ <i>thlA2</i>	110
Figure 4.20 Integration of CoA-dependent and CoA-independent 3-HP pathways in Δ <i>thlA2</i>	112
Figure 4.21 Effects of pH on product formation in Δ <i>thlA2</i> with 3-HP pathways integrated.	114

Figure 4.22 Effects of pH on product profile of $\Delta thlA2$ with 3-HP pathways integrated.	115
Figure 4.23 Comparing co-production of 3-HP and 1,3-PDO and product yield between engineered strains and wildtype.	116
Figure 5.1 Effects of acetophenone on growing cultures of <i>C. pasteurianum</i>	120
Figure 5.2 Initial screen for conversion of acetophenone (R)-1-phenylethanol. ...	122
Figure 5.3 Evaluating effects of premixing ethanol with acetophenone on conversion efficiency.	124
Figure 5.4 Effects of adding acetophenone or (R)-1-phenylethanol at different time points on growth of wildtype <i>C. pasteurianum</i>	126
Figure 5.5 Characterising the performance of the strain $\Delta thlA2$ harbouring LK-ADH for whole cell reduction of acetophenone to (R)-1-phenylethanol.	128
Figure 5.6 Expanding substrate scope for whole-cell biocatalytic reduction using wildtype <i>C. pasteurianum</i> expressing LK-ADH.	130

List of Tables

Table 2.1 CBMS agar components and instructions for preparation.	31
Table 2.2 Components and instructions for preparing Biebl minimal media.	32
Table 2.3 Components and instructions for preparation of SL7 trace element solution.	32
Table 2.4 Summary of products formed during the fermentation of glycerol by <i>C. pasteurianum</i>	48
Table 2.5 Strains, plasmids, and oligonucleotides, constructed, used, and designed in Chapter 3.	49
Table 2.6 Strains, plasmids, and oligonucleotides, constructed, used and designed in Chapter 4.	52
Table 2.7 Strains, plasmids, and oligonucleotides, constructed, used, and designed in Chapter 5.	58
Table 3.1 List of genome modification and gene expression tools developed by the CRG for the genus <i>Clostridium</i> and used in this work.	62
Table 3.2 List of annotated and verified RM systems in the genome of <i>C. pasteurianum</i> and method for circumvention permitting DNA transfer.	66
Table 4.1 Redox and energetic characteristics of products formed during the fermentation of glycerol by <i>C. pasteurianum</i>	77
Table 4.2 Carbon mass balance of fermentations for strains expressing the CoA-dependent (Pdu) 3-HP pathway.	82
Table 4.3 Carbon mass balance of fermentations for strains expressing the CoA-independent (AldH) 3-HP pathway.	86

Chapter 1

Introduction, Literature Review & Research Objectives

1.1.0 Introduction

1.1.1 Society is becoming more conscience about the environment

The signing of the Paris Agreement in 2016 was an important step in the fight for the environment. It follows on from the Kyoto Protocol signed in 1997 and is part of the United Nations Framework Convention on Climate Change (1). Although the effectiveness of the Paris Agreement has been called into question, it represents a concerted commitment by global states towards tracking and implementing changes to our economic models to stabilise the levels of greenhouse gases (GHGs) in the atmosphere and reduce anthropological damage on the climate system (2). These international commitments echo the increasing awareness of the current state of the environment by global citizens and the increasing demands to change the way we interact with the environment. In 2018 a young Swedish girl named Greta Thompson gained widespread media coverage as she began touring the world criticizing governments and leading politicians for their inaction and disregard for the environment in their policies (3). The rise of the international movement Extinction Rebellion also occurred in 2018, was started here in the UK and has expanded its protests internationally; they use non-violent civil disobedience to compel governments action towards “creating a world that is fit for generations to come” (4). Of course, there have been other players and organisations around the world fighting for the same cause, but the message is the same, the environment has been put in the forefronts of people minds.

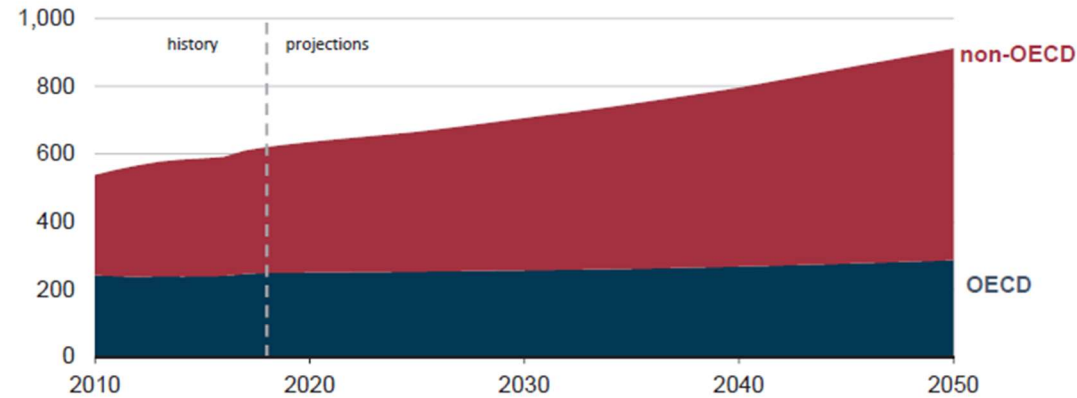
1.1.2 What are the main sources of GHG emissions?

The reality of our world economy is that we are still very much a fossil fuel-based economy. In terms of energy, the world consumes ≈ 600 quadrillion British thermal units (BTUs) of energy with non-renewables such as oil, coal and gas accounting for more than 80% in 2018; world energy consumption is expected to increase nearly 50% by 2050 (5) (Fig. 1.1A). Our increase in oil and energy consumption over the last 150 years has increased stepwise with atmospheric levels of carbon dioxide (CO₂) now over 400 parts per million (ppm) and the highest it has been in 800,000 years (6) This increase in atmospheric CO₂ is thought to be the cause of the global averaged combined land and ocean temperatures increasing 0.85 °C between 1880 and 2012 (7). Going forward, it will be difficult balance our increase in energy consumption with our goals of reducing GHG emissions at the global scale as the energy market contributes approximately two-thirds of all GHG emissions (5). The EU

has set its own targets for reducing GHG emissions; a reduction of 20% and 40% by 2020 and 2030, respectively, compared to 1990 levels (Fig. 1.2). Is the EU on pace to meet its ambitious targets? By 2017, the EU had reduced its GHG emissions by 21.9%, but is not projected to meet its 2030 target of 40% reduction (8) (Fig. 1.1B). One of the reasons for this is the difficulty in reducing GHG emissions from the burning of oil. In the EU, GHG emissions derived from fuel consumption including transportation is responsible for almost 80% of all GHG emissions, dwarfing emissions from industrial processes and agriculture (9). 2017 saw a small increase in overall GHG emissions in the EU as decreases in the energy sectors (caused by a continuing shift away from coal and towards renewables for heat and energy) were offset by increases in the transportation and manufacturing sectors (8). In contrast, the EU chemical industry, a pillar of the European economy with approximately €565 billion in annual sales (2018), has reduced GHG emissions by 58% from 1990 levels, yet still, annual emissions are 135 million tonnes of CO₂ equivalent (10). Given that significant long-term capital has been invested into the energy, petrochemical and transportation infrastructure and that shifts in energy systems have historically been slow, it is unlikely that these industries will significantly decrease their GHG emissions in the next decade (11). By moving from an oil-based economy to a bio-based economy, many of the same products and feedstocks can be produced renewably, integrating into existing infrastructure, thus decoupling economic growth from GHG emissions and facilitating the shift to a low carbon economy (12).

World energy consumption rises nearly 50% between 2018 and 2050 in the Reference case —

World energy consumption
quadrillion British thermal units

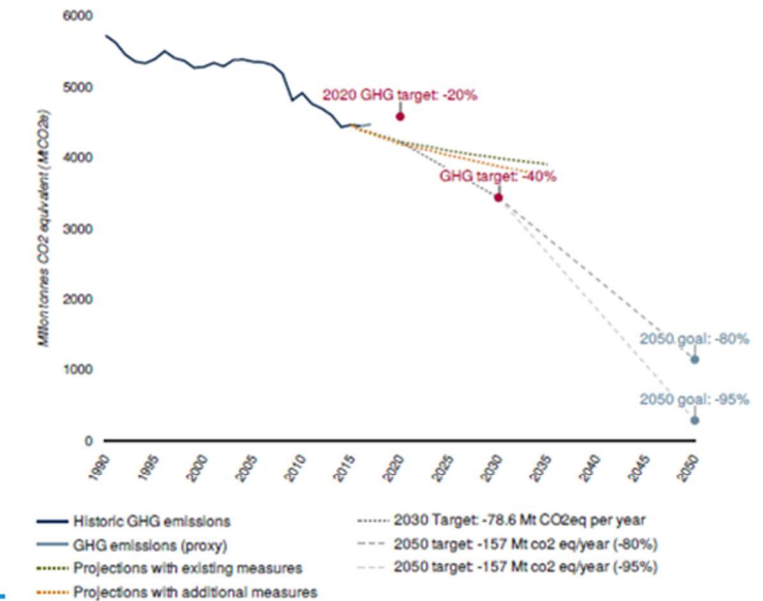


U.S. Energy Information Administration

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Climate change mitigation

Figure 1. Greenhouse gas emission trends, projections and targets in the EU



European Environment Agency

Figure 1.1 | World energy consumption and EU GHG emissions. (A) Projected world energy consumption from 2010 to 2050. Figure taken from the U.S. Energy Information Agency report: International Energy Outlook Report, 2019. **(B)** EU greenhouse gas emission trends, projections, and targets. Figure taken from the 2019 European Environment Agency report: Recent trends and projections in EU greenhouse gas emissions.

1.1.3 Bioeconomy and biotechnology

The EU defines the bioeconomy as the “the production of renewable biological resources and the conversion of these resources and waste streams into value-added products, such as food, feed, bio-based products and bioenergy”(13). This broad definition includes the agriculture, forestry, fishing and aquaculture, paper and wood product industries among others. The Organisation for Economic Co-operation and Development (OECD) defines the bioeconomy as an economy in which biotechnology contributes to a significant share of economic output and highlight the three main sectors in which biotechnology can be applied: agriculture, health and industry (14). While the EU definition includes all biologically linked industries in the bioeconomy, the OECD definition focuses on modern biological sciences and how those technologies can enhance those older industries as well as develop new ones.

To address both the future demand of goods and services required for an increasing population and meet future targets in terms of GHG emissions, our economies must become more efficient. This is echoed in the key challenges highlighted by the EU that must be addressed through the further development of the bioeconomy, mainly the development of a circular economy by using waste as a resource, implementing a systems approach to all forms of agriculture, educating consumers on scarcity and waste, incentivising purchase of sustainably produced goods and reducing GHG emissions by using biomass as a feedstock (15). The above can be achieved through increased integration of biotechnological processes into our modern economies. One of the most common and increasingly used biotechnological processes is fermentation.

1.1.4 A brief introduction into fermentation

In society and historically, fermentation is known for its role in the alcoholic beverage, fermented foods and baking industries. Although these ‘industries’ have been part of human culture for millennia, the underlying mechanism of fermentation wasn’t described until 1857, when Louis Pasteur discovered “life without air”. Biochemically, “life without air” refers to the set of biochemical reactions that describe the catabolism of different carbohydrates into different metabolites in the absence of air. This is catabolised by microorganisms, which grow by breaking down available sugars for carbon and energy and produce various end products, such as acetic acid, lactic acid and ethanol during the vinegar, cheese and beer making processes, respectively. Our increased understanding of the microbiology and biochemistry of the

process over the last 100 or so years has allowed us to harness the power of fermentation in many new ways. In modern times, the use of the word 'fermentation' has expanded to include the production of chemicals, biofuels and medicines in what is present day biotechnology or biomanufacturing.

1.2.0 Literature Review

1.2.1 *Modern biomanufacturing is centred on medicinal and food products*

The application of the fermentation process has progressed from the early 20th century and this term is now used to describe both anaerobic and aerobic bioprocesses catalysed by both prokaryotes and eukaryotes. In the 1912, the acetone-butanol-ethanol (ABE) fermentation was used to initially produce the solvent butanol but then used for production acetone during the first world war by fermentation of sugars using *Clostridium acetobutylicum* (16). Fermentation processes were developed for the production of penicillin between 1942 – 44 by isolating a hyperproducing strain of *Penicillium chrysogenum* (17). The production of amino acids such as L-glutamic acid L-lysine were first produced in 1958 using the fermentation of sugars by *Corynebacterium glutamicum* for use as food and feed additives (18,19). Over the last four decades, advancement in molecular biology and recombinant DNA technologies have allowed for an ever-increasing number of complex products to be produced using fermentation. These processes originally used cultures of wildtype microorganisms but have been mostly replaced by engineered or recombinant microorganisms to produce primarily medicines, fuels and industrial enzymes. The first recombinant protein produced commercially using recombinant DNA technologies was insulin in 1982, when the human insulin A and B chains were cloned into *E. coli* (20). The 1980s saw the onset of the biopharmaceutical industry as a number of peptide hormone products were commercially manufactured using recombinant organisms including erythropoietin and human growth hormone. This coincided with the production of industrial enzymes such as amylase and chymosin used in the brewing and cheese making industries (21). The late 20th century saw an explosion in the number of genetically modified (GM) crops such as corn, soybean and cotton which was possible due to advances in recombinant DNA technology and resulted in decreased pesticide use, increased drought resistance, increased yields and nutritional enhancements, the latter culminated with the engineering of 'golden rice' in 2000 (22–25). In more recent times, biomanufacturing has expanded into production of gene therapy products such as adeno-associated viruses (AAVs) vectors for the treatment of genetic disorders and

personalised medicines such as chimeric antigen receptor T (CAR-T) cells used in immunotherapies (26,27). The recent discovery of clustered regularly interspaced short palindromic repeats (CRISPR) systems will certainly lead to the development of novel gene therapy medicines (28). While biotechnology has the potential to deliver a wide variety of products, across a variety of industries, commercial success has been mostly concentrated in the healthcare, agricultural and food industries. While our societies have reaped the benefits of these innovative products, the commercial success of biotechnology has not translated over to the bulk commodity and fuel markets and thus have had limited effect on reducing GHG emissions.

1.2.2 Biocatalysis vs. biomanufacturing

One field that is considered biomanufacturing but has been intentionally omitted from the above section is biocatalysis. By the strictest definition, brewing, the production of penicillin or the making of cheese are all considered biocatalysis. There are more advanced examples of this type of first generation of biocatalysis such as the use of proteases in laundry detergents or the conversion of glucose into fructose; the commonality between them being the conversion of natural products into more natural products (29,30). The second generation of biocatalysis used wildtype and the first engineered enzymes for production of unnatural products or synthetic intermediates. For example, the production of enantiomerically pure cyanohydrins, important intermediates in the agrochemical and pharmaceutical industry were produced from hydrogen cyanide and aldehydes or ketones using purified bacterial hydroxynitrile lyases (31). Similarly, the commercial production of the commodity chemical acrylamide was developed in Japan in the 1980s using acrylonitrile as the feedstock and nitrile hydratase (32). The most recent or third generation of biocatalysis has been based on the engineering of enzymes through directed evolution; variants were created through an iterative process of mutant library creation followed by selection of mutants with desired characteristics. These efforts have been aided by the development of gene sequencing and synthesis technologies, bioinformatic and computer modelling tools and robotic automation (33). Two well-known examples include an engineered transaminase for the production of Sitagliptin and an engineered ketoreductase for the production of key intermediates of atorvastatin, used in the treatment of type II Diabetes and high blood cholesterol, respectively, each with markets worth many billions per year; development of these two biocatalysts has resulted in decreased waste, higher selectivity and overall higher

productivity of the overall process compared to their metal catalyst counterparts (34,35). Given the advantages and success stories of biocatalysts, one may presume that widespread use of biocatalysts is common practice in the pharmaceutical and chemical industries, but that is not the case (36,37). The motivation for the author differentiating between biocatalysis and biomanufacturing is due to the inputs of the process. While not true for all biocatalytic processes, many use petrochemical intermediates as feedstocks. In biomanufacturing, the feedstock which is typically powering the process is also used to physically build or produce the final products. For example, *C. acetubutylicum* ferments glucose into acetone, butanol and ethanol; the starting and end products are completely different types of molecules and the conversion requires tens of enzymatic steps, but the starting material is renewable. Similarly, the production of recombinant insulin using engineered *E. coli* uses the machinery found in the microorganism to convert sugars, a renewable substrate into a protein product. Thus, these products have lower GHG emissions associated with their manufacture.

1.2.3 Biomanufacturing for the fuel and bulk chemical industries

Unlike biocatalysis, there are relatively few success stories in the commercialisation of biomanufacturing type processes for production biofuels and chemical commodities. Historically, interest and investment in this application of biotechnology has risen and fallen with the price of oil. The last two decades has seen a number of high-profile companies receive large investments to commercialise the large-scale production of biofuels only to close their doors several years later, switch to producing higher value chemicals or get bought out. For example, LS9, which won the Presidential Green Chemistry Award in 2010 for developing renewable petroleum from fatty-acid metabolism intermediates using recombinant microorganisms had raised \$75 million over four funding rounds between 2007 – 2010 only to get bought out for \$61.5 million in 2014 (38,39). Many players in the biofuel industry were hoping for the success of Amyris, which was founded in 2003 and had received more than \$150 million to produce farnesene (40,41). Amyris went public in 2010 but has since pivoted to producing compounds for the food, health and beauty industries (42). Aurora Biofuels founded in 2007, later renamed Aurora Algae, had raised more than \$100 million to commercialise algae derived biofuels by cultivating algae in large outdoor ponds but ceased operations back in 2015 (43,44). TerraVia, originally named Solazyme and founded in 2003, started out by producing biofuels and had raised more

than \$170 million, but now creates algae derived health foods as many of its competitors have been forced to do (45–48) .

Two notable examples of biotechnological success in the chemical industries are the production of 1,3-propanediol (1,3-PDO) and 1,4-butanediol from glucose. Dupont developed a recombinant *E. coli* strain in 2004 that was able to produce upwards of 130 g/L of 1,3-PDO from glucose (49). In 2009, Genomatica developed a recombinant strain of *E. coli* used for the commercial production of 1,4 butanediol from carbohydrate mixtures (50,51). Although these bioprocesses are a step in the right direction, there are real societal trade-offs and environmental costs associated with bioprocesses that take food grade carbohydrate feedstocks and turn them into chemical commodities (52,53). Take the example of bioethanol production in the U.S.A., in some eyes considered a success story. Due to the Renewable Fuel Standard Program adopted in 2005, which forced a minimum amount of renewable fuel to be blended into transportation fuel, creating an artificial demand for biofuel and influencing the costs of agricultural prices (51,52). This approach to production of bioethanol is in contrast to that taken by Lanzatech. Founded in 2005, they have raised more than \$275 million to produce bioethanol using syngas, which can be generated from a variety of waste streams including biomass gasification and steel mills (56,57). This strategy does not require arable land, nor compete with food sources and represents the next generation of biofuels and biochemicals. Next generation biofuels, produced from cellulosic material, biomethane from waste or syngas produced from the gasification of biomass and the like result in less GHG emissions compared to petrochemical derived fuels (58,59).

1.2.4 The circular economy and using waste as a feedstock for bioprocesses

The viability of producing bulk commodities such as chemical feedstocks or fuels from biological sources (biofuels and biochemicals) depends largely on the feedstock being used as this typically represents 60-70% of the overall cost of the bioprocess (60). The production of bioethanol from sugar crops such as corn, sugar cane, sugar beet and starch crops are considered first-generation biofuel as they compete with the food and feed industries for their raw material or feedstock. The high and easily accessible sugar content of crops makes their conversion into biofuels relatively simple, although this strategy creates ethical, political and environmental concerns. The same is true of biodiesel, which is extracted from high fat content crops such as rapeseed, soybean, sunflower and palm oil through transesterification (61).

Next generation biofuels or biochemicals are those derived from the non-food crops, waste residues or waste gases. In contrast to first generation, where only a fraction of the biomass is used in the conversion to product thus generating waste residues, second generation biofuels or biochemicals rely on utilisation of the whole plant. This results in increased carbon yields, land usage efficiency and environmental performance (lower GHG emissions) (61). Typically, the carbon in the biomass is found as polysaccharides and must be broken down into their component sugars and is achieved by pre-treatment using either biochemical, chemical or thermochemical means. The use of strong acids or alkalis promote hydrolysis of cellulosic material and is one method of chemical pre-treatment, the other being transesterification of vegetable oils. Biochemical treatment of cellulosic biomass involves hydrolysis using industrial enzymes or gasification by anaerobic digestion. Pyrolysis or gasification of biomass produces bio-syngas and is achieved by heating substrates in the absence of oxygen at medium or high temperatures, respectively, and are common methods of thermochemical treatment (59). The advantage of bio-syngas is that it can be used as an intermediate feedstock as it can be easily converted into various other chemicals using chemical catalysts (62).

To compete with existing petroleum-based commodities, it is imperative that modern biorefineries be able to efficiently produce multiple chemicals from various waste or non-food feedstocks. One recent example is the case of glycerol. The large increase in biodiesel production has resulted in glycerol becoming a waste product rather than a by-product of the biodiesel industry as it is produced at a ratio of 10% w/v (63). Thus, the conversion of glycerol to higher value products has been highlighted as means to increasing economic viability of the biodiesel industry. Due to the reduced nature of glycerol, the theoretical yield for production of reduced compounds such as butanol, ethanol, succinic acid or propionic acid is higher than that possible using traditional sugars (64). Two compounds in particular have been highlighted for production from glycerol using microbial catalysts: 3-hydroxypropionic acid (3-HP), and 1,3-PDO (65). Although not the only usable waste stream available, the ability to convert a waste stream such as glycerol from the biodiesel industry into a valuable product is an example of the circular economy initiatives currently being enacted by governing bodies such as the EU. Indeed, turning waste into a resource is one of the key pillars of European Commission's circular economy approach (66). The EU projects that demand for biomass from increased population growth will increase

by 50% by 2050 and thus simultaneously decreasing GHG emissions and meeting the needs of the population will only be achieved through the efficient use of biomass (15).

1.2.5 Overview of *Clostridia* and *Clostridium pasteurianum*

Initially described in the late 1800s, the genus *Clostridium* includes a diverse variety of Gram positive, rod shaped mesophilic bacteria, characterised by having a low G-C content, being obligate anaerobes and having the capability of forming endospores. Furthermore, the genus also has a rich and important history for the investigation of microbial metabolism biochemistry, anaerobic fermentations and a variety of prokaryotic proteins and enzymes. Some influential findings made with *Clostridium* spp. include but are not limited to: the discovery of the Stickland reaction in proteolytic clostridia, demonstration that fermentations were not necessarily degradative in nature, involvement of an acyl-carrier protein in fatty acid biosynthesis, discovery of the coenzyme forms of vitamin B₁₂, discovery of major components and mechanisms of the Wood-Ljungdahl pathway, discovery of low potential electron donors such as ferredoxin, demonstration of nitrogen fixation by a cell free bacterial extract, demonstration of the catalytic versatility of metalloproteins, investigations of NADH and NADPH as electron carriers as well as highlighting the coupling between electron flow and free energy conservation (67).

Species of *Clostridia* can be broadly classified as being pathogenic and apathogenic, the former containing some very well-known species such as *C. tetani* and *C. botulinum*, responsible for causing tetanus and botulism, respectively (68). Perhaps even more well known among healthcare professionals is *C. difficile*, responsible for a large proportion of antibiotic associated diarrhoea and antibiotic-associated pseudo-membranous colitis (69).

Of industrial biotechnological relevance are the solventogenic and acid producing strains *C. acetobutylicum*, *C. butyricum* and *C. beijerinckii*, the cellulolytic strains *C. thermocellum* and *C. cellulolyticum* and the acetogenic strains *C. ljungdahlii*, *C. thermoaceticum* and *C. carboxydovorans* (67). The diversity of growth substrates that are accessible to *Clostridia* is a testament to their exquisite metabolic capabilities and highlights their potential for industrial biotechnology (70). Indeed, the use of *Clostridia* for industrial biotechnological applications is not new. One of the first modern industrial processes developed was that of the acetone-butanol-ethanol (ABE) fermentation catalysed by the fermentation of sugars by *C. acetobutylicum* (16). This

process was abandoned due to the rise of the petrochemical industry making the ABE process obsolete. We have come full circle as there is increasing interest in the use of fermentation-based technologies to produce biofuels and biochemicals from renewable and waste resources.

One such waste resource is glycerol, introduced above as a waste by product of the biodiesel industry. There are several species of Clostridia that can use glycerol as their sole carbon and energy source, including *C. butyricum*, *C. saccharobutylicum* and *C. pasteurianum*. In recent years, *C. pasteurianum* has gained renewed interest for its ability to ferment glycerol yielding a mixture of gases, acids, alcohols, and notably 1,3 propanediol and butanol. With the development of tools for genetic manipulation of the species, more research efforts have focused on the engineering of this host through targeted genetic modification (71–80). The availability of genetic tools and its ability to ferment glycerol make *C. pasteurianum* an interesting chassis from which to produce biofuels and biochemicals by applying synthetic biology and metabolic engineering.

1.2.6 Microbial cell factories and metabolic engineering

The term microbial cell factory describes the use of microorganisms for the production of natural or unnatural products, proteins, chemicals or any other compound of commercial interest. Historically achieved by growing wildtype microorganisms known to naturally produce a desired compound, the development of molecular biology and genetic tools have paved the way for the development of recombinant microorganisms. As research and commercial interest shifted towards manipulating the metabolism of organisms to overproduce compounds of interest, the field of metabolic engineering emerged. Even with the most modern genetic tools and with an ever expanding knowledge base, it has been frustratingly difficult to engineer new microbial cell factories with projects typically taking 6-8 years and costing in excess of \$50 million to reach commercialisation (81). This is due to the extensive amount of work that is required to successfully engineer a host for overproduction of a compound of interest. The motivation for these projects is usually two-fold: the ability to produce new chemicals and the fact that the process has the potential to be much more sustainable compared to petrochemical derived biochemicals.

Modern metabolic engineering typically involves use of ‘work horses’ such as *E. coli* or *S. cerevisiae* as the chassis, as they are the most studied and well understood

and have the most advanced genetic tools available. Although, in recent times, the development of tools in non-model organisms is increasing, allowing for researchers to exploit the natural differences in microorganisms towards specific purposes. Thus, the choice of the platform organism or chassis used in the development of a novel bioprocess is no longer restricted to either *E. coli* or *S. cerevisiae*.

To engineer an efficient and productive strain requires manipulating the host organisms' metabolism to redirect carbon flux towards a specific product of interest. To do this, a combination of *in silico* and 'omics' type approaches are taken. Genome scale models are built by genome sequencing of the organism and building of a metabolic model of the organism by introducing reaction rates to understand distribution and flux of carbon (82). Synthetic pathways are designed using retrosynthesis algorithms and selection of enzymes can now be done algorithmically (83,84). Machine learning is now being used in the aid of design of experiments facilitating workflows and exploration of design space, leading to quicker iterative design build test cycles with impressive results (85). The discovery of CRISPR-Cas9 systems has revolutionised genome editing across all domains of life and is being exploited for knock down studies, multiple gene editing, evolutionary studies, and in genetic circuits among others (86–90). The ever increasing tools available to researchers and the decreasing cost of DNA synthesis is facilitating the development of recombinant organisms for the biological production of biochemicals.

1.2.7 The metabolism of *Clostridium pasteurianum*

The attractiveness of *C. pasteurianum* as a host chassis is due to its highly flexible metabolism characterised by a unique combination of oxidative and product forming pathways common among clostridia. Oxidation of the growth substrate provides carbon and energy for biomass formation and reducing equivalents in the form of NADH, the latter of which must be recycled to maintain redox balance. The mechanism for recycling of reducing equivalents is one of the defining differences between aerobic and anaerobic fermentation. In aerobic fermentation, reducing equivalents are recycled by reduction of oxygen, the terminal electron acceptor, to water. In anaerobic fermentation, reducing equivalents are recycled through the formation of the fermentation products. Thus, in anaerobic fermentation, product formation can be influenced by the reduced nature of the oxidised substrate.

Like other ABE fermenting clostridia such as *C. acetobutylicum* and *C. beijerinckii*, fermentation of sugars by *C. pasteurianum* results in formation of acids acetate, lactate, butyrate, and solvents butanol and ethanol. However, there are several key differences between *C. pasteurianum* and other ABE fermenting clostridia. For example, *C. pasteurianum* does not exhibit a bi-phasic growth pattern (acidogenesis followed by solventogenesis) or acetone formation. Furthermore, ABE fermentation typically results in the formation of acetone-butanol-ethanol at a consistent ratio of 3:6:1 (91). *C. pasteurianum* has been known to form fermentation products in inconsistent ratios even under similar growth conditions, which has been attributed to its metabolic flexibility (80). *C. pasteurianum* is also able to grow solely on glycerol, a characteristic shared with only one other species of clostridia, *C. butyricum*, and which results in the formation of 1,3-PDO, in addition to the other fermentative products. The central carbon metabolism of *C. pasteurianum* is summarised in Figure 1.2.

In terms of metabolic pathways, *C. pasteurianum* oxidises growth substrates to pyruvate through the Embden-Meyerhof-Parnas (EMP) or glycolysis pathway. The genome of *C. pasteurianum* contains an incomplete pentose phosphate pathway (PPP) as well as a partial Entner-Doudoroff (ED) pathway, the latter of which is common among Gram-positive bacteria. Finally, like several other clostridia, *C. pasteurianum* contains a non-cyclic Krebs or citric acid cycle. See Pyne *et al.*, for a detailed summary of the genes present and missing from each respective pathway (73).

Most relevant to this thesis, are the reductive and oxidative pathways or branches of glycerol metabolism. The reductive pathway consists of glycerol dehydratase (DhaB) and 1,3-propanediol dehydrogenase (DhaT), which catalyse the dehydration of glycerol to 3-HPA and subsequent reduction to 1,3-PDO, respectively. Both *dhaB* and *dhaT* are found in the reductive operon, which also contains glycerol reactivating factor (*ddrA*) and other genes hypothesised to be involved with cobalamin (vitamin B₁₂) metabolism. The glycerol oxidative pathway consists of glycerol dehydrogenase (DhaD) and dihydroxyacetone kinase (DhaK), encoded in a single operon and which catalyse the oxidation of glycerol to dihydroxyacetone and subsequent phosphorylation to dihydroxyacetone phosphate (DHAP), respectively. The combined actions of DhaD and DhaK allow for glycerol metabolism to link into glucose metabolism as DHAP is an intermediate of glycolysis (Figure 1.2). It has been shown that disruption of the reductive glycerol operon prevents formation of 1,3-PDO,

while also impacting the ability to grow solely on glycerol (78). It is hypothesised that disruption of the oxidative glycerol operon will prevent growth solely on glycerol as there would be no link between glycerol and central carbon metabolism (glycolysis), however this has not yet been shown empirically.

There are several key metabolic branch points in the metabolism of *C. pasteurianum*. The importance of DHAP for linking glycerol and glucose metabolism and the role of the reductive operon for formation of 1,3-PDO have been described above. The other major branch point occurs at acetyl-CoA, formed from the decarboxylation of pyruvate (Figure 1.2). Both pyruvate and acetyl-CoA are essential as they are required for anabolic processes and biomass formation. Formation of C2 products acetate and ethanol occurs by the phosphorylation and reduction of acetyl-CoA, respectively. Acetyl-CoA is also important because it is the upstream metabolite for C4 products butyrate and butanol. Two acetyl-CoA molecules are combined by Claisen condensation to acetoacetyl-CoA catalysed by the thiolase (Thl) enzyme and represents the first committed step towards formation of C4 products. Thus, Thl is an obvious target for improving or abolishing formation of C4 products. The last important player in terms of metabolism is the high energy electron carrier ferredoxin (Fd). Ferredoxin is iron sulfur protein and is an essential cofactor for pyruvate ferredoxin oxidoreductase (PFOR) and the butyryl-CoA dehydrogenase electron transferring flavoprotein complex (BcDH/Etf). In both cases, reduced ferredoxin is co-formed and is recycled by the action of hydrogenase, generating hydrogen gas (Figure 1.2 inset). The availability of a genome sequence, a detailed understanding of the central carbon metabolism, a description of key enzymes and regulators, and the necessary tools and protocols makes *C. pasteurianum* an attractive host to which apply metabolic engineering strategies for the design and construction of an industrially relevant strain.

The following three sections (1.2.8 – 1.2.10) review the relevant literature for each results chapter and which provide context for the research objectives summarised at the end of this chapter, in section 1.3.

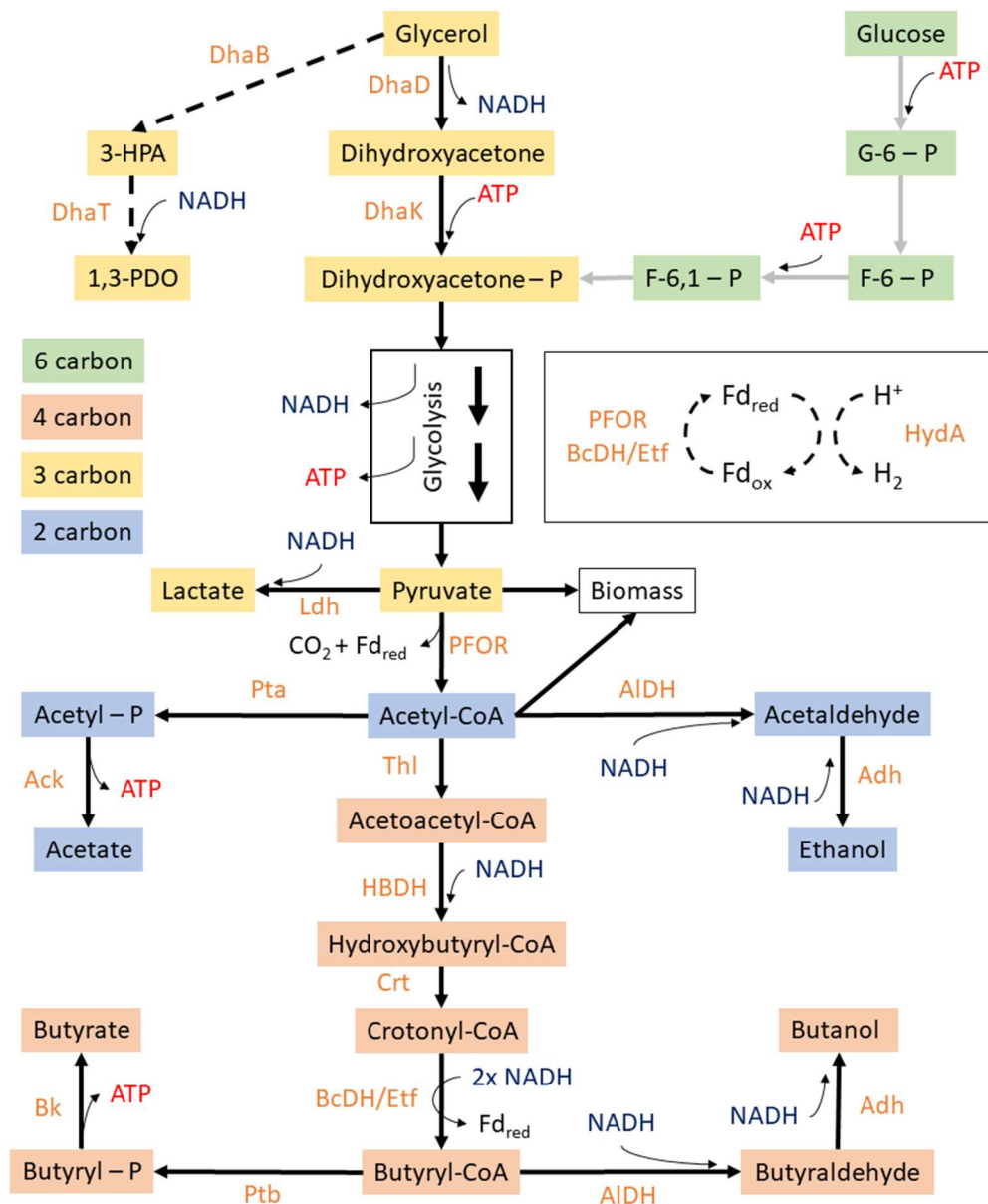


Figure 1.2 | Overview of central carbon metabolism of *C. pasteurianum*. Glycerol metabolism occurs through two pathways referred to as the reductive (dashed black) and oxidative (solid black) branches; glucose metabolism is shown in solid grey. The reductive branch consists of glycerol dehydratase (DhaB) and 1,3-propanediol dehydrogenase (DhaT) which catalyse the dehydration of glycerol to 3-hydroxypropionaldehyde (3-HPA) and subsequent reduction to 1,3-PDO, respectively. The oxidative branch consists of glycerol dehydrogenase (DhaD) and dihydroxyacetone kinase (DhaK) which catalyse oxidation of glycerol to dihydroxyacetone and subsequent phosphorylation to dihydroxyacetone phosphate (DHAP), respectively. DHAP is the metabolic branch point in which glycerol metabolism links into the EMP pathway, also known as glycolysis and ends with the formation of pyruvate. Pyruvate is reduced to lactate by lactate dehydrogenase (Ldh). Pyruvate is decarboxylated to acetyl-CoA by pyruvate ferredoxin oxidoreductase (PFOR) generating carbon dioxide and reduced ferredoxin (Fd_{red}). Acetyl-CoA is sequentially reduced to acetaldehyde and subsequently to ethanol by aldehyde dehydrogenase (AIDH) and alcohol dehydrogenase (Adh), respectively. Acetyl-CoA can also be used to generate ATP by acetate formation through substrate level phosphorylation (SLP) by combined actions

of phosphate acetyltransferase (Pta) and acetate kinase (Ack). Acetyl-CoA is an important metabolic branch point, serving as a precursor to biomass formation as well as the upstream metabolite to the C2 and C4 product forming pathways. The first committed step towards C4 product formation is the Claisen condensation of two acetyl-CoA molecules to acetoacetyl-CoA, catalysed by thiolase (Thl). Acetoacetyl-CoA is reduced to hydroxybutyryl-CoA and then to crotonyl-CoA by the sequential action of hydroxybutyryl-CoA dehydrogenase (HBDH) and 3-hydroxybutyryl-CoA dehydratase (Crt), respectively. Crotonyl-CoA is reduced to butyryl-CoA by the butyryl-CoA dehydrogenase electron transferring flavoprotein complex (BcDH/Etf) by electron bifurcation, consuming 2 moles of NADH and generating 1 mole of Fd_{red} per mole of reduced crotonyl-CoA. Butyryl-CoA can be used to generate ATP through SLP by combined actions of phosphate butyryltransferase (Ptb) and butyrate kinase (Bk), respectively. Butanol is formed from butyryl-CoA by the sequential reduction by AIDH and Adh. Inset: reduced ferredoxin (Fd_{red}) generated by PFOR and BcDH/Etf complex is recycled through formation of hydrogen, catalysed by hydrogenase (HydA).

1.2.8 Transformation efficiency and the restriction modification systems in *Clostridium pasteurianum*

The work contained in this thesis is centred on the study, modification and use of the microbial host *C. pasteurianum* for production of bulk industrial chemicals. The bacterium *C. pasteurianum* is an appealing host for industrial biotechnology applications as it naturally produces the biofuels ethanol and butanol, renewable hydrogen gas, as well as the bulk chemical 1,3-PDO, from a variety of substrates (73,80). By combining metabolic engineering and synthetic biology, the production of non-native compounds using this industrially relevant host can be explored. At the heart of this is the ability to genetically modify the host organism, which depends greatly on the genetic tools available. While the last 15-20 years has seen the development of a wide range of genetic tools for the genus *Clostridium*, the availability of tools for genome engineering of the genus have lagged behind those available to model organisms such as *E. coli* and *S. cerevisiae* and have thus hampered engineering efforts (92–94). One impediment to the utilization of more advanced genome engineering tools in the host *C. pasteurianum* is the low transformation efficiency (TE) by electroporation when compared to *E. coli*, but also to other solventogenic strains within *Clostridium*. The TE of *C. pasteurianum* was originally found to be in the order of 1.0×10^1 transformants μg^{-1} DNA and has recently been improved to 1.0×10^4 transformants μg^{-1} DNA through the isolation of hypertransformable mutants and the development of an electroporation protocol (95–98). The TE of the closely related *Clostridium acetobutylicum* was reported to be in the order of 1.0×10^6 transformants μg^{-1} DNA, two orders of magnitude higher than that achieved in *C. pasteurianum* (99).

In comparison, the TE of *E. coli* has been 1.0×10^9 transformants μg^{-1} DNA for more than two decades, three and five orders of magnitude higher than *C. acetobutylicum* and *C. pasteurianum*, respectively (100). For the use of advanced genome engineering techniques like multiplexing or the generation of large mutant libraries or introduction of very large (> 50 kbp) DNA fragments to be feasible in non-model organisms like *C. pasteurianum*, the TE must be increased to levels comparable to those of *E. coli*.

In prokaryotes, RM systems represent a natural barrier to the introduction of foreign DNA through self/non-self-determination. RM systems identify foreign DNA by the lack of specific DNA methylation patterns leading to DNA cleavage and degradation (101). With the publication of the fully sequenced genome of *C. pasteurianum*, a Type I RM system was identified and the presence of Type II RM systems was confirmed (102,103). According to the restriction enzyme database REBASE®, *C. pasteurianum* has genes encoding a total of six RM systems, of which there is a single Type I RM system and five Type II RM systems (104). The lone Type I RM system has both a methyltransferase and restriction enzyme, while of the five Type II RM systems, two consist of a restriction and associated methyltransferase enzyme, one consists of a single protein with dual restriction and methyltransferase activity and the last two have methyltransferase proteins without the associated restriction enzyme. (73,104). Thus, it is likely that the lone Type I and three of the Type II RM systems are active, resulting in a total of four potentially RM systems that are indeed active and affecting TE in *C. pasteurianum*.

Initial work at the Clostridia Research Group (CRG) with *C. pasteurianum* confirmed published data, that the Type II system encoding three putative N4-cytosine or N6-adenosine DNA methyltransferases and a single associated restriction protein, recognized the restriction site 5'-G^mATC-3'. These RM systems could be circumvented by *in vivo* methylation if shuttle vectors were assembled or propagated in a *dam*⁺ strain of *E. coli* (96,98). A second Type II RM system was revealed to recognize the CpaAIP restriction site (5'-C^mGCG-3') and could also be protected against restriction by *in vivo* methylation using an *E. coli* strain co-harboring a plasmid expressing the M.FnuDII methyltransferase (96,98). Thus, of the four putatively active RM systems, two could be circumvented and resulted in the previously commented transformation efficiency in the order of 1.0×10^4 transformants μg^{-1} DNA. Follow on work suggested that the Type I RM system present in *C. pasteurianum*, recognizing a predicted CpaAII sequence of 5'-AAGNNNNNCTCC-3' was also active, however removal of this site from

transformed plasmids failed to increase TE levels, suggesting that the true restriction site may be different to the one proposed (105). Although *in vivo* methylation can circumvent two RM systems resulting in a usable TE (1.0×10^4 transformants μg^{-1} DNA), there is room for improvement through targeted genome modification. The Type I system recognizing a predicted CpaAII cut site was shown to be active, and the presence of the third Type II RM system, encoding a putative dual active Type IIG restriction enzyme/ N6-adenine DNA methyltransferase has yet to be characterized or deleted.

1.2.9 Microbial production of the platform chemical 3-hydroxypropionic acid and 1,3-propanediol

Biological production of 3-HP by microbial means has been intensely pursued since its identification of as a top value-added chemical from biomass in 2007 by the US Department of Energy (106). The ability to easily convert it into various other chemical precursors make it an attractive commodity building block. Biological production of 3-HP offers a means to produce an alternative 'green' bulk commodity with lower energy and fewer GHG emissions (107). Another important industrial chemical is 1,3-PDO, used as a solvent and chemical building block of polymers and in formulations used in composites, adhesives and coatings with a market greater than 100 million pounds in 2008 (108). Biological production of 1,3-PDO consumes 40% less energy and reduces GHG emissions by 20% compared to petrochemical routes (109). The conversion of waste glycerol to higher value products such as 3-HP or 1,3-PDO has been highlighted as a means to increase the viability of the biodiesel industry (64).

Conversion of glycerol into 3-HP and 1,3-PDO is a two-step process that proceeds by dehydration of glycerol to the intermediate 3-hydroxypropionaldehyde (3-HPA) and is catalysed by glycerol dehydratase (DhaB). This is a key enzyme in glycerol and diol (1,2 or 1,3-PDO) metabolism and is typically found in the *dha* operon of various glycerol or propionic fermenting organisms (110). Conversion of glycerol to 3-HPA is thus a fundamental step towards the production of either 3-HP or 1,3-PDO. The DhaB found in *K. pneumonia* and *L. reuteri* are known to be B₁₂-dependent dehydratases while the DhaB from *C. butyricum* has been reported to be a B₁₂-independent variant (111–114). When expressing an B₁₂-dependent DhaB in a heterologous host that cannot biosynthesize vitamin B₁₂, it must be supplemented into the media, thus increasing the costs of the process. It is unclear why many of the studies focusing on production of 3-HP using *E. coli* have used the B₁₂-dependent

rather than the B₁₂-independent DhaB (115–118). Not only is vitamin B₁₂ required for continued activity of DhaB, but so is the glycerol reactivating factor (gdrA), a protein necessary for reactivation of DhaB following the dehydration of glycerol (119). When *gdrA* or *gdrAB* were co-expressed with *dhaB* titres of 3-HP improved from 0.45 g/L to 2.8 g/L in shake flask (116,120). Given these factors, overproduction of 3-HP using hosts that natively produce vitamin-B₁₂ and gdrA is logical. Indeed, production of 3-HP has been demonstrated in *K. pneumoniae*, *P. denitrificans* and *L. reuteri*, all of which natively produce vitamin-B₁₂ and some homolog of gdrA (121–123). Like these hosts, *C. pasteurianum* natively produces vitamin-B₁₂ and expresses *ddrA* (a homolog of *gdrA*) and thus activity of the native B₁₂-dependent *dhaB* was expected to be efficient for overproduction of 3-HP (73). Overexpression of the B₁₂-independent *dhaB* from *C. butyricum* was hypothesized to increase flux towards the reductive glycerol operon.

Following the dehydration of glycerol to 3-HPA, the second step is oxidation to 3-HP where one mole of NADH is generated per mole of 3-HP formed (Fig. 1A). This 3-HP pathway, considered the CoA-independent pathway, can be catalysed by an aldehyde dehydrogenase (ALDH), α -ketoglutaric semialdehyde dehydrogenase (KGSADH), a succinic aldehyde dehydrogenase (SSADH) or the like. The first non-patent report of 3-HP production using a microbial host was in 2008 and used a native ALDH from *E. coli* (120). Since then, various ALDHs from *Azospirillum sp.*, *Klebsiella spp.*, *Lactobacillus spp.*, *Escherichia sp.*, *Cupriavidus sp.*, *Gluconobacter sp.*, *Rhodobacter sp.*, *Pseudomonas spp.* and *Saccharomyces sp.* have been screened for their efficiency for catalysing the oxidation of 3-HPA to 3-HP (112,116,117,124). The most promising studies for 3-HP production utilised a KGSADH from *A. brasilense* expressed in *E. coli*, an *E. coli* ALDH overexpressed in *K. pneumoniae*, a mutated GabD4 (SSADH) from *C. necator* also expressed in *E. coli* and the native PuuC (ALDH) overexpressed in *K. pneumoniae* which resulted in titres of 56.4 g/L, 48.9 g/L, 71.9 g/L and 83.8 g/L of 3-HP, respectively (112,117,125,126). To guarantee expression of the key enzyme, well established commercial (*trc* / *tac* / *lac*) or synthetic promoters were used in the above studies (127). Although these promoters provide strong and inducible expression, they require the addition of lactose or IPTG for induction, increasing the cost of the bioprocess.

NB. When referring non-specifically to any aldehyde dehydrogenase, the acronym AldH is used.

A second 3-HP pathway from glycerol that has been described in the literature is a CoA-dependent pathway catalysed by three propanediol utilisation (Pdu) enzymes. These enzymes are native to both *L. reuteri* and *K. pneumoniae* and have been used for production of 3-HP using resting cells and expressed in *E. coli*, respectively (128,129). Like the CoA-independent ALDH pathway, the Pdu pathway oxidises 3-HPA to 3-HP, although in three steps. Although much less explored and more complex than the CoA-independent pathway described above, the Pdu pathway has the added benefit of generating one mole of ATP per mole of 3-HP and thus offers a growth advantage to the host. The Pdu pathway oxidises 3-HPA to 3-HP by CoA-dependent propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW) with CoA and NAD⁺ as cofactors and 3-HP-CoA and 3-HP-phosphate as intermediates (Fig. 1). Combined expression of the Pdu and ALDH pathways in *E. coli* resulted in high yield (54 moles of product per 100 moles of glycerol) of 3-HP production from glycerol (128). A second benefit of this pathway is that PduP, which catalyses the oxidation and acylation of 3-HPA uses both NAD⁺ and NADP⁺ as a cofactor (130). Although this pathway is relatively unexplored in comparison to the ALDH pathway it offers an alternative route to 3-HP production from glycerol (115).

The molecule 3-HPA, also known as reuterin, is a potent antimicrobial compound and its accumulation can cause inhibition of growth at low concentrations and cease production of downstream products such as 3-HP and 1,3-PDO (131–133). To prevent this, balanced expression of DhaB and ALDH has been optimised through engineering of the untranslated region (UTR) of the transcript and by utilising synthetic promoters and bicistronic ribosome binding sites (RBSs) (126,134). Another method for reducing accumulation of 3-HPA, is to generate mutants of the ALDH with higher activity towards substrate and co-factors. This approach was taken by Chu *et al.* who used *in silico* methods to guide mutation of GabD4 for increased activity and 3-HP production (117). A similar approach was taken by Park *et al.* who screened mutant libraries for a superior KGSADH. A variant was found to have a lower *K_m*, decreased inactivation of the enzyme, less inhibition by NADH and higher substrate specificity which resulted in increased 3-HP production (123).

Elimination of side products is a key strategy in metabolic engineering for enhancing the yield of the overall bioprocess (81). This is achieved through targeted deletion of enzymes catalysing side product formation or by changing expression of key regulators involved in pathway expression. In recombinant *E. coli* engineered for

3-HP production, genes responsible for acetate (*ackA-pta*) and ethanol (*yqhD*) formation, the glycerol-3-phosphate regulon repressor (*glpR*) were disrupted, as well as overexpression of the glycerol uptake facilitator protein (*glpF*) and conditional repression of glyceraldehyde-3-phosphate dehydrogenase (*gapA*) and triosephosphate isomerase (*tpiA*) (118,134,135). In *K. pneumoniae*, genes related to lactate (*ldhA*), acetate (*pta-ackA*), ethanol (*adhE*), succinate (*frdA*) and 1,3-PDO (*dhaT*) formation have been disrupted as well as those required for glycerol oxidation (*dhaD*, *glpK*) (112,136). Deletion of key enzymes resulted in increased product yield as expected, however results did not always translate to the same productivity when scaled from shake flask to fermenters. Fermentation of glycerol by *C. pasteurianum* typically results in many of the same products that are formed in *E. coli* and *K. pneumoniae*; 1,3-PDO, lactate, ethanol, butanol, butyrate and acetate (Fig. 1.2). Apart from 1,3-PDO, these products are considered by-products and their formation can be disrupted by targeting the key genes involved. The tools exist for disruption of endogenous genes in *C. pasteurianum*, opening the door for disruption of key enzymes in central carbon metabolism (78,137,138).

Various process parameters have been investigated for their effects on production of 3-HP. Oxygen is a potent inactivator of the glycerol dehydratase (DhaB) holoenzyme and must be considered when using a B₁₂-dependent DhaB. The amount of dissolved oxygen in the media is regulated by aeration of the fermentation, which influences growth by affecting flux through the tricarboxylic acid (TCA) cycle, carbon distribution and intracellular NADH levels (125). By extension, these factors influence the co-production of 3-HP and 1,3-PDO as product formation is dependent on co-factor availability. Huang *et al.* showed that 3-HP production improved linearly by increasing the aeration while completely aerobic conditions prevented any 3-HP formation (125). These results were echoed by Ko *et al.* who showed aeration and agitation speed inverse influenced 3-HP and 1,3-PDO production in *K. pneumoniae* (136). In *E. coli*, fermentations were typically completed under microaerobic conditions and process optimisation showed that 3-HP production was inhibited by pH ≤ 6.0 and by a low starting glycerol concentration in shake flasks (115,139).

Most studies using *E. coli* focused on sole production of 3-HP and considered 1,3-PDO an unwanted by-product (139). In contrast, co-production of 1,3-PDO and 3-HP has been pursued in *K. pneumoniae* as it is a native producer of 1,3-PDO. As mentioned above, 1,3-PDO is also an industrially relevant chemical and its

bioproduction has been investigated for many decades. Initially pursued by fermentation of native producers, such as *K. pneumoniae*, *L. reuteri*, *C. butyricum* and *C. pasteurianum*, with the rise of recombinant DNA technology, it has also been achieved in non-native producers such as *E. coli* and *S. cerevisiae* (140–146). Dupont famously developed a bioprocess for the production of 1,3-PDO from glucose in the early 2000s using recombinant *E. coli*, which is currently still in operation today (147). No similar process has yet to be developed using glycerol as a feedstock. Most of the works using *E. coli* use a co-substrate approach, whereby a sugar provides reducing power and carbon for growth and biomass formation while glycerol is solely used for conversion into 1,3-PDO. The same strategies used in the production of 3-HP have been employed to improve production of 1,3-PDO, namely, overexpression of the key enzymes glycerol dehydratase (DhaB) and 1,3-propanediol dehydrogenase (DhaT) and deletion of competing pathways. One important factor for the success of 1,3-PDO formation in the DuPont strain developed was the difference in the DhaT used. The DhaT from *K. pneumoniae* which is typically used is NADH dependent, while the YqhD from *E. coli* which was used in the DuPont patent was NADPH dependent. The DuPont strain was able to produce upwards of 130 g/L (≈ 1.7 M) and was attributed to the activity of YqhD and the intracellular ratios of reduced/oxidised co-factors (148).

Production of 1,3-PDO in a native producer has been focused on *K. pneumoniae* as it is the most well studied and the genetic tools are available for its manipulation (133). Extensive amounts of effort have been put into improving and optimising the 1,3-PDO producing ability of *K. pneumoniae* as wildtype titres can easily exceed 1 M (> 76 g/L) (133). In contrast, work focusing on *C. butyricum*, although a strong producer of 1,3-PDO from glycerol, has been limited to process manipulation and chemical mutagenic studies as the tools for its manipulation are not available (149–151). By transferring the 1,3-PDO pathway from *C. butyricum* into *C. acetobutylicum*, growth solely on glycerol was endowed as well as production of 1,3-PDO (152). Interestingly, many but not all microorganisms which can grow using glycerol as their sole carbon and energy source typically produce 1,3-PDO, regarded as a hallmark of growth on glycerol (140).

Production of both 3-HP and 1,3-PDO are greatly influenced by co-factor availability. Theoretically, they can be co-produced from glycerol with 100% yield as their co-production is redox balanced but would require a suitable host to do so. Using *C. pasteurianum* as a host for co-production of 3-HP and 1,3-PDO is attractive due to it

being a natural glycerol fermenting species, with excellent growing capacity on glycerol only and a natural producer of 1,3-PDO and vitamin-B₁₂. Most previous works using *C. pasteurianum* have focused on enhancing native butanol production, either through process optimisation, targeted modification or through disruption of the 1,3-PDO pathway (78–80,153,154). No genetic studies have as of yet looked at enhancing native 1,3-PDO production. Given that co-production of 1,3-PDO and 3-HP is redox balanced and the tools are available to genetically modify *C. pasteurianum*, there is an opportunity to develop a strain that could achieve conversion of glycerol into the useful industrial chemicals 3-HP and 1,3-PDO. Furthermore, such a project would entail expanding the product scope of *C. pasteurianum* by introduction of a heterologous pathway, something that has not been done before and would represent an important step for this industrially relevant microorganism.

1.2.10. Production of high value compounds through whole-cell biocatalysis

1.2.10.1 A short summary of enzyme biocatalysis

Enzyme biocatalysis is a well-established technology that has widespread use in the fine and bulk chemical, pharmaceutical, food, cosmetic, textile and pulp and paper industries. With growing emphasis on green or environmentally friendly processes, enzymes are increasingly being used due to mild reaction conditions and lack of toxic chemicals required for their use. Another advantage that enzymes provide over their chemical catalyst counterparts are the superior regio and stereo-selective catalytic properties. Nowhere is this most evident than in the preparation of chiral compounds. Organic chemists are frequently turning to enzyme biocatalysis to complete difficult chemical steps, which often also results in increased yields and productivities (155).

Chiral alcohols are one type of chiral compound that are important chemical building blocks in the chemical and pharmaceutical industry (156). Traditionally produced through chemical methods, the use of enzymes to generate stereocenters in molecules of interest has increased in recent times (157). Chiral alcohols are generated by reduction of a prochiral ketone and can be catalysed by several types of enzymes such as, oxidoreductases, hydrolases, lyases and alcohol dehydrogenases (ADH), the latter of which is the enzyme class tested in this thesis. The most common cofactors required by ADHs are β -1,4-nicotinamide adenine dinucleotide phosphate (NADPH) and β -1,4-nicotinamide adenine dinucleotide (NADH); both serve as a source of electrons for reduction of the prochiral ketone into its respective chiral alcohol. Chiral

alcohols are typically produced using either isolated enzymes in a 'one-pot' type of reaction or whole cells and can result in almost complete conversion (>99.5%) and an enantiometric excess (*ee*) of >95% (158).

The advantage of using isolated enzymes over whole cells is due to fewer side reactions resulting in higher enantioselectivity and increased volumetric productivity (159). One major drawback of using isolated enzymes is the requirement for the addition of expensive cofactors such as NADH or NADPH and has led to the development of *in situ* cofactor regeneration strategies to reduce costs (160). Cofactor regeneration can be accomplished by enzymatic means and can be classified as either enzyme coupled or substrate coupled, the former being much more common (161). Cofactor regeneration by enzyme coupling requires the addition of a second substrate and enzyme that function in the opposite redox direction; the substrate is oxidized by the second enzyme and NADH or NADPH is regenerated. A common NADPH enzyme coupled regeneration system consists of glucose dehydrogenase (GDH) and glucose; GDH oxidizes glucose to gluconolactone and reduces NADP⁺ to NADPH. A common NADH enzyme coupled regeneration system consists of formate dehydrogenase (FDH) and formate; FDH oxidizes formate to carbon dioxide and reduces NAD⁺ to NADH (158). Apart from high cofactor costs, isolated enzymes have also been reported to be sensitive to high concentrations of solvents and substrates (162).

Whole cell enzyme biocatalysis can also be applied to the production of chiral compounds. Typically, either resting cells or fermentative (growing) cells are utilized. Whole cell systems are generally more stable compared to isolated enzyme systems as the enzyme catalyst is held within the cell, which is a more natural environment. Furthermore, there is no need for an exogenous supply of expensive cofactor with whole cell systems. Initial efforts consisted of screening libraries of wildtype microorganisms and evaluating conversion and *ee* for selected compounds of interest. These wildtype strains often resulted in poor enantioselectivity and conversion. To improve on this, cofactor regeneration systems were employed and shown to increase conversion. As is the case with isolated enzyme systems, enzyme coupled regeneration systems are much more common than substrate coupled regeneration systems. In whole cell systems, native GDHs are stimulated by simply adding glucose, is referred to as an internal cofactor regeneration system and is the most commonly used system. This strategy, however, was not applicable to all microorganisms as it depends on the presence of GDH. The progression to recombinant whole cell biocatalysis allowed for

expression of GDH in hosts that did not natively harbour it, as well as expression of enzymes of interest in more suitable industrial strains. Co-expression of the enzyme of interest with GDH quickly followed, as the internal cofactor regeneration provided by the native GDH was usually not sufficient or fast enough for the desired application (155). The use of common industrial strains such as bakers' yeast and *E. coli* meant that competing enzymes could be removed through genetic engineering increasing the optical purity of the desired compound (163). Further cost reductions are achieved with whole cell systems as enzyme catalysts do not need to be purified prior to use. Taken together, whole cell biocatalysts offer various advantages over isolated enzyme systems (155).

NB. A reaction that uses an enzyme for biocatalysis, either in an isolated or whole cell system, is sometimes referred to as a biotransformation reaction, and is referred to as such in this work.

1.2.10.2 *C. pasteurianum* as a host for whole cell biocatalysis

As has been described, the host *C. pasteurianum* is an attractive industrial biotechnology microbial host strain. To broaden the industrial applications of this strain, whole cell biocatalysis was explored within the context of the topics described above. *C. pasteurianum* has various characteristics that make it an attractive strain to apply to whole cell biocatalysis and they are explored in this thesis.

To increase performance of whole cell biocatalysts, an internal cofactor regeneration system is normally employed. As fermentation of glycerol results in excess reducing equivalents generated compared to fermentation of glucose, it was hypothesized that these reducing equivalents could be siphoned to drive biocatalysis rather than product formation (80). Rather than employing an internal enzyme coupled cofactor regeneration system as is typically done, by fermenting glycerol, sufficient reducing equivalents (NADH) would be generated to drive biocatalysis. Furthermore, by using a strain that generates excess reducing equivalents, biocatalytic activity would be increased due to strong internal reducing power available. Indeed, similar metabolic engineering efforts have been pursued in *S. cerevisiae* and *E. coli* to increase cytosolic levels of NADH and NADPH and thus increasing the activity of the whole cell biocatalyst (164,165). Furthermore, like many Clostridia, *C. pasteurianum* naturally produces ethanol and butanol when fermenting glycerol (80). This characteristic is attractive, as some substrates have moderate to poor solubility and

require the addition of solvents for effective conversion (166). Lastly, the tools required for genome and metabolic engineering, and expression of heterologous enzyme expression via the inducible glycerol promoter are available for this strain and allow for effective building and testing of engineered strains.

It should be noted the genus of *Clostridium* has been previously explored for whole cell biocatalysis. Growing cultures of *C. acetobutylicum* were used to screen for reduction of various aldehydes and ketones to their respective alcohols with limited success (167). Cultures of *C. beijerinckii* were used to support the hypothesis that butanol dehydrogenases were responsible for the reduction of cyclohexane and benzaldehyde to cyclohexanol and benzylalcohol, respectively (168). Cultures of *C. saccharoperbutylacetonicum* were used to screen whole cell biotransformation of a group of 13 aldehydes and ketones to their respective alcohols (169). These studies have been limited to using wildtype strains without genetic modification and thus, the use of *Clostridium* for biocatalytic purposes has remained relatively unexplored. The next logical step is then to combine synthetic biology with biocatalysis to expand the potential uses of the industrially relevant genus *Clostridium*, and more specifically, *C. pasteurianum*.

1.2.10.3 Choosing an alcohol dehydrogenase and substrate for whole cell biocatalysis

As this work was intended as a proof of concept for the use of *C. pasteurianum* as a whole cell biocatalyst, a previously characterized enzyme was used. To this end, the alcohol dehydrogenase (ADH, EC 1.1.1.1) from *Lactobacillus kiefer* (LK-ADH) was provided (see pg. 118) and had been chosen due to its industrial relevance. This LK-ADH is an (R)-specific NADP-dependent ADH with broad substrate specificity and able to produce chiral alcohols with high ee (170). Although classified as an NADP – dependent ADH, LK-ADH was demonstrated to use NADH as a cofactor by colleagues in the O'Reilly group (personal communication). The industrial relevance of LK-ADH stems from the ability to generate (R) alcohols, a characteristic which is rare in nature and in demand in the pharmaceutical industry (171). Various works have focused on improving the performance of LK-ADH through mutational, immobilization and cofactor regeneration studies (172–175).

The reduction of acetophenone to (R)-1-phenylethanol is a model reaction in biocatalysis as it is the most basic aromatic ketone. This reaction has been used to study product formation kinetics, mechanisms of reduction by ADHs, used as a proxy

substrate for reduction of derivatives of aromatic ketones as well as to screen strains for reducing abilities (176–180). Due to its previously reported use, acetophenone was chosen as the primary substrate to which test with previous results serving as a benchmark for performance of the *Clostridium* whole cell biocatalyst tested in Chapter 5.

1.3 Research objectives of this thesis

The general objectives of this thesis can be briefly summarised as follows:

1. Exploit the previously developed genetic tools to further our understanding of the host *C. pasteurianum*.
2. Develop new gene expression tools and strains of *C. pasteurianum* that can be used for industrial purposes.

The above objectives will be pursued through the following:

1. Improve the transformation efficiency of *C. pasteurianum* through targeted genetic disruption of host restriction modification systems using the genetic tools developed at the SBRC/CRG.
2. Engineer *C. pasteurianum* to produce a non-native product for the first time. This will be achieved through introduction of two heterologous 3-HP pathways. The yield and titres of co-products 3-HP and 1,3-PDO will be improved by applying metabolic engineering and process optimisation.
3. Explore the use of developed strains of *C. pasteurianum* with enhanced metabolic properties for whole-cell biocatalysis. Reduction of ketones to their respective chiral alcohol will be achieved by introducing an R-specific alcohol dehydrogenase.

Chapter 2

Materials and Methods

2.1 Materials and Methods

2.1.1 Reagents, growth and maintenance conditions of bacterial strains

Assembly of all shuttle vectors was done in the cloning strain, *E. coli* DH5 α (New England Biolabs UK Ltd, NEB). *E. coli* was grown in lysogeny broth (LB) at 37 °C for plasmid propagation or on solidified agar (1.5% w/v) at 30 °C when plating transformations for cloning purposes (181). Media was supplemented with 25 μ g/ml chloramphenicol, 100 μ g/ml kanamycin or 500 μ g/ml erythromycin as required. *E. coli* TOP10 harbouring pMTL-CR1 was used for maintenance of *E. coli* – *C. pasteurianum* shuttle vectors (98). Long term storage of *E. coli* was done by adding 20% glycerol to exponential growing cultures and kept at -80 °C. Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Competent DH5 α *E. coli* cells were purchased from NEB.

The control strain of *C. pasteurianum* used throughout this thesis is H0D0R4 (H4), a hypertransformable variant of wildtype *C. pasteurianum* DSM 525 previously isolated (97). *Clostridium pasteurianum* was cultured in an anaerobic workstation (Don Whitley, Yorkshire), maintained with a gas mixture of carbon dioxide (CO₂), nitrogen (N₂) and hydrogen (H₂) at a ratio of 10:80:10, at a pressure of 1 atm and a temperature of 37 °C. Strains were stored either as spores at room temperature or were frozen by adding 20% DMSO to exponential growing cultures and keeping at -80 °C.

Depending on the application, different growth mediums were used when culturing *C. pasteurianum*. When reviving cultures from frozen stocks, tubes were transported into the cabinet in an ice block and a 1 μ l or 10 μ l loop was used to remove some frozen culture from the tube which was then streaked onto RCM agar (Oxoid Ltd). When growing strains in liquid culture in preparation for storage or for preparation of competent cells, 2X YPG media was used. 2X YPG media contains 16 g/l veggie peptone (Novagen, Merck), 10 g/l veggie yeast extract (Novagen, Merck) and 5 g/l NaCl. Both RCM agar and 2X YPG were supplemented with 7.5 μ g/ml thiamphenicol or 40 μ g/ml erythromycin as required; only 2X YPG media was supplemented with 60 μ g/ml uracil (96).

When *C. pasteurianum* was cultured for modifying its genome using uracil as a counter-selection marker, supplemented clostridia basal media (CBMS) agar was used, supplemented with 60 μ g/ml of uracil and/or 600 μ g/ml of 5-fluoroorotic acid

(FOA) as required. See Tables 2.1 and 2.2 below for components of CBMS agar and trace element solution SL7 (182).

Table 2.1 | CBMS agar components and instructions for preparation. This recipe is for preparation of 0.9 litre of CBMS agar, split into two 500 ml duran bottles (450 ml in each). Add glucose, K₂HPO₄, KH₂PO₄, biotin, p-aminobenzoic acid, thiamine HCl, uracil and FOA on the day the plates are poured after melting the agar.

To 0.7 litres of water, add the following:	
MgSO ₄ ·7H ₂ O	200 mg
MnSO ₄ ·H ₂ O	7.58 ml of a 1 mg/ml stock
FeSO ₄ ·7H ₂ O	10 ml of a 1 mg/ml stock
Casein hydrolysate (enzymatic)	4 g
After adding the above, make up volume to 0.735 litre with RO water	
Add 4.5 g bacteriological agar (technical #1 or low salt version) to each of the 500 ml duran bottles. Then add 367 ml of the above media to each bottle and autoclave. Following autoclave, bottles can be used directly for preparing plates or stored until use.	
Regardless if the agar was stored or not, before preparing plates, a melted agar solution is required. Before adding the remaining components, allow for the melted agar to cool by placing in a warm water bath (57°C). To each 500 ml duran bottle, add the following (giving a final volume of 450 ml) :	
Glucose (pre-warmed to 57°C)	45 ml of a 50% stock
K ₂ HPO ₄	4.5 ml of a 50 mg/ml stock
KH ₂ PO ₄	4.5 ml of a 50 mg/ml stock
p-aminobenzoic acid	0.45 ml of a 1 mg/ml stock
Biotin	9 µl of a 0.1 mg/ml stock
Thiamine.HCl	0.45 ml of a 1 mg/ml stock
Uracil (if not needed, add water!)	27 ml of a 1 mg/ml stock
Water (if no uracil added)	27 ml of RO water
FOA	2.7 ml of a 100 mg/ml stock
Mix each bottle well and pour plates. Plates are good for 30 days.	

Unless stated otherwise, fermentations of *C. pasteurianum* were completed in Biebl minimal media containing 6% glycerol and 1 g/l yeast extract, supplemented with 60 µg/ml uracil and antibiotics as required. See Table 2.2 and 2.3 below for components of Biebl media and the trace element solution, respectively (80). The pH of the media used for culturing of *C. pasteurianum* was adjusted to 6.2 by titration of 1 M HCl prior to autoclaving. It is normal for the media to look cloudy following autoclave, as some of the salts precipitate. Dissolve precipitated salts by shaking the closed bottles in a way that avoids water entering the bottle cap, as this is where contamination can derive from.

Table 2.2 | Components and instructions for preparing Biebl minimal media. Amounts listed are for preparing a solution with a final volume of 1 litre. Any added solution which is added AFTER autoclaving, but is not listed as autoclaved, was filtered using a syringe and a 0.45 µm filter using sterile technique. RO water was always used when preparing media and stock solutions.

Compound	Amount – add the following to 0.7 L of RO water	
KH ₂ PO ₄	0.5 g	
K ₂ HPO ₄	0.5 g	
(NH ₄) ₂ SO ₄	5.0 g	
MgSO ₄ x 7H ₂ O	0.2 g	
CaCl ₂ x 2H ₂ O	0.02 g	
FeSO ₄ x 7H ₂ O	9.14 mg	
Yeast extract	1 g / litre (add only if glycerol is the carbon source)	
Add the above and then adjust the pH to 6.2 using 1M HCl. Top up to 780 ml and autoclave. Add the following AFTER autoclaving.		
Component	Total Added	How to add
Trace elements solution SL7	2 ml	See Table 2.3 for SL7 prep.
Biotin	25 µg	250 µl of a 0.1 mg / ml solution
CaCO ₃	5 g	20 ml of a 250 g/l autoclaved solution
Resazurin	2 mg	1 ml of a 2 mg/ml autoclaved solution
Once the above is added into your 1 litre bottle of media, it can be stored on the shelf for 90 days. Add the following on the day you are going to use your solution to prevent contamination.		
L-cysteine	1 g	20 ml of 50 mg/ml (5%) stock solution. (store stock at -20°C and thaw prior to use)
6% Carbon - glucose or glycerol	60 g	120 ml of a 500 g/l autoclaved solution
Uracil	60 mg	60 ml of a 1 mg/ml solution

Table 2.3 | Components and instructions for preparation of SL7 trace element solution. Amounts shown are for preparation of a 1 litre solution. Solution container was wrapped in aluminium foil and stored at 4°C with a new stock prepared every 18 months.

Compound	Amount
MnCl ₂ x 4H ₂ O	100 mg
ZnCl ₂	70 mg

H ₃ BO ₃	60 mg
NaMoO ₂ x 2H ₂ O	40 mg
CoCl ₂ x 6H ₂ O	28.7 mg
CuCl ₂ x 2H ₂ O	20 mg
NiCl ₂ x 6H ₂ O	20 mg
HCl (25%)	1 ml

2.1.2 Standard Molecular Biology Techniques

Plasmid DNA was isolated using Monarch® Plasmid DNA Miniprep Kits (New England Biolabs – NEB). When amplifying DNA using polymerase chain reaction (PCR), genomic, plasmid, genomic or synthesized DNA was used as the template. Polymerases used for cloning and screening purposes were the Q5® High-Fidelity DNA polymerase and the DreamTaq Green PCR Master Mix (2X) purchased from NEB and Thermo Fisher Scientific, respectively. Amplified and digested DNA was visualised by gel electrophoresis with SYBR Safe DNA Gel Stain (1:10000 dilution in DMSO) mixed into the melted agarose (Thermo Fischer Scientific). For fragments of small (< 500 bp), normal (between 500 – 3000 bp) and large (> 3000 bp) size, agarose concentrations (w/v) of 1.5 %, 0.8 % and 0.4 % were used, respectively. Gels were run at 100 volts with TAE as the running buffer between 20 – 50 minutes, depending on the band size. DNA samples were pre-mixed with 6X Gel Loading Dye from NEB and a 2-log ladder was used to determine band size. To extract a DNA fragment of interest, the Monarch® DNA Gel Extraction Kit was used (NEB). For extraction of genomic DNA (gDNA) from *C. pasteurianum*, the GenElute™ Bacterial Genomic DNA Kit was used, according to the manufacturer's instructions for gram-positive bacteria (Sigma-Aldrich, Merck).

For DNA amplification, two types of PCR were performed using the Q5® High-Fidelity DNA polymerase from NEB. A standard version consisting of an initial denaturation at 98 °C for 30 seconds, followed by 25 cycles of the following three steps, denaturation at 98 °C for 10 seconds, annealing at an appropriate temperature for the primer pair for 20 seconds, extension at 72 °C for 30 seconds for every kb, ending with a final extension at 72 °C for 3-5 minutes. The annealing temperature of primers was determined using the online Tm calculator at www.tmcaculator.neb.com. Where the standard PCR protocol did not yield a strong band when visualised on an agarose gel, a touch down PCR was attempted. In this protocol, the annealing temperature (Tm) of the primer pair is decreased by 0.5 – 1.0 °C per cycle, starting at

5 °C above the predicted T_m until a temperature 5 °C less than predicted T_m is reached. This T_m is cycled until the 25 cycles are reached. If isolating a DNA fragment directly from a PCR reaction, the Monarch® PCR and DNA Cleanup kit was utilised (NEB).

Vectors and DNA fragments were pre-assembled and cloned into destination backbones using a combination of traditional restriction enzyme and isothermal cloning methods. Plasmid DNA and amplified DNA fragments were first digested and purified individually, and depending on the method of assembly, were mixed at the appropriate ratios as described by the manufacturers' instruction. Individual restriction enzymes used were the FastDigest restriction enzymes purchased from Thermo Fisher Scientific. For ligation of DNA fragments, the Rapid DNA Ligation kit from Thermo Fisher Scientific was used. For isothermal assembly, the NEBuilder HiFi DNA Assembly Master Mix was used (NEB). Sanger sequencing was carried out to verify the sequences of all assembled constructs by Eurofins Genomics (Ebersberg, Germany). Synthetic DNA fragments and oligonucleotides were purchased from GeneArt® Gene Synthesis (Thermo Fisher Scientific) and Sigma Aldrich (Merck), respectively. All kits and reagents were used according to the manufacturers' instructions.

To aid with visualising plasmids, designing primers, designing plasmids, assembly workflows and confirming sequencing results, DNA software was required. An academic software license was purchased from GSL Biotech for SnapGene, which was used throughout the thesis. SnapGene allows for sequence alignment, viewing of genomic sequences, visualising cut sites and primer binding sites among a host of other useful functions. There is a much better selection of software now, including Benchling and Geneious. Note that SnapGene automatically calculates the annealing temperature for any primer sequence that is found in a plasmid map. The annealing temperature which is used during a PCR reaction depends on the polymerase being used and SnapGene does not take this into account. It is advised that you use the NEB annealing temperature calculator available on their website, which takes into account the polymerase being used. The same can be said when using their HiFi assembly primer design function, the lengths and temperatures of the primers should be confirmed using third party software such as the NEB temperature calculator followed by manual adjustment of the length and sequence of the primers to match the required temperatures. This is especially important when working with organisms such as Clostridia, which have low GC content.

2.1.3 Construction of expression plasmids

All expression plasmids and genetic modification plasmids used were derivatives of the modular pMTL-series shuttle vectors (183). The vectors contain four modules which are described in the name of plasmid pMTL_XXXX; description of each module is available at www.clostron.com. The list of plasmids used in each chapter are listed in the tables at the end of this chapter with the relevant primers used in their construction and required for screening listed as well. The two main plasmids used in this work are pMTL_85151 (85151) and pMTL_Q5_82251 (Q5_82251) or derivatives of the same. The XXX1 module of the above plasmids contains a multiple cloning site (MCS) that is flanked by terminators allowing for the insertion of promoters, genes or expression constructs. The plasmid pMTL_Q5_82251 is a derivative of pMTL_82251 with a putative Type I restriction modification site reported by Pyne *et al.* and removed by site directed mutagenesis (SDM) (105).

To demonstrate the workflow for cloning a plasmid, the assembly of the plasmid Q5_82251_*P_{dhaBL}_dhaB₁B₂* is described. A circular and linear plasmid map of the starting plasmid, Q5_82251, with a size of 5,367 bp, is shown in Figure 2.1A and 2.1B. Of the cut sites available in the MCS of the pMTL-series of vectors, the most commonly used in this work were NotI, NdeI, FspI and NheI (Fig. 2.1C). In this example, assembly of the final plasmid was done in two steps. First, the plasmid Q5_82251_*P_{dhaBL}* was constructed by inserting the promoter fragment *P_{dhaBL}* into the destination backbone Q5_82251, at cut sites NotI/NdeI using restriction cloning, resulting in plasmid Q5_82251_*P_{dhaBL}*. The second step involved inserting the *dhaB₁B₂* gene at the NdeI/FspI cut sites using HiFi assembly into the previously assembled plasmid Q5_82251_*P_{dhaBL}*, resulting in plasmid Q5_82251_*P_{dhaBL}_dhaB₁B₂*.

Using SnapGene, primers (F2 and F4) were designed to amplify a DNA fragment of 219 bp directly upstream of the *dhaB* gene found in the genome of *C. pasteurianum*, resulting in the DNA fragment *P_{dhaBL}* (Fig. 2.2A). The primers were designed to have overhangs to allow for digestion using NotI/NdeI. This fragment was amplified from genomic DNA using Q5® High-Fidelity DNA polymerase and a fraction (1/5 or 5 µl) of the PCR reaction was run on a 0.8% agarose gel to confirm the band size (Fig. 2.2B). The remaining DNA fragment in the PCR reaction was purified using a Monarch® PCR and DNA Cleanup kit and the quality of the DNA fragment was assessed using a NanoDrop™ Lite spectrophotometer (Thermo Scientific). An absorbance ratio (280/260) above 1.8 indicated sufficient purity and quality for subsequent cloning

procedures; the same quality and purity threshold applies for plasmid DNA. The purified fragment P_{dhaBL} was digested using the restriction enzymes NotI/NdeI. Following digestion, the fragment P_{dhaBL} was again purified using a Monarch® PCR and DNA Cleanup kit and quality of the DNA fragment again assessed using a NanoDrop™. Next, a frozen *E. coli* stock harbouring plasmid Q5_82251 was inoculated into LB media supplemented with erythromycin (500 µg/ml) and grown overnight at 37 °C. The next morning, before the culture reached stationary phase, the plasmid was isolated using a Monarch® Plasmid DNA Miniprep Kit (NEB). Both plasmid concentration and quality were measured using a NanoDrop™. Between 1 – 5 ng of plasmid DNA was subsequently linearized using the restriction enzymes NotI/NdeI. The entire digestion reaction was run on a 0.5% agarose gel to ensure proper cutting and to separate the undigested plasmid from the linearized plasmid (Fig. 2.2B). The linearized plasmid was cut from the agarose gel using a knife and the DNA was extracted using a Monarch® Gel Extraction Kit (NEB). Both quality and concentration of the linearized plasmid were measured by NanoDrop™. Finally, the digested fragment P_{dhaBL} and linearized plasmid were ligated together using a Rapid Ligation Kit (Thermo Fisher Scientific). A portion (1-5 µl) of the ligation reaction was chemically transformed into competent *E. coli* DH5α (NEB). Following recovery, the entirety of the transformation mixture was plated onto LB agar plates supplemented with 500 µg/ml of erythromycin and placed in an incubator at 30 °C. Plating was done at three dilutions to avoid a 'lawn' growing on the plate. Once colonies were present, colony PCR was used to screen colonies for successful assembly of the plasmid Q5_82251_ P_{dhaBL} . The primers C3 and F4 were used to screen for successful insertion of the fragment P_{dhaBL} into the backbone Q5_82251. Only successful insertion of the fragment would yield the correct PCR product of size 489 bp and would indicate that P_{dhaBL} was correctly inserted upstream of the MCS. Colonies (usually 3) that yielded the correct band size from the colony PCR reactions were grown overnight in liquid LB supplemented with 500 µg/ml of erythromycin at 37°C. The following day, the plasmids were isolated from the cultures using a Monarch® Plasmid DNA Miniprep Kit and were sent for Sanger Sequencing (Eurofins). Sequencing results were aligned to the expected plasmid map of Q5_82251_ P_{dhaBL} using Snapgene. One of the clones containing the correct sequence for the plasmid were stored in glycerol stocks at -80 °C. This completed step one of the cloning procedure, resulting in the plasmid Q5_82251_ P_{dhaBL} with a size of 5573 bp.

The second step in our cloning workflow was the insertion of the *dhaB₁B₂* gene into the newly cloned plasmid Q5_82251_P_{dhaBL} yielding the desired plasmid Q5_82251_P_{dhaBL}_*dhaB₁B₂*. Unlike the insertion of the promoter fragment P_{dhaBL} into the backbone, which was done using restriction enzyme cloning, insertion of the *dhaB₁B₂* gene into the plasmid Q5_82251_P_{dhaBL} was done using HiFi assembly. Two primers (1B and 2B) were designed using SnapGene to amplify the *dhaB₁B₂* gene (which had previously been cloned from *C. butyricum*) with overhands that were homologous to the regions on the plasmid where the NdeI and FspI cut sites were located. The *dhaB₁B₂* gene was visualised by running a portion of the PCR reaction (5 µl) on a 0.8% agarose gel to confirm the correct band size of 3378 bp and quality of the PCR reaction. The remaining portion of the PCR reaction was purified using a Monarch® DNA and PCR Cleanup kit. The purified DNA fragment was assessed for concentration and quality by Nanodrop™. Next, using the newly made *E. coli* glycerol stock of Q5_82251_P_{dhaBL}, an overnight culture was prepared by inoculating the frozen stock into liquid LB supplemented with 500 µg/ml of erythromycin. The next morning, the plasmid DNA was isolated using a Monarch® Plasmid DNA Miniprep Kit. The plasmid was subsequently linearized using the restriction enzymes NdeI and FspI. The entire volume of the reaction was visualised on a 0.5% agarose gel and the correct band was cut out of the gel using a scalpel. The linearized plasmid was purified by using a Monarch® Gel Extraction Kit and the quality and concentration assessed using a Nanodrop™. Having the linearized plasmid and the purified *dhaB₁B₂* gene fragment, the NEBuilder® HiFi DNA Assembly was used to assemble the fragment into the plasmid. As in step one, a portion of the assembly mix was transformed into chemically competent *E. coli* DH5α with the culture plated following the recovery period onto LB agar supplemented with 500 µg/ml of erythromycin and allowed to grow at 30 °C. Once colonies had grown, successful assembly was screened using colony PCR with the primers F2 and I1, which resulted in a PCR product of 911 bp. Overnight cultures of successfully screened colonies (usually 3) were inoculated into liquid LB supplemented with 500 µg/ml of erythromycin. Plasmids were isolated the next morning using a Monarch® Plasmid DNA Miniprep Kit and sent for Sanger sequencing. Sequence results were aligned to the expected plasmid map using SnapGene to determine which colonies harboured the correct plasmid (Fig. 2.2D). Glycerol stocks of colonies harbouring Q5_82251_P_{dhaBL}_*dhaB₁B₂* were then prepared.

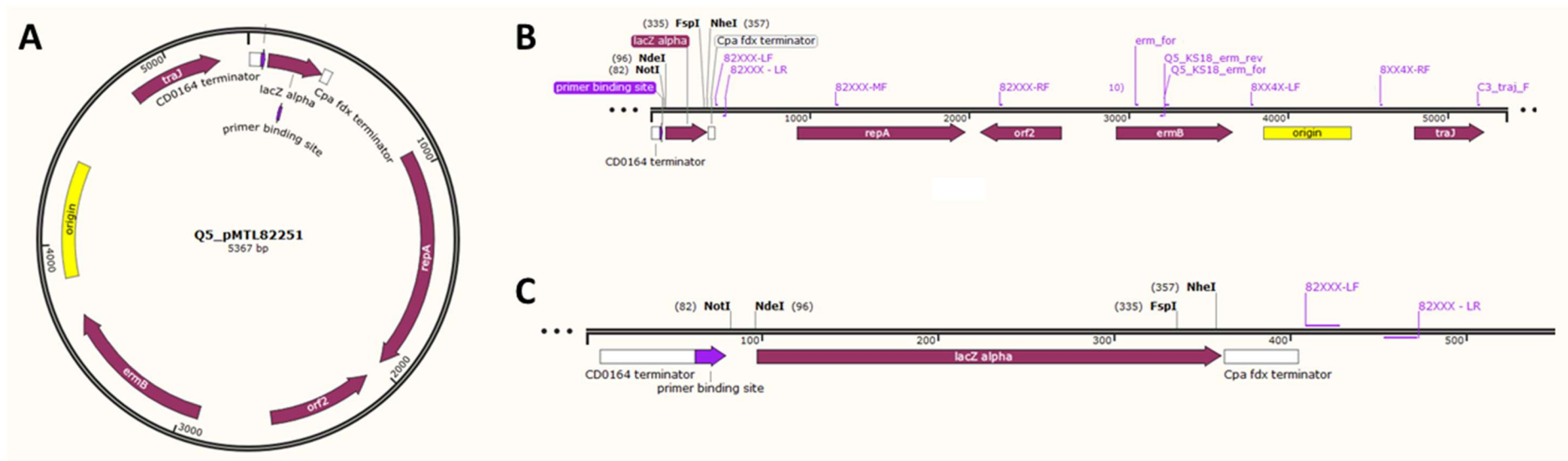


Figure 2.1 | Circular and linear maps of the expression plasmid Q5_82251. **(A)** Circular plasmid map of Q5_82251 of size 5367 bp containing the four interchangeable modules of pMTL-series plasmids, with the 2-2-5-1 modules corresponding to: *repA*, *ermB*, *ColE1* & *traJ* and MCS (*lacZ alpha*). **(B)** Linear plasmid map of A with primers used for screening of **(C)** Linear map of A/B, zoomed into the MCS, illustrating the flanking terminators and the four primary cut sites used for cloning during this work.

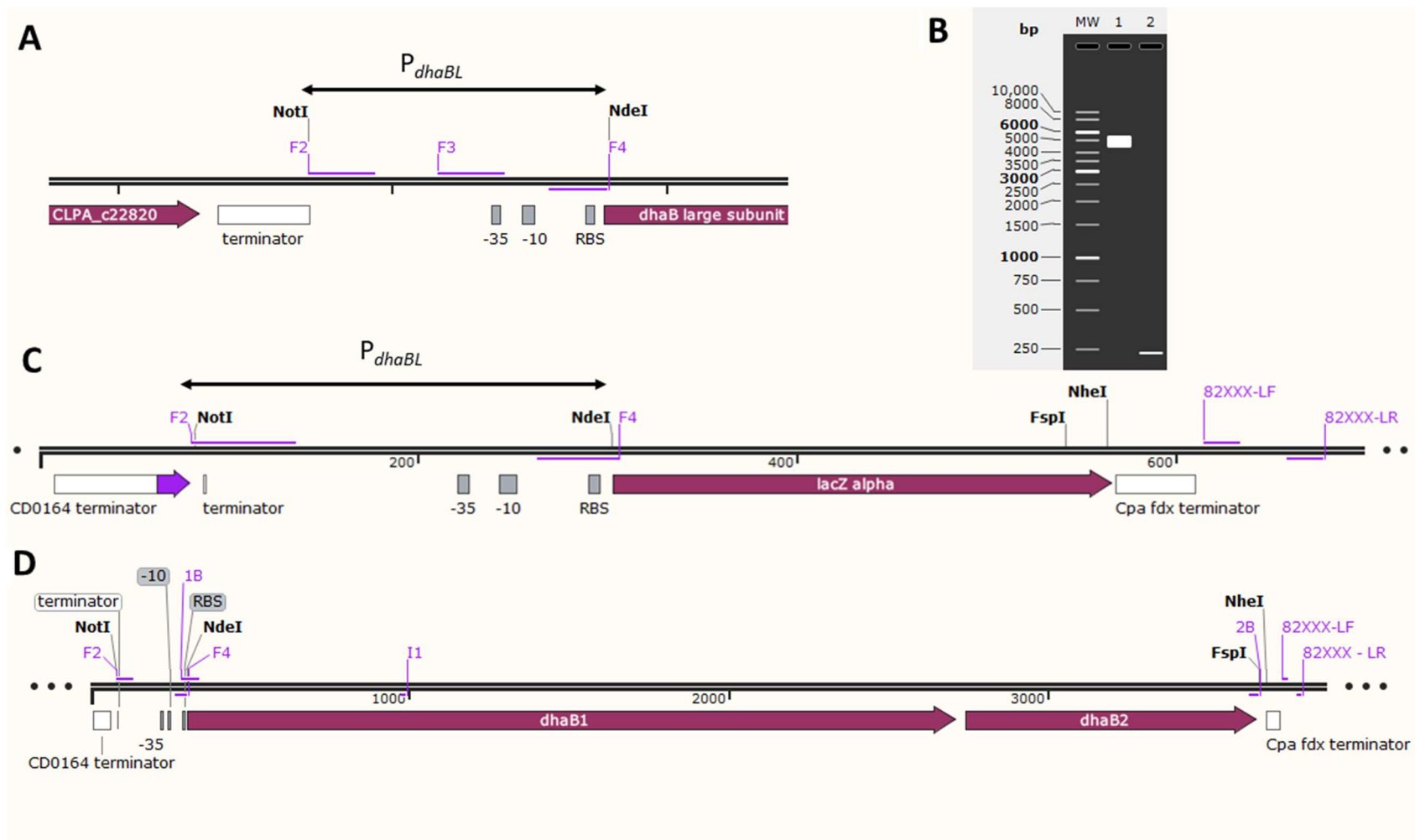


Figure 2.2 | Illustration of cloning procedure for insertion of *dhaB₁B₂* downstream of inducible glycerol promoter P_{dhaBL} in plasmid Q5_82251_ P_{dhaBL} . **(A)** Genome of *C. pasteurianum* focused on the region directly upstream of the reductive glycerol operon showing binding sites for F2 and F4 primers used for amplifying P_{dhaBL} with overhangs for NotI and NdeI cut sites. **(B)** Simulated agarose gel with Q5_82251 linearized by digestion using NotI and NdeI in lane 1, and the PCR product of P_{dhaBL} DNA fragment in lane 2. **(C)** Plasmid map following insertion of P_{dhaBL} into Q5_82251 upstream of MCS and zoomed in. **(D)** Plasmid map of Q5_82251_ P_{dhaBL} _dhaB₁B₂ showing insertion of *dhaB₁B₂* (amplified using 1B and 2B) downstream of P_{dhaBL} , screened with primers F2 and I1.

The above workflow was used for constructing most expression plasmids. Depending on the DNA fragment being inserted, sometimes HiFi assembly was used while in other instances restriction cloning was used. However, in all cases, DNA fragments were inserted into Q5_82251_P_{dhaBL} at NdeI/NheI or NdeI/FspI. The other promoter frequently used in this work was the ferredoxin promoter (P_{fdx}) from *Clostridium kluyveri*, sourced from internal libraries at the CRG/SBRC. Like the promoter P_{dhaBL}, it was cloned into the destination backbone using NotI/NdeI cut sites with subsequent genes or pathways inserted downstream at NdeI/NheI or NdeI/FspI cut sites, using a similar workflow as the one described above. Enzymes and pathways were inserted by amplifying the source DNA with primers containing overhangs with the NdeI and NheI cut sites at the 5' and 3' ends, respectively as described above. Some proteins or enzymes were sourced from the CRG culture collection and cloned into target backbones using digested fragments and not with amplified DNA. For example, the *catP* gene was cut out of other reporter plasmids using NdeI and NheI restriction enzymes and ligated into the destination reporter plasmid using the same restriction sites. The exception to the above was the cloning of the Pdu pathway into plasmid Q5_82251 which was carried out by Dr. Schuchmann. The two Pdu pathway variants, where each enzyme was expressed individually (PduI) or in an operon format (PduO), were cloned into Q5_82251 plasmid using the NotI/NheI cut sites rather than NdeI/NheI or NdeI/FspI cut sites. Promoters, RBSs and terminators for all constructs are listed in Tables 2.5 – 2.7.

2.1.4 Construction of genome modification plasmids and associated methods

Genome modification plasmids were previously published for *C. pasteurianum* (78,95). The methods are based on the work developed at the CRG by Heap *et al.* and Ng *et al.* with deletion of a target DNA sequence called allele coupled exchange (ACE) (184,185). In brief, the plasmid pMTL_KS15 was used for selection of double crossover events by using *pyrE*-based allelic exchange. The target host requires partial disruption of the *pyrE* gene for this method. Thus, the parent strain for all mutants was H4 Δ *pyrE* and was used for all deletions, as well as integrations into *C. pasteurianum* without a selectable marker (see below, plasmid pMTL_KS18 and method for integration using a selectable marker). The plasmid pMTL_KS15 contains a functional copy of *pyrE* from *C. acetobutylicum* which allows for use of FOA as a counter selection marker and was used for in-frame deletion of target genes. Regions of homology were combined by using SOE PCR and cloned into pMTL_KS15 using SbfI and NheI. Following successful

deletion of a target gene, the truncated *pyrE* was corrected using the plasmid pMTL_AGH12. This plasmid contains a functional copy of the wildtype *pyrE* gene from *C. pasteurianum* and was transformed into the isolated mutant and used to repair the truncated *pyrE* to wildtype. For chromosomal complementation of a deleted gene or operon, the plasmid pMTL_KS12 was used. The native promoter along with the deleted DNA was amplified using genomic DNA with primers containing overhangs for the NotI and NheI cut sites. The gene to be complemented was then cloned into pMTL_KS12 and then transformed into the mutant. The protocol for integration at the *pyrE* locus was the same as that for restoration of the *pyrE* allele. Colonies formed following transformation were screened for the presence of the plasmid and restreaked on RCM agar supplemented with Tm and screened for single crossover by colony PCR. Single crossover colonies were then restreaked on CBMS agar; only clones where *pyrE* was restored would grow on CBMS agar without uracil. These colonies were then confirmed using colony PCR first and then sequenced to confirm restoration of the *pyrE* allele and integration of the cargo.

For example, a typical high-level workflow for creating mutants was the following: the mutant $\Delta dhaDK \Delta pyrE$ which is a derivative of H4 $\Delta pyrE$, was created by transforming pMTL_KS12_ *dhaDK* into H4 $\Delta pyrE$. Using FOA as a counter selection marker, the mutant $\Delta dhaDK \Delta pyrE$ was isolated and confirmed by PCR. Next, the plasmid pMTL_AGH12 was transformed into $\Delta dhaDK \Delta pyrE$ to correct the truncated *pyrE* generating the strain $\Delta dhaDK$. To create a complementation strain of $\Delta dhaDK$, pMTL_KS12_ *dhaDK* was created and transformed into $\Delta dhaDK \Delta pyrE$. Following successful integration of the cargo at the *pyrE* locus a complemented strain $\Delta dhaDK pyrE::dhaDK$ was isolated.

The plasmid pMTL_KS12 was also used for integration of pathways. For example, the constructs $P_{dhaBL_dhaB_1B_2_pduO}$ and $P_{dhaBL_dhaB_1B_2_gabD4}$ were integrated into the mutant $\Delta thIA2 \Delta pyrE$, creating the strains cpDO-421 and cpDO-422, respectively. This was accomplished by digesting each construct out of plasmids pDO-21 and pDO-22 using the NotI and NheI cut sites and cloning into pMTL_KS12 digested with the same. Using the same protocol for correction of the truncated *pyrE*, these constructs were successfully integrated into the genome at the *pyrE* locus.

The plasmid pMTL_KS18 was used for stably integrating the variants of the Pdu pathway, PduI and PduO into the genome of the wildtype at the *thIA2* locus generating

strains cpDO-201 and cpDO-202, respectively. The protocol and plasmids were published for *C. acetobutylicum* and were adapted for *C. pasteurianum* by former colleagues at the CRG but is unpublished (184). In brief, the protocol relies on selecting for double crossover events by activation of the *ermB* gene. The *ermB* is cloned into the plasmid without a promoter and the plasmid is designed such that activation of the *ermB* marker occurs only after the second crossover event and thus is expressed due to read through transcription of the upstream *thlA2* gene upon integration. The Pdul and PduO constructs were cloned into pMTL_KS18 using the NotI and NheI cut sites. The plasmids were then transformed into wildtype *C. pasteurianum* and integration was selected for by streaking colonies containing the plasmid onto RCM plates supplemented with erythromycin and confirmed by colony PCR.

2.1.5 *In vivo* plasmid methylation, DNA transfer into *C. pasteurianum* and screening transformants

Prior to transformation, constructed plasmids were propagated in *E. coli* TOP10 harbouring plasmid pMTL_CR1 which expressed the M.BepI methyltransferase from *Brevibacterium epidermidus* conferring protection (*dcm+*) against restriction by the Type II CpaAIP system (95,98,105). This of course required the transformation of the constructed plasmid into cells of *E. coli* TOP10 harbouring pMTL_CR1 followed by plating onto LB agar containing kanamycin and a second antibiotic appropriate to the plasmid being transformed.

Chemically competent *E. coli* prepared by the methods of Mandel and Higa (1970) (186). Selection of colonies containing the plasmid of interest was then verified by colony PCR using primers pairs specific to the transformed plasmid. Plasmid to be transformed into *C. pasteurianum* was then isolated following overnight culture of the TOP10 *E. coli* colony screened for presence of the desired plasmid.

The methods for preparation of competent cells of *C. pasteurianum* and the electroporation protocol used were published by Pyne *et al.* (96). Presence of the transformed plasmids was detected in single colonies following plating on RCM supplemented with the appropriate antibiotic using colony PCR. The colony PCR protocol published by Pyne was found to be unreliable (96). The following protocol was found to be more robust: a tiny amount of singly colony to be screened was resuspended in 10 µl of water and 1 µl of this mixture was added to a PCR master mix containing the appropriate primers. The master mix was prepared using DreamTaq

Green PCR master mix with a final volume between 10 – 15 μ l. The PCR steps (denaturation, annealing and extension) were designed as per the manufacturers' instructions and pertinent to the primers being used, except that a ten-minute initial denaturation step was used to ensure cell lysis.

2.1.6 Calculating transformation efficiency

The transformation efficiency (TE) of various strains of *C. pasteurianum* used in this thesis was assessed. To calculate the TE of each respective strain, 5 μ g of plasmid DNA was transformed using the above protocol. Following recovery, various dilutions (a range from $\times 10^0$ – $\times 10^{-6}$) were made and 100 μ l of the diluted recovery culture was plated onto RCM agar with the appropriate antibiotic supplemented. Three separate transformations using the same plasmid and the same cells were done on the day (three technical replicates) and each dilution was plated three times; a range of dilution was plated as the expected TE was unknown. Colonies were allowed to grow for 3 – 5 days before counting. This procedure was repeated on two separate occasions. The number of colonies counted was then multiplied by the dilution factor. The final CFU / μ g of DNA transformed was calculated by taking the colonies formed and dividing by 5. The CFU / μ g of DNA for each dilution of each transformation counted was averaged before inclusion into a comparative graph.

2.1.7 Analysis of fermentation products

Detection and quantification of fermentation substrates and products 1,3-PDO, 3-HP, acetate, acetone, butanol, butyrate, ethanol, glucose, glycerol and lactate were analysed by HPLC. Samples consisted of two ml of media taken at appropriate timepoints from each respective fermentation (flask or fermenter), centrifuged (10 min, 16,000 $\times$ g, at 4 °C) with cell-free supernatants stored at -80 °C until analysed. At time of analysis, samples were thawed on ice, mixed with an equal volume (200 μ l) of internal standard solution (80mM valeric acid in 5mM H₂SO₄), filtered through a 0.22 μ m HPLC certified syringe filter (Whatman® Spartan® 13/0.2 RC; GE Healthcare Life Sciences, Little Chalfont, UK) and transferred to a HPLC vial with a 100 μ l insert. Samples were analysed with a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific) equipped with a Bio-Rad Aminex HPX-87H column (Hertfordshire, UK), a refractive index 35 °C (RI) and diode array detector (DAD) at 210 nm UV-VIS, at an isocratic flow rate of 0.5 ml/min with 5mM H₂SO₄ as the mobile phase and a column temperature of 55 °C with a run time of 55 min. Standard concentrations for all products ranged from 0.49 mM to 500 mM with an additional concentration of 750

mM for glycerol. Signal data was analysed using Chromeleon 7.2 Chromatography Data System (Thermo Fisher Scientific). Detection of all metabolites was completed using the RI channel except 3-HP, which was detected using DAD. Glycerol elutes at the same retention time as 3-HP, and at high concentrations glycerol generates a small response in the UV-VIS channel, which when measuring 3-HP must be factored in. To do this, glycerol was also measured in the UV-VIS channel generating a standard curve for the background signal. All quantities of 3-HP measured were adjusted by the corresponding quantity of glycerol in each respective sample.

2.1.8 Measuring activity of AldHs

Activities of the aldehyde dehydrogenases KGSADH and GabD4 were measured as was reported with purified enzyme previously, except using crude cell lysates and with slight modifications (187). Ten ml of exponentially growing culture harbouring plasmids with each respective enzyme were harvested after 24 h by centrifugation at 10,000 x g for 10 min at 4 °C. The control was 10 ml of exponentially growing wildtype *C. pasteurianum* harbouring no plasmid. Cell pellets were washed with 50 mM potassium phosphate buffer pH 7.0 containing 1 mM dithiothreitol (DTT) and resuspended in 1 ml of the same buffer containing 20 mg/ml lysozyme and incubated for 30 min at 37 °C. For measuring enzyme activity, a 2 ml cuvette was prepared containing a reaction mixture of 1 ml of 50 mM potassium phosphate buffer pH 7.0 1 mM DTT with 2 mM NAD⁺ and variable amounts of sample (2 – 50 µl), prewarmed to 37 °C. The reaction was initiated by addition of 2 mM propionaldehyde or 3-HPA. Enzyme activity was measured by following the reduction of NAD⁺ to NADH by monitoring the absorbance of the reaction mixtures at 340 nm. The amount of NADH formed was determined using the molar extinction coefficient ($\Delta\epsilon_{340}$) = 6.22 mM⁻¹ cm⁻¹. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 µmole of NAD⁺ to NADH in 1 min. All enzyme activities were determined in triplicate and normalized to the calculated protein concentration of the sample. The protein concentration of each sample was calculated according to Bradford with Coomassie Protein Assay (Thermo Fisher Scientific, UK) used according to the manufacturer's instructions (188). Since 3-HPA is not commercially available, it was synthesized accordingly (189).

2.1.9 Promoter identification, cloning and characterization by chloramphenicol acetyltransferase assay

The intergenic region between the start codon (3' end of the sequence) of the first gene in the reductive glycerol operon (*dhaB*) and the stop codon of the upstream gene (CLPA_c22820, 5' end of the sequence) contains 294 bp. This sequence was analysed using the online bacterial promoter analysis tool BPROM and the bacterial terminator analysis tool FindTerm, both available at www.softberry.com (190). A single terminator of length 54 was detected at position 25 of the sequence (from the 5' end of the sequence) and a single promoter was detected with putative -10 and -35 boxes at positions 235 and 213 (from the 5' end of the sequence), respectively. Two pairs of primers (F2 and F4, and F3 and F4) were used to include the regions between the end of the terminator or 40 bp upstream of the -35 box and the start codon of *dhaB*, generating two fragments of 215 bp and 122 bp and designated P_{dhaBI} and P_{dhaBS}, respectively. Forward and reverse primers were designed to include a NotI and NdeI recognition sites, respectively, and were cloned into the plasmid Q5_82251 digested with the same enzymes. The cloning procedure is described in detail in the section 2.1.3.

To characterize the strength and potential induction by glycerol of each promoter fragment, the chloramphenicol acetyltransferase (CAT) assay was used (191). A promoterless copy of the *catP* gene was cloned into Q5_82251 containing the *fdx* promoter (P_{fdx}) from *C. kluyveri*, P_{dhaBI} or P_{dhaBS} using the cut sites NdeI and XhoI. The plasmid containing the P_{fdx} promoter was used as the positive control. For the negative control, *catP* was cloned directly into Q5_82251 without a promoter using the cut sites NotI and XhoI. The *catP* gene was sourced from internal culture collection libraries. Cell lysates were obtained by harvesting 10 ml of exponentially growing cultures supplemented with either glucose or glycerol at 10,000 x g for 30 min at 4 °C, followed by disruption using BugBuster® (Novagen) according to the manufacturer's instructions. Enzymatic activity was determined using a Tecan Infinite® M1000 Pro (TECAN, CH) in a 96-well clear bottom plate (Grenier Bio One International) as described (95). In brief, a final reaction mixture of 200 µl was prepared containing 10 µl of sample with 100 mM of Tris-HCl, 0.1 mM acetyl-CoA and 0.4 mg/ml 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). Promoter strength was measured as function of enzyme activity by measuring the reaction of reduced CoA with DTNB, yielding free 5-thio-2-nitrobenzoate (5T2N). The amount of free 5T2N formed was determined using the

molar extinction coefficient ($\Delta\epsilon_{340}$) = 13.6 mM⁻¹ cm⁻¹ at 412 nm. Units of CAT activity per ml of lysate were calculated and subsequently normalized to protein concentration of each sample giving a final activity in units / mg protein. Protein concentration for each sample was determined by Bradford method with Coomassie Protein Assay (Thermo Fisher Scientific, UK) used according to the supplier's instructions (188).

2.1.10 Batch fermentations

All batch fermentations were completed anaerobically in 100 ml Erlenmeyer flasks with a working volume of 50 ml in Biebl minimal media adjusted to a starting pH of 6.2 and supplemented with the appropriate antibiotic or uracil as required (80). The carbon source supplied was maintained at 6% (w/v), either purely of glucose or glycerol or a mixture of the two, except in the carbon limited growth assay. Cultures of glycerol only were additionally supplemented with 1 g/l of veggie yeast extract (Novagen). Cultures were initiated by streaking stored strains onto RCM plates with antibiotic where appropriate. Following growth, several colonies were resuspended in 1 ml of Biebl media containing the required carbon source and antibiotic where appropriate and transferred into 10 ml of Biebl media of the same and incubated for 6h. Overnight cultures were initiated by taking 1 ml of the previously incubated culture and diluting up to 10⁻³ in 10 ml of the same Biebl media. After overnight incubation, cultures that were in mid-exponential growth phase were used to inoculate 50 ml of the same Biebl media to an approximate starting OD₆₀₀ of 0.10 using the required volume. Fermentations were run for at least 72 h with samples taken for OD₆₀₀, pH and product profile analysis as required.

2.1.11 pH controlled fermentations

Static pH fermentations refer to fermentations where the pH of the media is artificially adjusted or set to a specific value through continuous monitoring and automatic addition of concentrated base or acid. To do this, fermentations were completed using Multifors 2 parallel reactor system (Infors UK, Reigate, UK) in 500 ml fermenters with a working volume of 350 ml. Fermenters were continuously sparged (1 L/min) with sterile N₂ gas for at least 2 h prior inoculation and throughout the fermentation. Pre-cultures and minimal media were prepared in the same manner as described in section 2.1.10 except the overnight cultures were scaled from 10 ml to 40 ml. Cultures were inoculated to a starting OD₆₀₀ between 0.10 – 0.15 and maintained at 37 °C with agitation at 250 RPM. The temperature and pH were measured online with the pH being adjusted by automatic addition of 4M KOH or 2M HCl as required.

Fermentations were run for at least 72 h with samples taken for OD₆₀₀ and product profile analysis as required.

2.1.12 Whole cell biocatalysis fermentations

Strains used for whole cell biocatalysis were cultured in the same minimal media described above containing 6% glycerol supplemented with the appropriate antibiotic. Precultures and cultures were prepared in the same manner as described above for batch fermentations (2.1.10). To accommodate the large number of samples used, two changes were made to the protocol. First, the culture volume was reduced from 50 ml to 20 ml and 50 ml falcon tubes were used rather than 100 ml Erlenmeyer flasks. The second change was that tubes were continuously shaken following inoculation of the main culture. Tubes were sealed shut and placed into an incubator shaker rotating at 200 RPM with an internal temperature of 37 °C.

2.1.13 Measuring conversion efficiency of biotransformations

To measure the amount of substrate and product following whole cell biocatalysis, a total solvent extraction was completed. Following the incubation period, 10 ml of ethyl-acetate was added to the culture and vortexed for 30 seconds. This was then centrifuged for 10 minutes at 10,000 x g and the aqueous phase was aspirated out. One ml from ethyl acetate sample was then used to quantify the amount of substrate and product in the mixture.

To quantify the amount of substrate and product remaining after the incubation period, samples were analysed using a GC-FID on an Agilent 6850 equipped with a CP Chirasil-Dex CB chiral column (Agilent) using Helium as the carrier gas (flow at 1 ml/min). Standards of substrates and products were run individually and together to establish average intensities and retention times. Samples were then quantified following establishment of standard curves. Conversion were calculated by the ratio of remaining substrate to product in the sample.

2.1.14 Calculations for mass balance

Stoichiometric carbon mass balancing was used to evaluate shifts in product formation as well as to determine product yield. Carbon consumed and products generated were quantified by HPLC as described in section 2.1.7. Based on the central carbon metabolism of *C. pasteurianum* summarised in Figure 1.2, Table 2.4 lists the moles of substrate (glycerol) required for the formation of one mole of each

fermentation product and the moles of carbon per mole of product. Biomass was calculated by assuming 3.0 g/L dry-weight per 10 OD₆₀₀ values and assuming dry mass composition of C₄H₇O₂N (80,192).

Table 2.4 | Summary of products formed during the fermentation of glycerol by *C. pasteurianum*. Based on the central carbon metabolism and conservation of mass, the moles of glycerol required to form one mole of each fermentation product is shown. The moles of carbon atoms per mole of product and the associated moles of CO₂ generated per mole of each product is also shown.

Moles of Glycerol / Mole of Product	Product	Moles of Carbon / Mole of Product	Moles of CO ₂ / Mole of Product
1	acetate	2	1
1	ethanol	2	1
1	lactate	3	n/a
1	1,3-PDO	3	n/a
2	butyrate	4	2
2	Butanol	4	2

2.2.1 Tables of strains, plasmids and oligonucleotides used in this thesis

Table 2.5 | Strains, plasmids, and oligonucleotides, constructed, used, and designed in Chapter 3.

Strain	Relevant Characteristics	Relevant Primers	Source or Reference
Wildtype, (cpDO-1XX)	<i>C. pasteurianum</i> DSM 525 H0D0R4	N/A	(97)
H4P5	<i>C. pasteurianum</i> DSM 525 H0D0R4 Δ <i>pyrE</i>	Screening <i>pyrE</i> locus: 2R, 9Q	(95)
Δ <i>cpaAIP</i> Δ <i>pyrE</i>	H4P5 with in-frame deletion of CPAST_c05920	Screening for deletion: 2A, 3A	This study
Δ <i>cpaAIP</i> Δ <i>cpaAII</i> Δ <i>pyrE</i>	H4P5 Δ <i>cpaAIP</i> with in-frame deletion of CLPA_c28470, CLPA_c28480 and CLPA_c28490	Screening for deletion: 5B, 6B	This study

Plasmid	Relevant Characteristics	Relevant primers (assembly / screening) and sources
pMTL_85151	<i>E. coli</i> -Clostridium shuttle vector (pIM13, <i>catP</i> , ColE1 <i>traJ</i> , <i>lacZα</i> ORF/MCS, T _{Cpa fdx})	(183)
pMTL_82251	<i>E. coli</i> -Clostridium shuttle vector (pBP1, <i>ermB</i> , ColE1 <i>traJ</i> , <i>lacZα</i> ORF/MCS, T _{Cpa fdx})	(183)
Q5_pMTL_82251	Same as pMTL_82251, except the Type I RM site in the <i>ermB</i> gene is removed by SDM,	E9, F1, (105), this study.
pMTL_85251	<i>E. coli</i> -Clostridium shuttle vector (pIM13, <i>ermB</i> , ColE1 <i>traJ</i> , <i>lacZα</i> ORF/MCS, T _{Cpa fdx})	(183)
pMTL_82151	<i>E. coli</i> -Clostridium shuttle vector (pBP1, <i>catP</i> , ColE1 <i>traJ</i> , <i>lacZα</i> ORF/MCS, T _{Cpa fdx})	(183)
pMTL_KS15	pMTL_85151, <i>pyrE</i> (<i>C. acetobutylicum</i>), T _{Ctet gluRS} , T _{Cpa fdx} , 300-bp SHA <i>pyrE</i> , <i>lacZα</i> ORF/MCS	(78)
Q5_pMTL_KS15	Derived from pMTL_KS15, has the Type I RM site located in the <i>pyrE</i> from <i>C. acetobutylicum</i> removed by SDM	E7, E8, (105) ,this study
pMTL_KS15_ <i>cpaAIP</i>	Plasmid was cloned by previous students of a colleague.	(95)
pMTL_KS15_ <i>cpaAII</i>	Regions of homology directly upstream and downstream of Type I RM system operon <i>hsdR</i> , <i>hsdM</i> and <i>hsdS</i> were cloned into KS15 using SbfI / NheI	F5, F6, F7, F8, this study

Oligonucleotide Designation	Oligonucleotide Name	Sequence
2A	cpaAIP_KO_fwd	ctgaagcttcagactatcctca
3A	cpaAIP_KO_rev	aagcaatggaggttgtgtatagtt
5B	TIRS_KO_fwd	aagaatagtaaatgaagtaagggtgtaa
6B	TIRS_KO_rev	ctgaaaatcaccaaaaatcatattagatt
E9	Q5_82251_ter_rev	gctagcgccattcgccatt
F1	Q5_82251_ter_fwd	ggcgcgcgcttctgaatc
E7	Q5_KS15_pyrE_fwd	aagttagaagaaggagacagattataattg
E8	Q5_KS15_pyrE_rev	tgcgccaagaagtattcccttatcacc
F5	KO_TIRS_F1_fwd	ATAGCCTGCAGGaagctttccggggttagtga
F6	KO_TIRS_F1_rev	ttcaagatcatatataattacatcctaataccccctcagtttaact
F7	KO_TIRS_F2_rev	AAGCGCTAGCtgcaccattattccatcgcc
F8	KO_TIRS_F2_fwd	aactgagggggattagatgtaattatatatgatcttgaagtaattaccattaacttaatttcaaacagg

Table 2.6 | Strains, plasmids, and oligonucleotides, constructed, used and designed in Chapter 4.

Strain	Relevant Characteristics	Relevant Primers	Source or Reference
Wildtype, H0D0R 4 (H4) (cpDO-1XX)	<i>C. pasteurianum</i> DSM 525 H0D0R4	N/A	(97)
H4P5	<i>C. pasteurianum</i> DSM 525 H0D0R4 Δ <i>pyrE</i>	Screening <i>pyrE</i> locus: 2R, 9Q	(95)
Δ <i>dhaDK</i> (cpDO-2XX)	Δ <i>dhaDK</i> – in frame deletion of <i>dhaD</i> and <i>dhaK</i> with <i>pyrE</i> repaired	Confirming <i>pyrE</i> repair: 2R, 9Q	This study
Δ <i>dhaDK</i> Δ <i>pyrE</i>	Δ <i>dhaDK</i> Δ <i>pyrE</i> – in frame deletion of <i>dhaD</i> and <i>dhaK</i>	Screening deletion: K2, K4	This study
Δ <i>dhaDK</i> <i>pyrE::dhaDK</i>	complementation of <i>dhaD</i> and <i>dhaK</i> at <i>pyrE</i> locus	Confirming integration: 9Q, G7	This study
Δ <i>thlA2</i> (cpDO-3XX)	In frame deletion of active thiolase (<i>thlA2</i>) with <i>pyrE</i> repaired	Confirming <i>pyrE</i> repair: 2R, 9Q	This study
Δ <i>thlA2</i> Δ <i>pyrE</i>	In frame deletion of active thiolase (<i>thlA2</i>)	Screening deletion: 1A, D8	This study
Δ <i>thlA2</i> <i>pyrE::thlA2</i>	complementation of <i>thlA2</i> at <i>pyrE</i> locus	Confirming integration: 9Q, 2R	This study
cpDO-201	WT <i>thlA2::pdul</i>	Confirming integration: D7, E1	This study
cpDO-202	WT <i>thlA2::pduO</i>	Confirming integration: D7, E1	This study
cpDO-101	WT harbouring pDO-01	Screening for presence: C3, E1	This study
cpDO-102	WT harbouring pDO-02	Screening for presence: C3, E1	This study
cpDO-103	WT harbouring pDO-03	Screening for presence: C3, 82XXX-LR	This study
cpDO-104	WT harbouring pDO-04	Screening for presence: C3, 82XXX-LR	This study
coDO-105	WT harbouring pDO-05	Screening for presence: C3, 85XXX-LR	This study
cpDO-106	WT harbouring pDO-06	Screening for presence: C3, 85XXX-LR	This study
cpDO-111	WT harbouring pDO-11	Screening for presence: C3, I1	This study
cpDO-115	WT harbouring pDO-11 and pDO-05	Screening for presence: C3, 85XXX-LR and C3 and I1	This study

cpDO-116	WT harbouring pDO-11 and pDO-06	Screening for presence: C3, 85XXX-LR and C3 and I1	This study
cpDO-121	WT harbouring pDO-21	Screening for presence: C3, I1	This study
cpDO-122	WT harbouring pDO-22	Screening for presence: C3, I1	This study
cpDO-123	WT harbouring pDO-23	Screening for presence: C3, I1	This study
cpDO-124	WT harbouring pDO-24	Screening for presence: C3, I1	This study
cpDO-302	$\Delta dhaDK$ harbouring pDO-02	Screening for presence: C3, E1	This study
cpDO-303	$\Delta dhaDK$ harbouring pDO-03	Screening for presence: C3, 82XXX-LR	This study
cpDO-304	$\Delta dhaDK$ harbouring pDO-04	Screening for presence: C3, 82XXX-LR	This study
cpDO-315	$\Delta dhaDK$ harbouring pDO-11 and pDO-05	Screening for presence: C3, 85XXX-LR and C3 and I1	This study
cpDO-316	$\Delta dhaDK$ harbouring pDO-11 and pDO-06	Screening for presence: C3, 85XXX-LR and C3 and I1	This study
cpDO-321	$\Delta dhaDK$ harbouring pDO-21	Screening for presence: C3, I1	This study
cpDO-401	$\Delta thlA2$ harbouring pDO-01	Screening for presence: C3, E1	This study
cpDO-402	$\Delta thlA2$ harbouring pDO-02	Screening for presence: C3, E1	This study
cpDO-411	$\Delta thlA2$ harbouring pDO-11	Screening for presence: C3, I1	This study
cpDO-421	$\Delta thlA2$ <i>pyrE::P_{dhaBL} dhaB₁B₂ pduO</i>	Screening for presence: 9Q, I1	This study
cpDO-422	$\Delta thlA2$ <i>pyrE::P_{dhaBL} dhaB₁B₂ gabD4</i>	Screening for presence: 9Q, I1	This study

Plasmid	Relevant Characteristics	primers for assembly or screening; source plasmid
pMTL_CR1	<i>M.Bepl</i> , <i>Kan^R</i>	N/A, (98)
pMTL_KS12	pMTL_85151, 548-bp internal <i>pyrE</i> fragment, 937-bp fragment immediately downstream of <i>pyrE</i> , <i>T_{CtetgluRS}</i> , <i>T_{Cp fdx}</i> , <i>AsiSI</i>	N/A, (78)
pMTL_AGH12	1748-bp fragment comprising <i>pyrE</i> (35–582 nt) and 1200-bp immediately downstream of <i>pyrE</i> cloned into <i>SbfI</i> / <i>Ascl</i> recognition sites of pMTL-KS12	N/A, (78)
pMTL_KS12_ <i>dhaDK</i>	The <i>dhaD</i> and <i>dhaK</i> genes and native promoter were cloned into KS12 using <i>NotI</i> / <i>NheI</i>	6AC, 7AC, this study
pMTL_KS12_ <i>thIA2</i>	The <i>thIA2</i> gene and native promoter were cloned into KS12 using <i>NotI</i> / <i>NheI</i>	4R, 5R, this study
pMTL_KS12_ <i>P_{dhaBL} dhaB₁B₂ pduO</i>	<i>P_{dhaBL} dhaB₁B₂ pduO</i> fragment obtained from pDO-21 and cloned into KS12 using <i>NotI</i> / <i>NheI</i>	This study
pMTL_KS12_ <i>P_{dhaBL} dhaB₁B₂ gabD4</i>	<i>P_{dhaBL} dhaB₁B₂ gabD4</i> fragment obtained from pDO-22 and cloned into KS12 using <i>NotI</i> / <i>NheI</i>	This study
pMTL_KS15	pMTL_85151, <i>pyrE</i> (<i>C. acetobutylicum</i>), <i>T_{Ctet gluRS}</i> , <i>T_{Cpa fdx}</i> , 300-bp SHA <i>pyrE</i> , <i>lacZα</i> ORF/MCS	N/A, (78)
pMTL_KS15_ <i>dhaDK</i>	Regions of homology directly upstream and downstream of <i>dhaD</i> and <i>dhaK</i> genes were cloned into KS15 using <i>SbfI</i> / <i>NheI</i>	G6, H1, H2, H3, this study
pMTL_KS15_ <i>thIA2</i>	Regions of homology directly upstream and downstream <i>thIA2</i> were cloned into KS15 using <i>SbfI</i> / <i>NheI</i>	B4, B5, B6, B7, this study
pMTL_KS18_ <i>pduI</i>	KS18 adapted for <i>C. pasteurianum</i> is unpublished and was provided by colleagues at the CRG. <i>pduI</i> was cloned into KS18 by Dr. Schuchmann. <i>PduI</i> construct: <i>P_{CP fdx} pduP T_{CP pfo1} P_{CP perf fdx} pduL T_{CSporo fdx} P_{Caceto thl} pduW T_{CP lsc}</i>	Schwarz, K. and Schuchmann K.
pMTL_KS18_ <i>pduO</i>	KS18 is unpublished and was provided by colleagues at the CRG. Assembly was completed by Dr. Schuchmann. <i>PduO</i> construct: <i>P_{CP fdx} pduP pduL pduW T_{CP lsc}</i>	Schwarz, K. and Schuchmann K.
pMTL_82251	<i>E. coli</i> -Clostridium shuttle vector (pBP1, <i>ermB</i> , <i>ColE1 traj</i> , <i>lacZα</i> ORF/MCS, <i>T_{Cpa fdx}</i>)	(183)
Q5_pMTL_82251	Same as pMTL_82251, except with removal of Type I RM site by SDM.	(105), E9, F1, this study.
pMTL_85151	<i>E. coli</i> -Clostridium shuttle vector (pIM13, <i>catP</i> , <i>ColE1 traj</i> , <i>lacZα</i> ORF/MCS, <i>T_{Cpa fdx}</i>)	(183)

pDO-01	Q5_82251 containing <i>pduI</i> fragment, cloned from KS18_ <i>pduI</i> using NotI / NheI	This study
pDO-02	Q5_82251 containing <i>pduO</i> fragment, cloned from KS18_ <i>pduO</i> using NotI / NheI	This study
pDO-03	Q5_82251, P _{Ckl_fdx} <i>kgs</i>	C3, 82XXX-LR, this study
pDO-04	Q5_82251, P _{Ckl_fdx} <i>gabD4</i>	C3, 82XXX-LR, this study
pDO-05	85151, P _{Ckl_fdx} <i>kgs</i>	C3, 85XXX-LR, this study
pDO-06	85151, P _{Ckl_fdx} <i>gabD4</i>	C3, 85XXX-LR, this study
pDO-07	Q5_82251, <i>catP</i> cloned promoter-less using NotI / NheI, sourced from internal libraries	C3, 82XXX-LR, this study
pDO-08	Q5_82251, P _{fdx} <i>catP</i> , cloned using NotI / NheI, sourced from internal libraries	C3, 82XXX-LR, this study
pDO-09	Q5_82251, P _{dhabL} <i>catP</i> , <i>catP</i> cloned in using NdeI / NheI cut sites	F2, F4, C3, 82XXX-LR, this study
pDO-10	Q5_82251, P _{dhabS} <i>catP</i> , <i>catP</i> cloned in using NdeI / NheI cut sites	F3, F4, C3, 82XXX-LR, this study
pDO-11	Q5_82251 P _{dhabL} <i>dhaB₁B₂</i> cloned into NotI / FspI	F2, F4, 1B, 2B, this study
pDO-21	Q5_82251 P _{dhabL} <i>dhaB₁B₂</i> <i>pduO</i> ; <i>pduO</i> amplified from pDO-02 cloned into pDO-11 at FspI / NheI	4M, 5M, this study
pDO-22	Q5_82251 P _{dhabL} <i>dhaB₁B₂</i> <i>gabD4</i> ; <i>gabD4</i> amplified from pDO-04 cloned into pDO-11 at FspI / NheI	4Q, 5Q, this study
pDO-23	Q5_82251 P _{dhabL} <i>dhaB₁B₂</i> <i>gabD4i</i> ; (E269Q) mutation generated by cloning <i>gabD4</i> from pDO-04 using SDM, cloned into pDO-11 at FspI / NheI	8L, 9L, 4Q, 5Q, this study
pDO-24	Q5_82251 P _{dhabL} <i>dhaB₁B₂</i> <i>gabD4ii</i> ; (E219Q) mutation generated from <i>gabD4i</i> fragment using SDM, cloned into pDO-11 at FspI / NheI	1Q, 2Q, 4Q, 5Q, this study

Oligonucleotide Designation	Oligonucleotide Name	Sequence
N/A	82XXX-LR	tatccagggtgctatcttcg
N/A	85XXX-LR	ttccgacgcttattcgcttc
C3	<i>traj_fwd</i>	gcttggcaaggtcatgatg
E1	<i>pduL_rev</i>	tggtcccataagccttacatgt
I1	<i>dhaB₁_rev</i>	cttcgcatgagataataac
6AC	<i>dhaDK_fwd</i>	cggctcggccgctcattagaaatttaaagctacttgaaagtg
7AC	<i>dhaDK_rev</i>	ggccggctagcctactttataacctctgaaatctctgtgaata
2R	<i>pyrE_downstream_rev</i>	accaccactgatgagctgtt
9Q	<i>pyrE_upstream_rev</i>	tagaatttatcatggcaggagcaac
K2	<i>dhaDK_DCO_L</i>	gcttggattcagtgagtcagg
K4	<i>dhaDK_DCO_R2</i>	ggggcccagatctcttactg
G7	<i>dhaD_F1_fwd</i>	tttctggtttatttattatcttttcatattactttccccttctcttataaaaattttttatttttaacttat
1A	KO_ <i>thlA2_fwd</i>	agataatttgtaagagaaggtgtaaatgaaaa
D7	<i>thlA2_SCO_rev</i>	ggttctacgcctaccaatcc
4R	<i>thlA2_NotI_F</i>	gcgcgcgctagcttatcttttaactataagagcagttaccttgctccacctatac
5R	<i>thlA2_NheI_R</i>	gcgcgcgctagccttcataatccataagatttactaaatccattgcat
G6	<i>dhaD_F1_for</i>	ATAGCCTGCAGGttggagcagttatggcaggt
H1	<i>dhaDK_F1_rev</i>	aatttaattataatatttctttaaacattctaactacatattactttccccttctcttataaaaattttttatttttaact
H2	<i>dhaDK_F2_for</i>	aaaaaaaaatttataagagaaggggaaagtaatatgtagttagaatgtttaagaaatattataattaaattaataataacaat
H3	<i>dhaDK_F2_rev</i>	AAGCGCTAGCtaccaggcacaaggtt
B4	AE_ <i>thlA2_F1_for</i>	ATAGCCTGCAGGagaaaattgggagagggca
B5	AE_ <i>thlA2_F1_rev</i>	cacctttttgcaatagaattacataaaaagtaacctcctaaagtttatattcttataaaacacaaatcg
B6	AE_ <i>thlA2_F2_for</i>	tttaggaggttactttttatgtaattctattgcaaaaaaggtgaagatttagtcttcacct
B7	AE_ <i>thlA2_F2_rev</i>	AAGCGCTAGCtctccattttaatcgccgtagt
E9	Q5_82251_ <i>ter_rev</i>	gctagcgccattcgccatt
F1	Q5_82251_ <i>ter_fwd</i>	ggcgcgcggttctgaatc

F2	Pglycerol_long_fwd	ATGCGGCCGCaaaccttttcaatagctatattttatgtaaaattatgttgtaaatagc
F4	Pglycerol_short_fwd	ATGCGGCCGCaaataataagcaaaaaagtataaaaaataagacaaaattatttgataatat
F3	Pglycerol_rev	ACAcatatgtaatcctcctttatcaaatctctgttgttcttatctgt
1B	<i>dhaB₁B₂</i> _fwd_Pgl	gataaaggaggattacatatgataagattagataaaccaagtaagaatgtgattggag
2B	<i>dhaB₁B₂</i> _rev_Pgl	gcgccattcgccattcaggctgcgcattactcagctccaattgtgcaaggatttcc
4M	<i>pduO_dhaB₁B₂</i> _fwd	caattggagctgagtaatgcttgtaatcatcatattaaataaatataattttaatttctaggaggaattataaaATGCAAATAAATGAT ATAGAATCTGCTG
5M	<i>pduO_dhaB₁B₂</i> _rev	ccattcgccattcaggctgcGCATTATTGTTGAGTTACATTACATTACATCTCTAGCAATTATGATT
4Q	<i>gabD4_dhaB₁B₂</i> _fwd	attggagctgagtaatgcaatttaggaggttagttcaTATGTATCAAGATTTAG
5Q	<i>gabD4_dhaB₁B₂</i> _rev	cgccattcaggctgcTTAAGCTTGAGTTATGAATTTAGTTACTAAATATCCATCAA
8L	<i>gabD4i_E269Q</i> _fwd	tcctgctgctcaaatgcttgc
9L	<i>gabD4i_E269Q</i> _rev	tctatatcagcatcatcaaataactaatac
1Q	<i>gabD4ii_E219Q</i> _fwd	tggggagtaccatccaagtatctac
2Q	<i>gabD4ii_E219Q</i> _rev	gtagatacttgggatggtactcccca

Table 2.7 | Strains, plasmids, and oligonucleotides, constructed, used, and designed in Chapter 5.

Strain	Relevant Characteristics	Relevant Primers	Source or Reference
Wildtype	<i>C. pasteurianum</i> DSM 525 H0D0R4 (H4)	N/A	(97)
$\Delta thIA2$	In frame deletion of active thiolase (<i>thIA2</i>) with <i>pyrE</i> repaired	N/A	This study
H4 + pMTL_85151_LK-ADH	Harbours LK-ADH expression plasmid	C3, 85XXX-LR	This study
H4 + Q5_pMTL_82251_LK-ADH		C3, 82XXX-LR	This study
$\Delta thIA2$ + Q5_pMTL_82251_LK-ADH		3, 82XXX-LR	This study
Plasmid	Relevant Characteristics	primers for assembly or screening; source plasmid	
pMTL_85151	<i>E. coli</i> -Clostridium shuttle vector (pIM13, <i>catP</i> , ColE1 <i>traJ</i> , <i>lacZ</i> α ORF/MCS, T _{Cpa fdx})	(183)	
Q5_pMTL_82251	Same as pMTL_82251, except with removal of Type I RM site by SDM.	(183)	
pMTL_85151_LK-ADH	85151 P _{dhabL} LK-ADH, LK-ADH cloned in using NdeI / NcoI cut sites	5S, 6S, C3, 85XXX-LR, this study	
Q5_pMTL_82251_LK-ADH	Q5_82251 P _{dhabL} LK-ADH, LK-ADH cloned in using NdeI / NcoI cut sites	5S, 6S, C3, 82XXX-LR, this study	
Oligonucleotide Designation	Oligonucleotide Name	Sequence	
C3	<i>traj_fwd</i>	gcttggaaggtcatgatg	
82XXX-LR	82XXX-LR	tatccagggtgctatcttcg	
85XXX-LR	85XXX-LR	ttccgacgcttattcgcttc	
5S	LK-ADH_Nde_fwd	gcgcatATGACCGATCGTCTGAAAGGTAAA	
6S	LK-ADH_NcoI_rev	gccggccatggTTACTGTGCGGTATAACCA	

Chapter 3

Improving Transformation Efficiency in *Clostridium* *pasteurianum*

3.1 Chapter Introduction

Construction of a microbial strain for industrial production of fuels, chemicals, or other compounds, typically requires significant genetic modifications. Modifying the host genome depends on the availability of a DNA transfer protocol as well as the necessary tools that allow for deletion or insertion of DNA sequences. Currently, there is a robust DNA transfer protocol for *C. pasteurianum* published, as well as the necessary tools that allow for modification of the genome. Unfortunately, the transformation efficiency (TE) achieved with the reported protocol, is 1.0×10^4 transformants μg^{-1} DNA, 100-fold lower than that achieved with *C. acetobutylicum* and 10^5 -fold lower than that achieved with *E. coli*. The protocol also requires methylation of the DNA (usually a plasmid) to be transformed prior to electroporation, adding an unnecessary step, and slowing the workflow by several days. The current method also results in transformation of an unknown mix of two plasmids, as the desired plasmid is isolated from an *E. coli* donor strain coharbouring the methylation plasmid. Furthermore, the TE currently achievable with the protocol does not allow for use of suicide vectors or for genetic studies using transposons, as the TE is too low. In light of the above, there is a need to increase the TE achievable with *C. pasteurianum* to facilitate and quicken genome modifications protocols as well as open the door for those tools which require high TE to be effective.

The objectives of this chapter are to increase the TE of *C. pasteurianum* through successive deletion of restriction modification (RM) systems. Using established knock out procedures, two RM systems, a Type I and Type II were targeted for in-frame marker-less deletion in *C. pasteurianum* H0D0R4 (H4) ΔpyrE . The effects on the TE of deleting two RM systems are reported, as are the effects on the fermentation profile.

3.2 Results

3.2.1 Transformation Efficiency (TE) in *C. pasteurianum*

As described in the introduction, and summarised in the background section, DNA transfer by electroporation has been established in *C. pasteurianum*. However, the achieved TE is lower than that of other solventogenic *Clostridium* such as *C. acetobutylicum* and model organisms such as *E. coli*. Through the development of an electroporation protocol, a TE on the order of 1.0×10^4 transformants μg^{-1} DNA has been reported (96). This TE is similar to that achieved by the isolation of hypertransformable mutants, which is one order of magnitude higher at $2.0\text{-}3.0 \times 10^5$ transformants μg^{-1} DNA (97). Both values are an improvement on the initial TE of 2.4×10^1 transformants μg^{-1} DNA reported for the wildtype strain. See Figure 3.1A for a visual comparison of reported TE between *E. coli*, *C. acetobutylicum*, and the various TE achieved with *C. pasteurianum*.

It is relevant to note, that the TE values reported in *C. pasteurianum* were achieved using one of two almost identical plasmids (pMTL_85151 and pMTL_85141). Interestingly, the use of other plasmids resulted in large drops of TE by several orders of magnitude, suggesting that other RM systems might be active (105). Reporting TE using a single plasmid is misleading from a practical perspective since molecular and genetic work will usually require the use of various plasmids depending on the objective that researchers are trying to achieve. The work encompassed in this thesis uses a variety of genetic tools adapted from other *Clostridium* for expression of heterologous pathways, characterization of promoters and genome modifications. The genetic tools are summarized in Table 3.1 with their associated vectors. Since the TE in *C. pasteurianum* was only reported using the previously mentioned plasmids, we sought to verify if the TE changed when using other plasmids. Plasmids expected to be utilized frequently in this work were chosen to assay the TE of *C. pasteurianum*; they are expression plasmids pMTL_85151 and pMTL_82251 and plasmid pMTL_KS15, which is required for creating marker-less in-frame deletion mutants via allelic exchange. The plasmid pMTL_KS12 is used for genome integration of cargo without a counter-selection marker by restoring the *pyrE* allele and was frequently used. However, since plasmids pMTL_KS12 and pMTL_KS15 are similar due to both being derivatives of pMTL_85151, only pMTL_KS12 was tested. The TE of the selected plasmids are shown in Figure 3.1B.

Plasmid pRECas1, used in CRISPR/Cas9 genome modification was not tested because it was not available until several years after the start of this work (138). The endogenous CRISPR/Cas3 system while published, was not used as it had not been tested in our group (193). Gene disruption using ClosTron was not utilized because allelic exchange was the preferred method for genome deletions (185,194). The plasmid pMTL_KS18 was used for chromosomal integration of cargo using the activation of a selectable marker, but was not tested because integration restoration of the *pyrE* allele was the preferred method of choice as it was simpler and did not require further steps to remove the antibiotic gene following integration.

Table 3.1 | List of genome modification and gene expression tools developed by the CRG and other for the genus *Clostridium* and used in this work.

Tool Name	Description	Host Required	Plasmid required	Used? (Y/N) / Source
Allelic Exchange	Marker-less in-frame deletion using uracil as counter-selection marker	<i>pyrE</i> truncation in wildtype	pMTL_KS15, based on pMTL_85151 with <i>pyrE</i> from <i>C. acetobutylicum</i>	Y - (185)
Integration without counter-selection marker	Chromosomal integration by restoring uracil autotrophy	<i>pyrE</i> truncation in wildtype	pMTL_KS12, based on pMTL_85151 with homology arms restoring <i>pyrE</i> allele	Y - (184)
ClosTron	Retro-homing mediated gene disruption by group II intron	Any	N/A	N - (194)
CRISPR/Cas3 – Type I-B	Endogenous CRISPR mediated genome modifications	wildtype	pCParray	N - (193)
CRISPR/Cas9 – Type II	Heterologous CRISPR mediated genome modifications	Any	pRECas1	Y – (138)
pMTL vector series	Overexpression of homologous and heterologous gene(s)	Any	Q5_pMTL_82251, pMTL_85151	Y - (183,195)
	Reporter assays	Any	pMTL_82251 or Q5_pMTL_82251	Y - (183,195)
Integration without counter-selection marker	Chromosomal integration by activation of selectable marker – erythromycin resistance (<i>ermB</i>)	Any	pMTL_KS18	Y - (184)

The control strain used throughout this work is H0D04 (H4), a hypertransformable derivative of the type strain *C. pasteurianum* DSM 525. It was isolated by the methods described and carries a single nucleotide polymorphism (SNP) that was shown to increase TE (97). The wildtype sequence of *pyrE* in H4 was truncated by a previous researcher, generating the strain H4 $\Delta pyrE$ and was the base strain utilized for making in-frame marker-less deletions using allelic exchange (95). The two expression and one knock-out (KO) plasmids pMTL_85151, pMTL_82251 and pMTL_KS15 respectively, were tested for TE in *C. pasteurianum* (Fig. 3.1B). The TE of the control plasmid (pMTL_85151) achieved in this work was 1.0×10^4 transformants μg^{-1} DNA (Fig. 3.1B), one order of magnitude lower than that reported by colleagues (Fig. 3.1A)(95). The TE of pMTL_82251 and pMTL_KS15 however were much lower, one and four orders of magnitude lower than the control pMTL_85151, respectively, suggesting that either plasmid size or the other RM systems were active and affecting the TE. It was previously shown that the Type I RM system was active and that when the predicted restriction sequence (5'-AAGNNNNNCTCC-3') was removed, TE could be recovered (105). When this predicted the Type I recognition sequence was searched against each plasmid, it was found in both pMTL_KS15 and pMTL_82251 plasmids, localised to the *pyrE* gene from *C. acetobutylicum* and the *ermB* gene encoding erythromycin resistance, respectively. This was hypothesized to be the cause for the low TE observed with these plasmids relative to pMTL_85151. Using site directed mutagenesis (SDM), silent mutations were introduced to both *pyrE* and *ermB* genes to remove the predicted sequence and were subsequently tested; the prefix 'Q5_' was used to differentiate the mutated plasmids from the original plasmids (Fig. 3.1C). Removal of the recognition site caused no change to the TE of Q5_pMTL_KS15 while an increase of two orders of magnitude was observed with Q5_pMTL_82251, although still two orders of magnitude lower than the control (Fig. 3.1B).

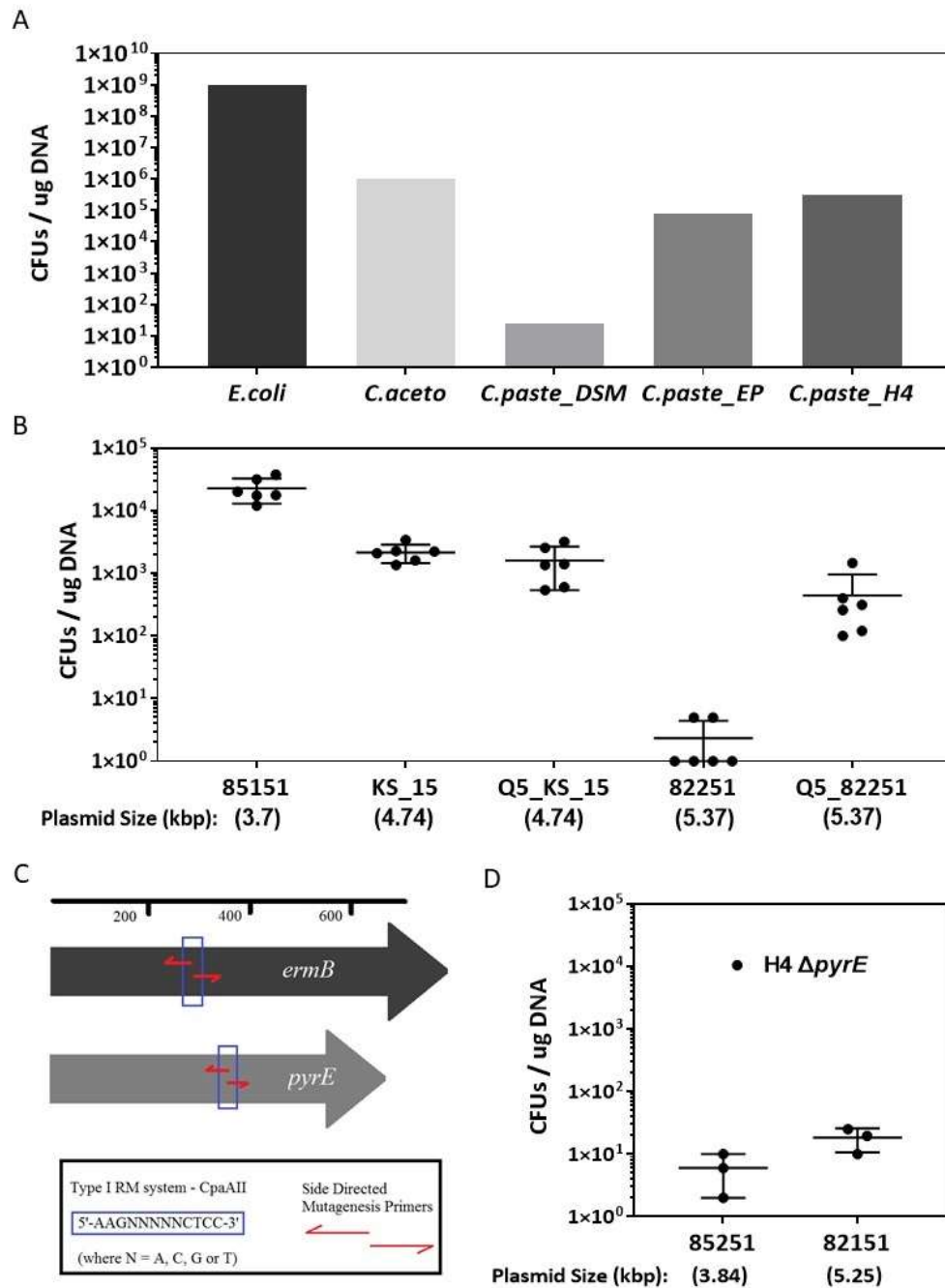


Figure 3.1 | Probing the TE of commonly used plasmids in *C. pasteurianum*. **(A)** Comparison of the reported TE using electroporation for *E. coli*, *C. acetobutylicum* (*C. aceto*), wildtype *C. pasteurianum* (*C.paste_DSM*), *C. pasteurianum* using the high level electroporation protocol (*C.paste_EP*) and the hypertransformable strain H4 (*C.paste_H4*). **(B)** Comparison of the TE of plasmids utilized in this work. The prefix 'Q5_' denotes plasmids that have had the Type I RM recognition sequence removed by SDM. **(C)** The predicted Type I restriction site was found in the *pyrE* allele from *C. acetobutylicum* and the *ermB* gene in plasmids KS_15 and pMTL_82251, respectively. The approximate location of the restriction site and the orientation of the primers used to introduce silent mutations into each respective gene are shown. **(D)** TE of the plasmids pMTL_85251 and pMTL_82151. These plasmids have modules 1 and 2, encoding the gram + replicon (1) and selection marker (2) swapped relative to the plasmids pMTL_85151 and pMTL_82251.

Although plasmid size influences TE, it is unlikely to be cause of the differing TE of each plasmid as they were all of similar size (Fig. 3.1B) (100). The difference between plasmids pMTL_85151 and pMTL_82251 are modules one (pMTL_81XXX) and two (pMTL_8X2XX), encoding the Gram+ replicon (module 1) and selection marker (module 2), respectively. Since removal of the Type I recognition site did not affect the TE of pMTL_KS15, the plasmids pMTL_82151 and pMTL_85251 were transformed in attempt to isolate which module was causing the drop in TE. Both plasmids had TE four orders of magnitude lower than the control (Fig. 3.1D). Since plasmid pMTL_85251 carries the *ermB* gene with the predicted Type I restriction site, it was expected to have a low TE. Plasmid pMTL_82151 however, did not have the Type I restriction site present anywhere on the plasmid yet still had a low TE. This suggests that other RM systems are also active, that the reported predicted restriction site for the Type I RM system is not entirely accurate or some other unknown factor is causing the observed decrease in TE. To increase TE in *C. pasteurianum*, deletion of the RM systems by in-frame marker-less deletion was explored.

3.2.2 Deletion of the Type II CpaAIP RM system in *C. pasteurianum*

Of the six genome encoded RM systems in *C. pasteurianum*, only four have associated restriction enzymes, suggesting that only four are active; the six annotated RM systems are detailed in Table 3.2. In the introduction (1.2.8), the recognition site and strategy for circumvention of the CpaAII, CpaAIP and CpaAIII RM systems were summarised. In the above section, it was shown that the use of plasmids other than pMTL_85151 resulted in decreases in TE. In this section, genome deletion of the Type II CpaAIP RM system was pursued and the TE phenotype assessed.

Genome deletion of the Type II CpaAIP RM system was expected to increase TE and streamline cloning and genetic modification procedures. Currently, to circumvent the Type II – CpaAIP RM system in *C. pasteurianum*, *in vivo* methylation is required prior to electrotransformation. This is accomplished by propagating the desired plasmid in a strain of *E. coli* co-harboring a plasmid encoding the M.BepI methyltransferase. While not difficult, these extra steps result in wasted lab time. Plasmids must first be transformed into *E. coli* and clones then screened to verify that the plasmid is present followed by a second plasmid (mini) prep or isolation step. This second miniprep is not ideal as it results in a mixture of the two plasmids, and thus transformation into *C. pasteurianum* consists of two plasmids, of unknown ratio of

methylation plasmid and the plasmid of interest. To create a marker-less in-frame deletion strain of the Type II CpaAIP RM system, the background strain $\Delta pyrE$ was utilized to allow for use of a heterologous *pyrE* as a counter-selection marker in the presence of FOA (78,185). A KO cassette consisting of approximately two 800 bp regions of homology to regions flanking the restriction protein (CPAST_c05920) were joined using sowing by overlap extension (SOE) PCR. These homology arms were cloned into the KO plasmid pMTL_KS15 generating the plasmid pDO-31. This plasmid was then transformed into *C. pasteurianum* $\Delta pyrE$ and the methods for isolating deletion mutants using allelic exchange (Materials and Methods 2.1.4) were applied generating the mutant $\Delta pyrE \Delta cpaAIP$. Four mutants were isolated and confirmed by PCR; bands corresponding to a deletion of the appropriate size were observed (Fig. 3.2A). To confirm the deletion of the Type II CpaAIP restriction protein, genomic DNA was isolated from each clone and a PCR was completed targeting the locus of interest, and the PCR product sequenced. Of the four mutants isolated, only one was not confirmed by sequencing and was thus discarded.

Table 3.2 | List of annotated and verified RM systems in the genome of *C. pasteurianum* and method for circumvention permitting DNA transfer.

Restriction System Type	Methyl-transferase	Restriction Enzyme	Recognition Site	Circumvention	Reference
Type I - CpaAII	CPAST_c28480	CPAST_c28490	5'-AAGNNNNNCTC C-3'	Site removal	(105)
Type II - CpaAIP	CPAST_c05910	CPAST_c05920	5'-C ^m GCG-3'	<i>In vivo</i> methylation by M.FnuDII	(96,98)
Type II - CpaAIII	CPAST_c17720	N/A	Unknown	N/A	(104)
Type II – CpaAORF24410P	CPAST_c24410	N/A	Unknown	N/A	(104)
Type IIG / N6-adenine	CPAST_c24520	CPAST_c24520	Unknown	Unknown	(104)
Type IIS N4-cytosine or N6-adenine	CPAST_c30880, c30890, c30900	CPAST_c30870	5'-G ^m ATC-3',	<i>in vivo dam</i> ⁺ methylation	(96,98)

Following confirmation by sequencing, the transformation efficiency was assessed. Competent cells were prepared of clone 8.4 and were transformed with the control plasmid pMTL_85151 in both methylated (m85151) and unmethylated forms (85151) (Fig. 3.2B). Deletion of the Type II CpaAIP RM system resulted in successful transformation of unmethylated plasmid with a TE of 2.1×10^4 CFUs/ μ g DNA in Δ pyrE Δ cpaAIP. The TE of methylated pMTL_85151 increased slightly to 4.7×10^4 CFUs/ μ g DNA in Δ pyrE Δ cpaAIP compared to 3.4×10^4 CFUs/ μ g DNA in Δ pyrE. This suggested that plasmid methylation still provided some benefit towards TE. To further increase the TE in *C. pasteurianum*, deletion of the other RM systems seemed necessary.

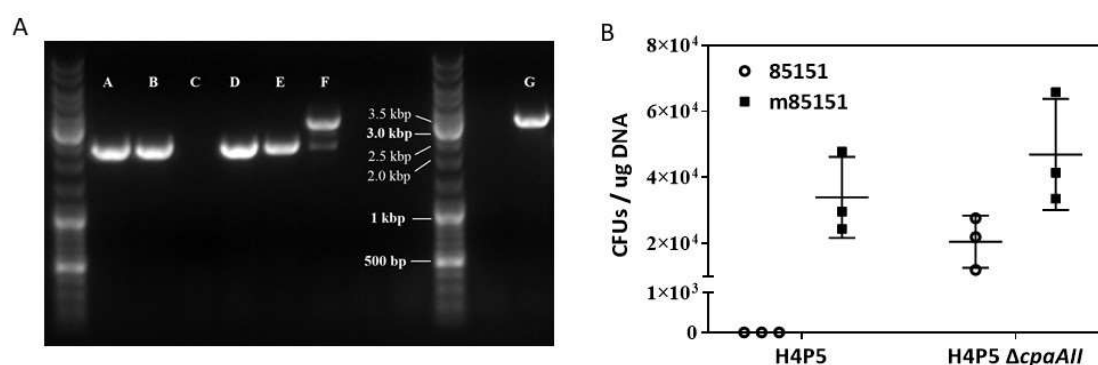


Figure 3.2 | Confirming and characterizing the TE of the mutant H4 Δ pyrE Δ cpaAIP. **(A)** Screening for the mutant Δ pyrE Δ cpaAIP. Lanes A-F are 6 clones that were screened using primers outside of the regions of homology used for deletion of *cpaAIP*. Lanes A, B, D and E correspond to the correct KO band size of 2.36 kbp and represents the mutant clones 2.1, 4.3, 7.2, 8.4, respectively. Lane G corresponds to the positive control with an expected wildtype band size of 3.22 kbp. **(B)** TE of the mutant Δ pyrE Δ cpaAIP (clone 8.4) evaluated by electroporating methylated (m85151) and unmethylated (85151) control plasmid. The control used was H4 Δ pyrE (H4P5).

Prior to pursuing the deletion of the Type I CpaAII RM system, the fermentation phenotypes of three isolated clones of Δ pyrE Δ cpaAIP (clones 4.3, 7.2, 8.4) were assessed. Fermentations under glucose or glycerol only conditions in minimal media were completed; the growth parameters were characterised by measuring the growth and pH curves of the fermentation as well as the 24 h product profile. The mutant Δ pyrE Δ cpaAIP had similar biomass production relative to the control strain Δ pyrE under either glucose or glycerol only conditions. The pH curve of the mutant Δ pyrE Δ cpaAIP also followed that of the control Δ pyrE closely with the pH remaining low under glucose conditions and increasing after 8 h under glycerol conditions (Fig. 3.3A, 3.3B). When fermenting either glucose or glycerol only, total

carbon consumption was very similar between the control and $\Delta pyrE \Delta cpaAIP$. Both total solvent production and the distribution of products was also very similar between mutant and control strains. For example, under glucose conditions, acetate production was greater than butanol production in both strains and at comparable titres, and under glycerol conditions, butanol production was greater than 1,3-PDO production and also at comparable titres (Fig. 3.3C, 3.3D). These results suggest that phenotypically the mutants behave comparably to the control strain and were suitable for subsequent genetic engineering.

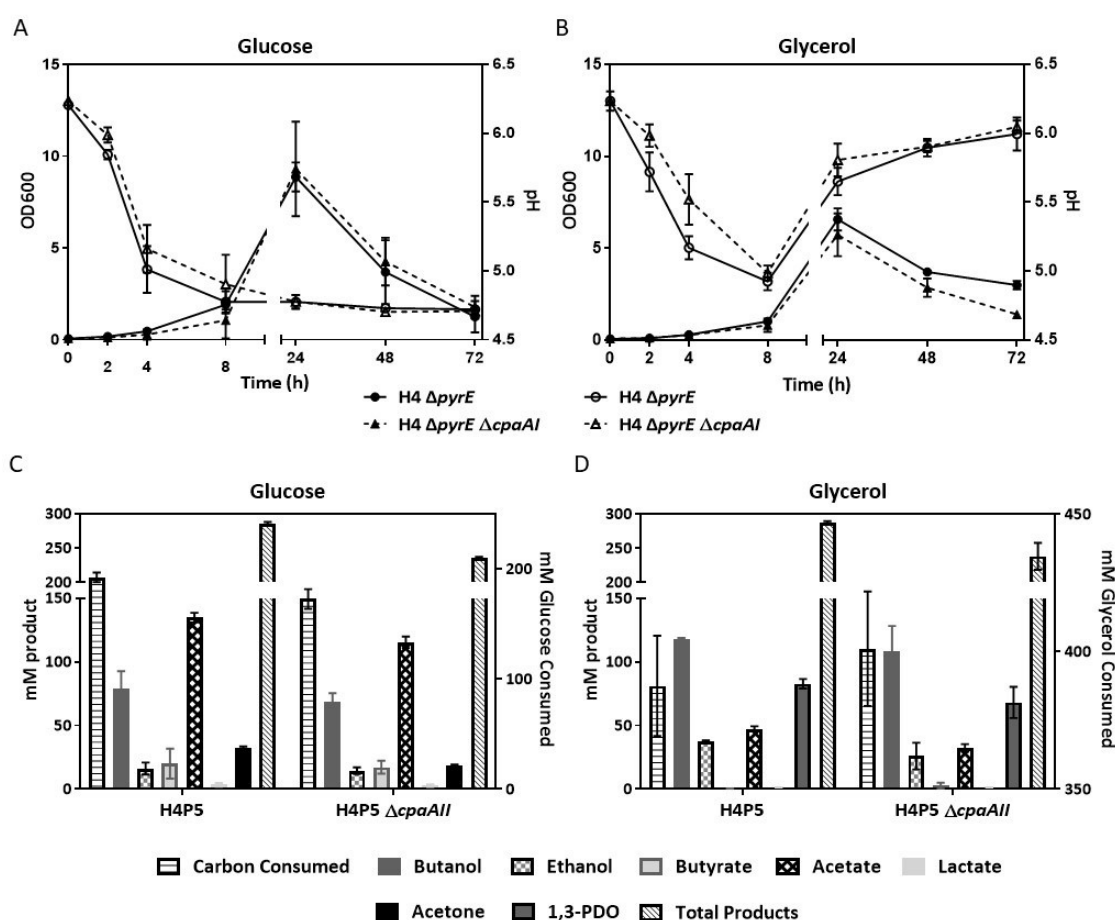


Figure 3.3 | Fermentation phenotype of mutant H4 $\Delta pyrE \Delta cpaAIP$. Growth parameters of control and mutant when grown on glucose (A) or glycerol (C) only in minimal media as measured by OD₆₀₀ (left y-axis) and pH (right y-axis). Twenty-four-hour product profile of control and mutant when grown on glucose (C) or glycerol (D) only. Individual products and carbon consumed are shown on the left and right y-axis, respectively. Error bars represent two technical replicates and three biological replicates for control and mutant strains, respectively.

3.2.3 Successive deletion of the Type I CpaAII RM system in *C. pasteurianum*

Although the phenotype of the mutant $\Delta pyrE \Delta cpaAIP$ was comparable to the control and parent strain H4 $\Delta pyrE$, deletion of the Type II CpaAIP RM system only marginally increased the TE of the control plasmid pMTL_85151. The Type I CpaAII RM system was reported to be active and to decrease the TE of any plasmid with the predicted recognition sequence 5'-AAGNNNNNCTCC-3' (105). As described in section 3.2.1, removal of the predicted recognition sequence by SDM did not increase TE in the plasmid pMTL_KS15 but did increase the TE in the plasmids pMTL_82251. In either case, the TE achieved did not reach that achieved of the control plasmid pMTL_85151 and suggested that the predicted restriction site was perhaps incorrect. The successive deletion of the Type I CpaAII RM system in the strain $\Delta pyrE \Delta cpaAIP$ was then pursued in an effort to further increase the TE.

At the genomic level, the Type I CpaAII locus is organised into a single operon consisting of a restriction enzyme, a methyltransferase and a specificity protein corresponding to genes CFAST_c28470, CFAST_c28480 and CFAST_c28490, respectively (Table 3.2). A KO cassette was constructed by cloning two regions of homology flanking the entire operon together and inserting into the KO vector pMTL_KS15 generating the plasmid pMTL_KS15_ CpaAII. This plasmid was not methylated *in vivo* prior to electroporation as the Type II CpaAIP RM system had been deleted in the strain $\Delta pyrE \Delta cpaAIP$. Following successful transformation of pMTL_KS15_ CpaAII into the mutant $\Delta pyrE \Delta cpaAIP$, the same screening and allelic exchange procedures were applied as previously described and the mutant $\Delta pyrE \Delta cpaAIP \Delta cpaAII$ was isolated. The deletion efficiency was lower when isolating this mutant than is typically observed with the procedure as 32 clones were screened to isolate a single mutant (Fig. 3.4A). Once isolated and cured of the KO vector, genomic DNA was isolated and the same primers binding outside the region of homology were used to sequence the region of interest and confirm the in-frame deletion. A second PCR was completed using isolated genomic DNA to demonstrate the deletion of the both the Type II CpaAIP and the Type I CpaAII RM systems. As expected, bands corresponding to deletion of both the restriction protein of the Type II CpaAIP and deletion of the entire Type I CpaAII locus were observed (Fig. 3.4B).

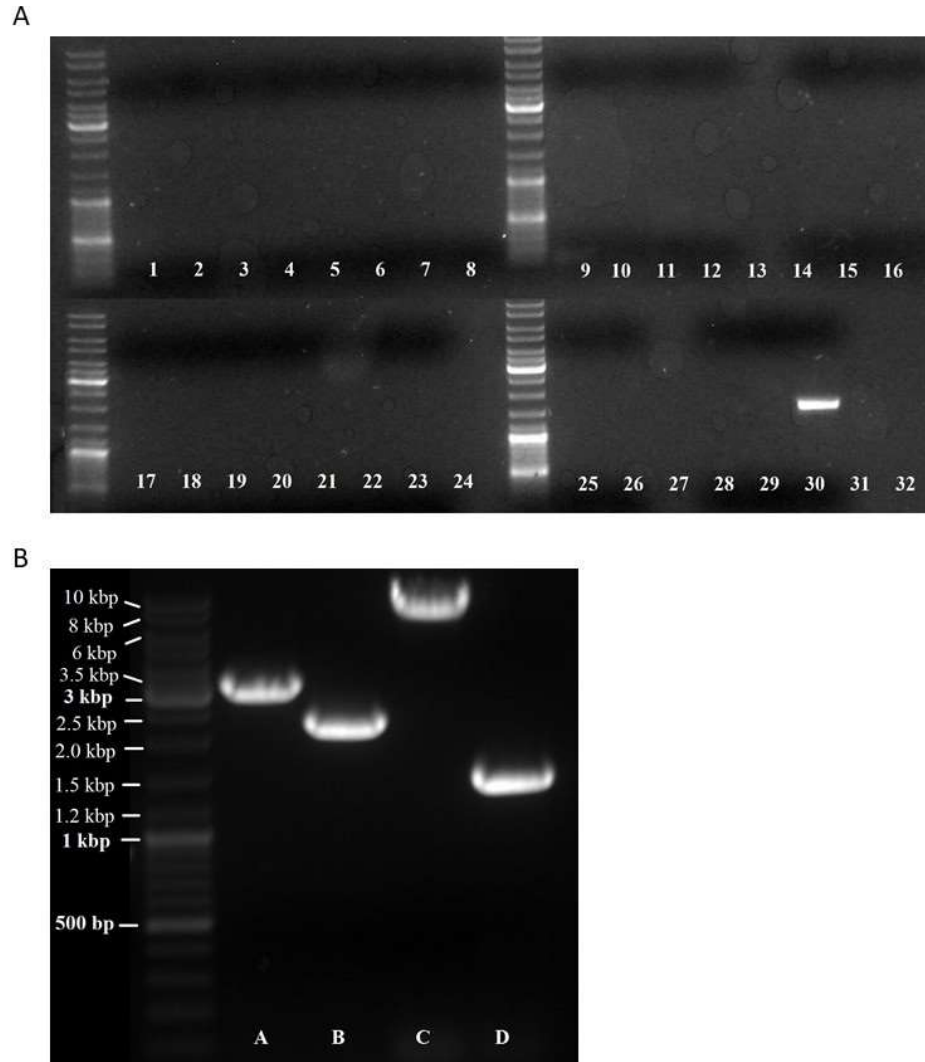


Figure 3.4 | Confirming the isolation of the mutant $\Delta pyrE \Delta cpaAIP \Delta cpaAII$. **(A)** Colony PCR screen for deletion of *cpaAII*. Of 32 colonies screened, only one band was present, suggesting either low efficiency in generating mutant, or the efficiency of the PCR reaction was poor. Since colony PCR had been a reliable technique, it was thought to be due to a low frequency of recombination and thus an unfavourable target. **(B)** PCRs demonstrating that the mutant $\Delta pyrE \Delta cpaAIP \Delta cpaAII$ had both CpaAIP and CpaAII RM systems deleted. Lanes A and C are the positive control bands obtained using H4 genomic DNA targeting the Type II CpaAIP and Type I CpaAII loci corresponding to a size of 3.22 kbp and 7.61 kbp, respectively, using primer pairs 2A and 3A, and 5B and 6B. Lanes B and D show bands obtained using genomic DNA from $\Delta pyrE \Delta cpaAIP \Delta cpaAII$ with the same primers used as the control PCRs targeting the Type II CpaAIP and Type I CpaAII loci corresponding to sizes of 2.36 kbp and 1.56 kbp, respectively.

Next, the strain $\Delta pyrE \Delta cpaAIP \Delta cpaAII$ was evaluated for its TE. The control plasmid pMTL_85151 was first tested, with both methylated and unmethylated versions of pMTL_85151 transformed. Deletion of *cpaAII* resulted in an increase in TE of the methylated plasmid pMTL_85151 to 5.67×10^5 CFUs/ μ g DNA from 1.6×10^4

CFUs/ μ g DNA in H4 Δ *pyrE*, an increase of 35-fold. The TE of unmethylated plasmid also increased to 1.68×10^5 CFUs/ μ g DNA from 2.05×10^4 CFUs/ μ g DNA in the mutant Δ *pyrE* Δ *cpaAIP*, an increase of 8-fold (Fig. 3.5A). The TE of the plasmids pMTL_82251 and Q5_82251 increased relative to the efficiencies observed in Δ *pyrE* but were still lower than those achieved using pMTL_85151. The TE of pMTL_82251 increased to 3.79×10^3 CFUs/ μ g DNA in the mutant Δ *pyrE* Δ *cpaAIP* Δ *cpaAII* compared to 2.3 CFUs/ μ g DNA in Δ *pyrE*, an increase of more than 1000-fold. The TE of Q5L_82251 increased to 4.75×10^4 CFUs/ μ g DNA in the mutant Δ *pyrE* Δ *cpaAIP* Δ *cpaAII* compared to 2.25×10^2 CFUs/ μ g DNA in the Δ *pyrE*, an increase of more than 200-fold (Fig. 3.5B).

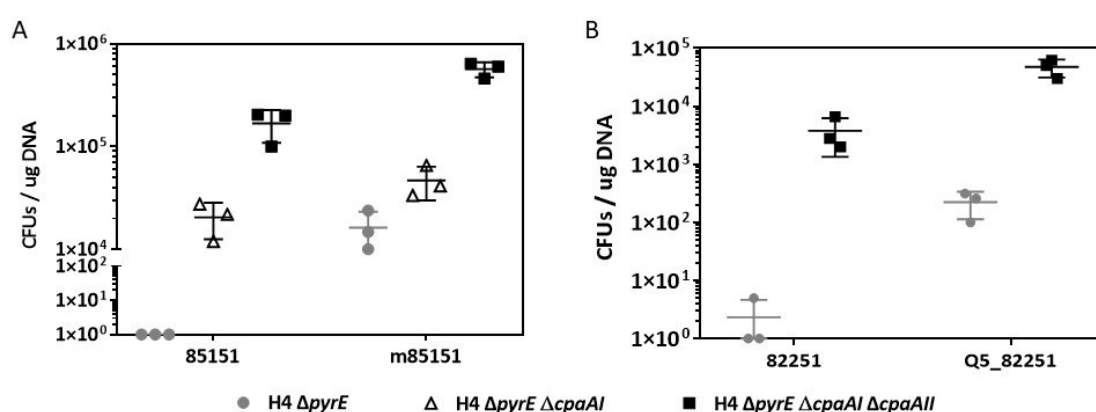


Figure 3.5 | Transformation efficiency of H4 Δ *pyrE* Δ *cpaAIP* Δ *cpaAII*. (A) TE of plasmid pMTL_85151 in unmethylated (85151) and methylated (m85151) forms in Δ *pyrE* Δ *cpaAIP* Δ *cpaAII*. For comparison purposes, the TE achieved with the same plasmid in the Δ *pyrE* Δ *cpaAIP* are also shown. (B) TE of the unmodified plasmid pMTL_82251 (82251) and with the Type I restriction site removed (Q5_82251) in H4 Δ *pyrE* Δ *cpaAIP* Δ *cpaAII*.

The fermentation phenotype of H4 Δ *pyrE* Δ *cpaAIP* Δ *cpaAII* was assessed next. Since only a single clone of the mutant was isolated, technical replicates were used instead of biological replicates. As done previously, the mutant was grown in minimal media on glucose or glycerol only and the growth and product profile were measured. The mutant generated similar amounts of biomass when grown on glucose only, but less biomass was generated when grown on glycerol. The pH curve of the fermentation in Δ *pyrE* Δ *cpaAIP* Δ *cpaAII* followed that of Δ *pyrE*; the pH remained below 5.0 after 8 h when grown on glucose and began to increase after 8 h when grown on glycerol (Fig. 3.6A, 3.6B). The product profile Δ *pyrE* Δ *cpaAIP* Δ *cpaAII* remained similar to the control Δ *pyrE* when grown on glucose; butanol and acetate were the main solvent and acid produced, respectively. From 190 mM of glucose consumed, 250 mM of products were formed after 24 h (Fig. 3.6C). When grown on glycerol, the

proportions and titres of the three main products, butanol, acetate and 1,3-PDO were the same between $\Delta pyrE \Delta cpaAIP \Delta cpaAII$ and $\Delta pyrE$. The only slight difference between the control and $\Delta pyrE \Delta cpaAIP \Delta cpaAII$ was observed when growth occurred on glycerol. Although similar amounts of products were formed (≈ 280 mM), $\Delta pyrE \Delta cpaAIP \Delta cpaAII$ consumed slightly more glycerol, 420 mM compared to 390 mM that the control consumed (Fig. 3.6D).

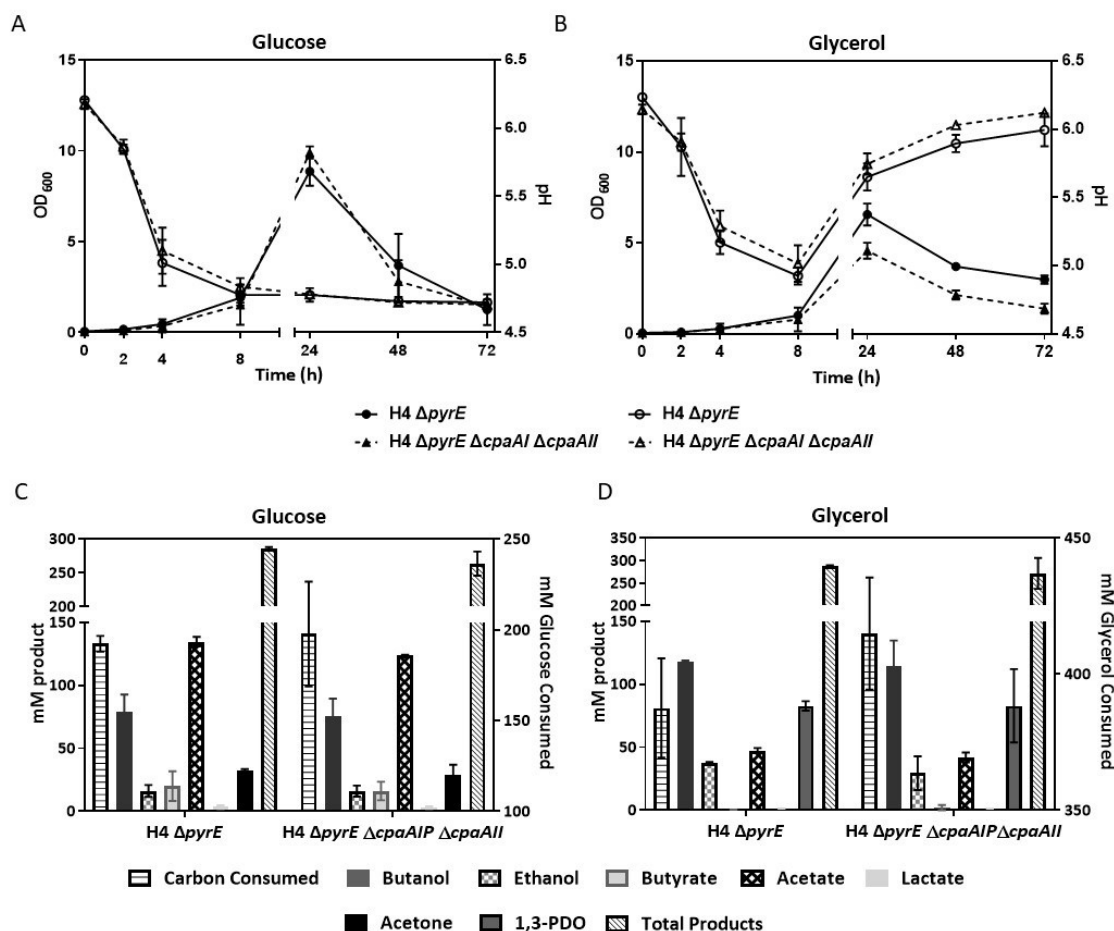


Figure 3.6 | Fermentation phenotype of $\Delta pyrE \Delta cpaAIP \Delta cpaAII$. Growth kinetics of control and mutant when grown on glucose (A) or glycerol (B) only in minimal media as measured by OD₆₀₀ (left y-axis) and pH (right y-axis). Twenty-four-hour product profile of control and mutant when grown on glucose (C) or glycerol (D) only. Products generated and substrate consumed are shown on the left and right y-axis, respectively. Error bars represent three technical replicates for control and mutant strains, respectively.

In summary, the TE of the *C. pasteurianum* was increased by successive deletion of two RM systems. A discussion on the significance and the impacts of the strains developed and the improvement in TE achieved for the work described in this chapter is included in Chapter 6.

Chapter 4

Co-production of 3- Hydroxypropionic Acid and 1,3- Propanediol From Glycerol

Project originality and accrediting statement:

The bulk of the work completed in this thesis will be found in this chapter. The idea for the co-production of 3-HP and 1,3-PDO from glycerol using *C. pasteurianum* was originally conceived by Dr. Kai Schuchmann, a visiting German Fellow. Dr. Schuchmann was my bench supervisor and we worked on the project together for approximately fifteen months. Unfortunately, Dr. Schuchmann was unable to continue in the lab after the first year and so I continued with the project, which became the bulk of the work I completed during my PhD.

Although much of the work described in this chapter was completed by me, many of the ideas and strategies must be credited to Dr. Schuchmann as he was the one who conceived the project and attained funding for the work. Furthermore, during the first year of the project, two visiting summer students also contributed to the project over the course of several months. Their names are Alexander Katsyv and Fabian M. Schwarz and are listed as co-authors in the expected publication. Alex was responsible for isolating the $\Delta dhaDK$ mutant and Fabian was responsible for cloning the *dhaB₁B₂* gene from *C. butyricum*. Having said that, as is the case with most research, projects evolve, unforeseen issues and expected or unexpected data was taken into account and thus I had some influence on the direction of the project as well.

4.1 Chapter Introduction

To date, *C. pasteurianum* has never been engineered to form a non-native product, with research efforts primarily focused on enhancing production of butanol from glycerol. Over the last ten years, glycerol has become a waste product of the biodiesel industry and thus there has been considerable effort to convert it into a higher value product to improve the economics of biorefineries producing biodiesel. The platform chemical 3-HP is a higher value product that can be produced from glycerol using either a three step CoA-dependent pathway or a one-step CoA-independent pathway. Both pathways have been previously used to produce 3-HP from glycerol in *E. coli*, with the progression of the research over the last ten years summarised in Chapter 1 (Section 1.2.9).

Clostridium pasteurianum is able to grow on glycerol as its sole carbon and energy source and thus is an attractive host for introducing both heterologous 3-HP forming pathways. Conversion of glycerol to 3-HP results in reducing equivalents generating in the form of NADH, which must be recycled to maintain redox balance. Growth on glycerol by *C. pasteurianum* results in formation of 1,3-PDO, consuming NADH and serving as a metabolic mechanism for maintaining redox balance, an attractive characteristic if introducing a heterologous 3-HP pathway which results in extra reducing equivalents being generated.

This chapter explores the co-production of the non-native product 3-HP and the native product 1,3-PDO, whose co-production is redox balanced. The two heterologous 3-HP pathways are introduced into *C. pasteurianum* through a variety of methods with resulting strains evaluated for their ability to co-produce 3-HP and 1,3-PDO from glycerol. The objectives of this chapter are to characterise the fermentation phenotypes of *C. pasteurianum* expressing the heterologous 3-HP pathways, as well as increase the titres of 3-HP and 1,3-PDO and overall product yield by applying synthetic biology and metabolic engineering strategies. The synthetic biology strategies will focus on characterising novel inducible promoters for overproduction of key enzymes to increase carbon flux towards product forming pathways as well as investigating different strategies for pathway expression. The metabolic engineering strategies will focus on decreasing carbon flux towards pathway competing pathways by deletion of enzymes responsible for by-product formation. Finally, to evaluate the effects of pH on product formation, fermentations were also completed at constant pH.

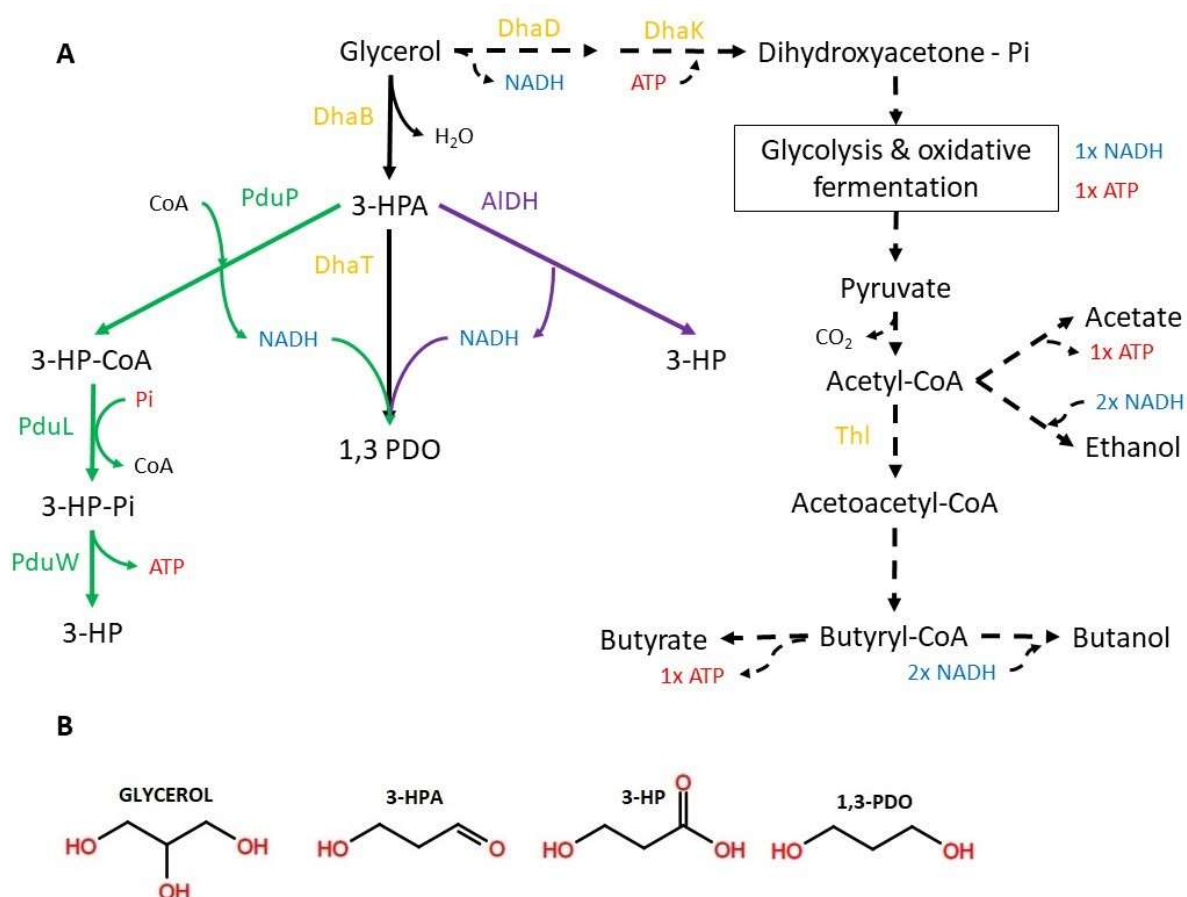


Figure 4.1 | Glycerol metabolism in *C. pasteurianum*, key metabolites and the two 3-HP formation pathways. **(A)** Glycerol is catabolised by two pathways referred to as the oxidative (dashed black) and reductive (solid black) branches. Oxidation and subsequent phosphorylation by glycerol dehydrogenase (DhaD) and dihydroxyacetone kinase (DhaK), respectively, to dihydroxyacetone phosphate allow for entry into the EMP pathway to allow for growth and biomass formation. As *C. pasteurianum* is an anaerobic organism, NADH is recycled through product formation. The main fermentation products are shown and the net ATP and NADH formation of each is shown in Table 4.1. The key step in formation of C4 products butyrate and butanol is the Claisen condensation of two acetyl-CoA molecules into acetoacetyl-CoA which is catalysed by the thiolase (Thl) enzyme. Not shown are ferredoxin linked reactions involved in decarboxylation of pyruvate to acetyl-CoA, the sequential reductions of acetoacetyl-CoA to butyryl-CoA and hydrogen evolution (see Fig. 1.2 for these). In the reductive branch glycerol is dehydrated to 3-HPA by glycerol dehydratase (GDHt) followed by reduction to 1,3-PDO by 1,3-propanediol dehydrogenase (DhaT) consuming 1 mole of NADH per mole of 1,3-PDO. The CoA-independent 3-HP pathway (purple) consists of oxidation of 3-HPA to 3-HP by an aldehyde dehydrogenase (AldH) generating 1 mole of NADH per mole of 3-HP. The CoA-dependent 3-HP pathway (green) consists of three reactions catalysed by CoA-dependent propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW), with 3-HPA-CoA and 3-HP-Pi as intermediates. The direction of arrows indicates whether substrates are released or included in the reactions and whether co-factors are consumed or formed. CoA = coenzyme A, Pi = inorganic phosphate, NADH = nicotinamide adenine dinucleotide (reduced) and ATP = adenine triphosphate. **(B)** Chemical structures of key molecules in the 3-HP and 1,3-PDO pathways.

Table 4.1 | Redox and energetic characteristics of products formed during the fermentation of glycerol by *C. pasteurianum*. Acids formed are butyrate, lactate and acetate, solvents formed are butanol, 1,3-PDO and ethanol; included as well is 3-HP. Gibbs free energy of formation from each product is shown, calculated by using the online tool eQuilibrator, a biochemical thermodynamics calculator (196). Net reducing equivalents are calculated by subtracting the NADH consumed during the formation of each product from the NADH generated from glycerol oxidation. Net ATP is calculated by summing all ATP generating reactions for formation of each product. Values of net reducing equivalents and net ATP are shown as moles per mol of fermentation products. Each mole of glycerol oxidised via glycolysis generates 1 mole of ATP, 2 moles of NADH and 1 mole of pyruvate. Production of C4 products butyrate and butanol each require 2 moles of glycerol per mole of product. All other fermentation products are produced in a 1:1 ratio per mole of glycerol consumed.

Fermentation Product	Chemical Formula	Gibbs Free Energy [KJ / mole]	Net Reducing Equivalents	Net ATP	Gas By-product
1,3-propanediol	C ₃ H ₈ O ₂	-63.9 ± 4.6	- 1 NADH	0 ATP	N/A
3-hydroxypropionic acid	C ₃ H ₆ O ₂	-71.4 ± 6.9	+ 1 NADH	0 or 1 ATP	N/A
Lactate	C ₃ H ₆ O ₃	-75.8 ± 4.2	+ 1 NADH	1 ATP	N/A
Acetate	C ₂ H ₃ O ₂ ⁻	-90.7 ± 7.6	+ 2 NADH	2 ATP	1 CO ₂ , H ₂
Ethanol	C ₂ H ₆ O	-82.5 ± 7.5	0 NADH	1 ATP	1 CO ₂ , H ₂
Butyrate	C ₄ H ₇ O ₂ ⁻	-217.7 ± 15.2	+ 1 NADH	3 ATP	2 CO ₂ , 3 H ₂
Butanol	C ₄ H ₁₀ O	-217.3 ± 14.7	- 1 NADH	2 ATP	2 CO ₂ , 3 H ₂

4.2 Results

4.2.1 3-HP is not consumed by nor is it toxic to *C. pasteurianum*

The reference or control strain used throughout this chapter is the hypertransformable variant H0D0R4 (H4) of *C. pasteurianum* previously isolated by a colleague in the CRG and is referred to as wildtype (97). Traditionally, *C. pasteurianum* has been investigated for production of native products butanol and 1,3-PDO and to date has not been exploited for production of any non-native compound such as 3-HP. To evaluate potential toxic effects of 3-HP on the host, *C. pasteurianum* was grown with an additional 50 mM 3-HP added at time of inoculation and compared to the growth of the wildtype. An almost identical growth rate was observed between the control culture and the culture with added 3-HP; only a slight decrease in overall biomass formation was observed after 24 h, as measured by OD₆₀₀. Furthermore, the concentration of 3-HP did not decrease after 24 h, suggesting that 3-HP cannot be metabolised by *C. pasteurianum* (Fig. 4.2). With these results, the next step was to introduce the two heterologous 3-HP pathways into *C. pasteurianum*, the three step CoA-dependent Pdu pathway, and the one step CoA-independent ALDH pathway (Fig. 4.1).

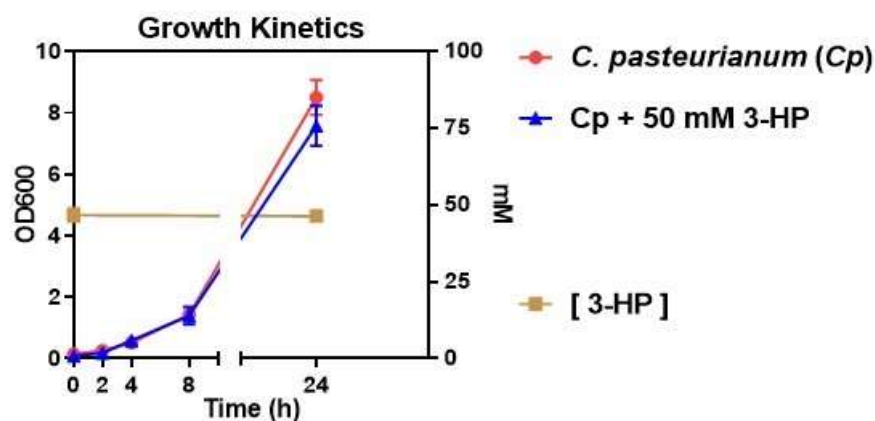


Figure 4.2 | Evaluating toxicity and consumption of 3-HP on growing cultures of *C. pasteurianum*. Wildtype *C. pasteurianum* was inoculated into 50 ml of minimal media containing 6% glycerol with and without 50 mM 3-HP. Growth was monitored for 24 h and 3-HP measured by HPLC. Errors bars represent standard error of three technical replicates for both the culture containing 3-HP and the control culture. 3-HP concentration is shown on the right y-axis.

4.2.2 Expression plasmids do not cause significant effects on fermentation

Heterologous enzymes and pathways used in this chapter were introduced into *C. pasteurianum* by integrating the genes into the genome or by expressing them from a plasmid. Introduction of plasmids is known to cause metabolic burden on hosts with unexpected changes to the solvent profile and growth phenotype possible. To ensure that shifts in the product profile of the fermentation are attributed to the genes and pathways introduced, it is necessary to demonstrate that the expression plasmids used do not cause any meaningful change to the product profile and growth patterns. To this end, control fermentations were carried out using the two expression plasmids used in this work, 85151 and Q5_82251. Both plasmids were transformed into wildtype *C. pasteurianum* without any cargo and colonies were screened for presence of the plasmid using colony PCR. Three colonies harbouring each plasmid were selected for a batch fermentation in minimal media supplemented with the appropriate antibiotic and 6% glycerol. The fermentation was characterised by measuring growth, pH, and product formation, as was done in the rest of the fermentations in this work.

Introduction of the empty expression plasmids 85151 and Q5_82251 resulted in a general slowing of the fermentation, likely due to the metabolic burden of harbouring a plasmid. The growth rate decreased with introduction of both plasmids, although more markedly with plasmid Q5_82251 than 85151, with biomass formation peaking after 48 h rather than 24 h (Fig. 4.3A). The pH of the media was observed to decrease faster than with wildtype and to trough close to pH 5.0 rather than 5.4 – 5.5, although the overall curve was relatively the same (Fig. 3.4B). Interestingly, harbouring plasmid 85151 caused formation of 1,3-PDO to increase almost 50% from 43 mM to 62.3 mM after 48 h, while plasmid Q5_82251 caused almost no change to 1,3-PDO (Fig. 4.3C). Ethanol production increased with both plasmids by almost 50%, from 23.8 mM in the wildtype to 38.5 mM, although it peaked after 48 h rather than 24 h. In contrast acetate production decreased slightly for both empty plasmids (Fig. 4.3D). Relative to the wildtype, glycerol consumed decreased with plasmid Q5_82251 but increased with plasmid 85151. Butanol produced increased slightly from 90 mM in the wildtype to 106 mM, with both plasmids. The most notable difference between the plasmid controls and the wildtype was in lactate production, which increased from 1.3 mM to 12.4 mM and 15.8 mM for plasmids Q5_82251 and 85151, respectively. Overall, the cumulation of small increases to various solvents resulted in total product formed

to increase from 168 mM in the wildtype to 206 mM and 233 mM for plasmids Q5_82251 and 85151, respectively (Fig. 4.3E). Taken together, introduction of the empty expression plasmids into *C. pasteurianum* were deemed to have had limited effects on solvent production within the context of the work in this chapter.

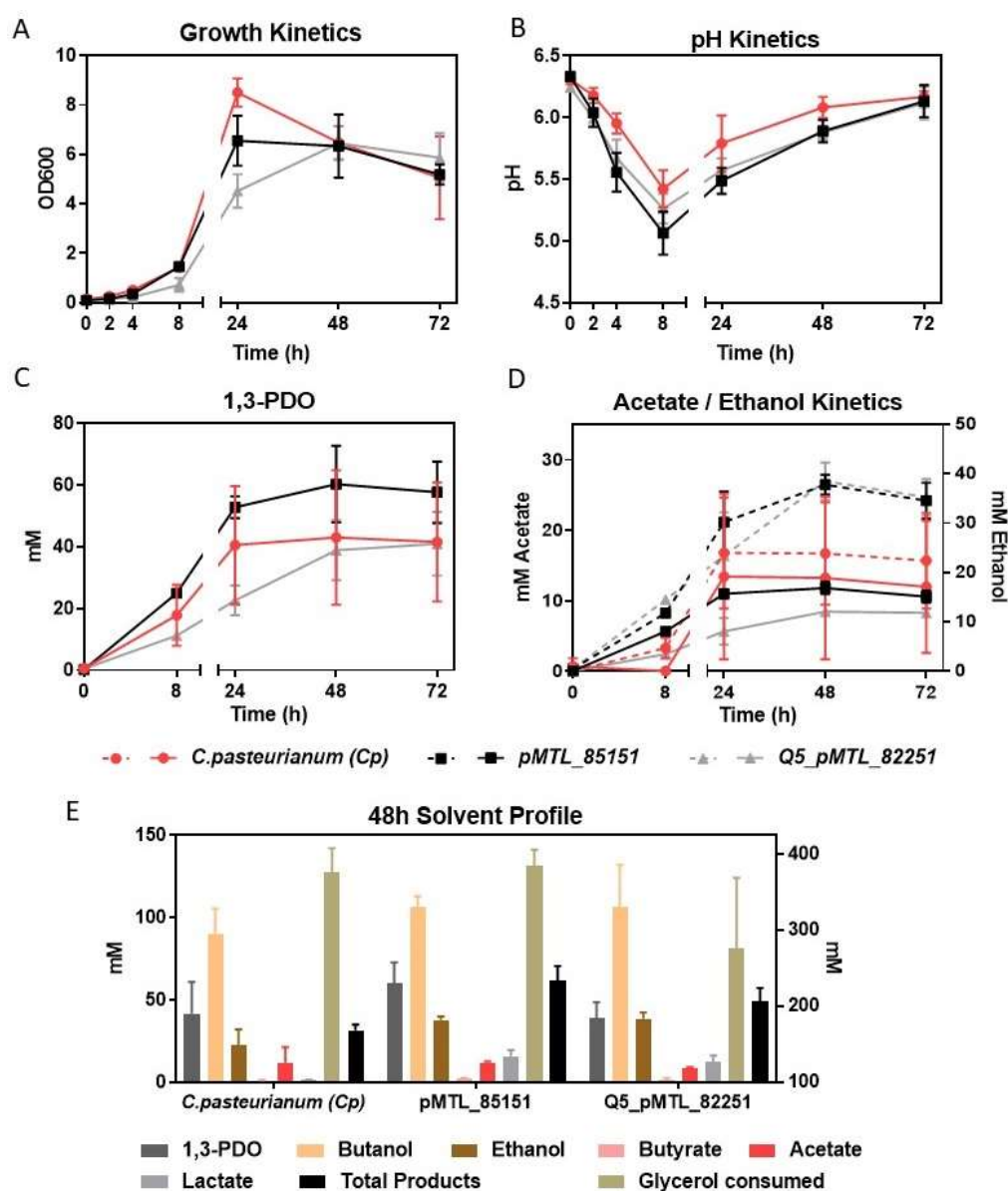


Figure 4.3 | Control fermentations with *C. pasteurianum* harbouring empty expression plasmids The expression plasmids 85151 and Q5_82251 were introduced into wildtype *C. pasteurianum* without any cargo to assess potential impact on solvent profile and growth phenotype. The growth (A) and pH (B) kinetics for glycerol only fermentation. (C) Production of 1,3-PDO. (D) Production of acetate (solid lines) and ethanol (dashed lines), shown on the left y-axis and right y-axis, respectively. (E) Solvent profile after 48h with solvents titres and glycerol consumed shown on the left y-axis and right y-axis, respectively. Error bars represent standard error for three technical replicates for plasmid controls and wildtype.

4.2.2 The CoA-dependent (Pdu) 3-HP pathway

The CoA-dependent 3-HP pathway consists of three Pdu enzymes (PduP, PduL and PduW) that catalyse the conversion of 3-HPA into 3-HP. The advantage of this pathway is that ATP is generated in a 1:1 ratio with 3-HP and would provide the host with an additional source of ATP for biomass formation (Fig. 4.1). These genes were ordered codon optimised for *C. pasteurianum* and assembled into two variants, as individually expressed genes (Pdul) and as an operon under the control of one promoter (PduO). The promoters, RBSs and terminators used in the assembly of both variants are shown in Table 2.6. Both Pdul and PduO variants were cloned into the expression plasmid Q5_pMTL_82251 generating the plasmids pDO-1 and pDO-2, respectively and were transformed into *C. pasteurianum*. Colonies were screened for presence of each plasmid by colony PCR, generating the strains cpDO-101 and cpDO-102. Both Pdul and PduO variants were also integrated at the *thlA2* locus using activation of a selectable marker, in this case erythromycin (*ermB*), leading to the generation of the strains cpDO-201 and cpDO-202, respectively. The *thlA2* locus was chosen as reporter assays conducted at the CRG by previous members had shown strong expression from this locus. The fermentation phenotype of the four resulting strains was evaluated by conducting 72 h fermentations in 50 ml of minimal media containing 6% glycerol. Cultures were inoculated to a starting $OD_{600} \approx 0.1$ and samples were taken at time of inoculation (0 h), 2 h, 4 h, 8 h, 24 h, 48 h and 72 h to measure growth parameters, OD_{600} and pH; metabolites were measured at 0 h, 8 h, 24 h, 48 h and 72 h. Unless otherwise stated, these are the conditions for all fermentations used in this chapter.

Strain cpDO-201 had a lower growth rate and generated lower overall biomass than the wildtype. Similar growth rates were observed for strains cpDO-101, cpDO-102 and cpDO-201 as well as an increase in overall biomass generated relative to the wildtype (Fig. 4.4A). Except for strain cpDO-201, the pH curves of all strains followed the same pattern as that of the wildtype, with pH dropping below 5.5 after 8 h and increasing back above 6.0 after 48 h (Fig. 4.4B). Introduction of the Pdu enzymes into *C. pasteurianum* resulted in large increases to 1,3-PDO production, between 1.7 and 3.6-fold, relative to the wildtype (Fig. 4.4C). Only strain cpDO-201 resulted in 3-HP production, with 0.4 mM 3-HP generated after 48 h (Fig. 4.4D). Production of acids, acetate and butyrate was elevated in strains cpDO-201 and cpDO-202. Butanol and ethanol production were relatively unchanged in all strains. Although total solvent

production increased in all Pdu expressing strains, increased glycerol consumption was only observed in strains cpDO-102 and cpDO-202, where the Pdu genes were cloned as an operon (Fig. 4.4E). The best performing strain in terms of co-production of 3-HP and 1,3-PDO was cpDO-201, where the Pdu enzymes were individually expressed and integrated at the *thlA2* locus. To evaluate the distribution of products, carbon mass balancing was completed.

Table 4.2 | Carbon mass balance of fermentations for strains expressing the CoA-dependent (Pdu) 3-HP pathway. Acids produced are acetate, lactate, and butyrate. Solvents produced are ethanol and butanol. Total products are the sum of acids, solvents and 1,3-PDO. The time at which solvent and acid production ceased was taken as the fermentation end point. 3-HP was excluded as it was negligible for the calculations.

Strain (48 h)	Glycerol Consumed (moles)	Total Products (moles)	Product Yield [moles / 100 moles glycerol]			Carbon Recovery [%]
			1,3-PDO	Acids	Solvents	
<i>C. pasteurianum</i>	0.37	0.19	11.4	4.1	36.3	85
cpDO-101	0.45	0.25	16.8	2.5	35.5	85
cpDO-102	0.45	0.24	17.9	2.9	32.3	80
cpDO-201	0.50	0.36	31.5	7.9	32.0	101
cpDO-202	0.66	0.29	14.9	4.1	24.1	63

The data retrieved from carbon mass balancing echoed the fermentation data generated and presented in Fig. 4.4 and Table 4.2. Strains expressing the CoA-dependent 3-HP pathway generated higher amounts of total products and consumed higher amounts of glycerol. The best performing strain in terms of 1,3-PDO titres also had the highest product yield (cpDO-201). It is unclear why the carbon recovery was so low with strain cpDO-202. The CoA-independent 3-HP pathway was assessed next for production of 3-HP.

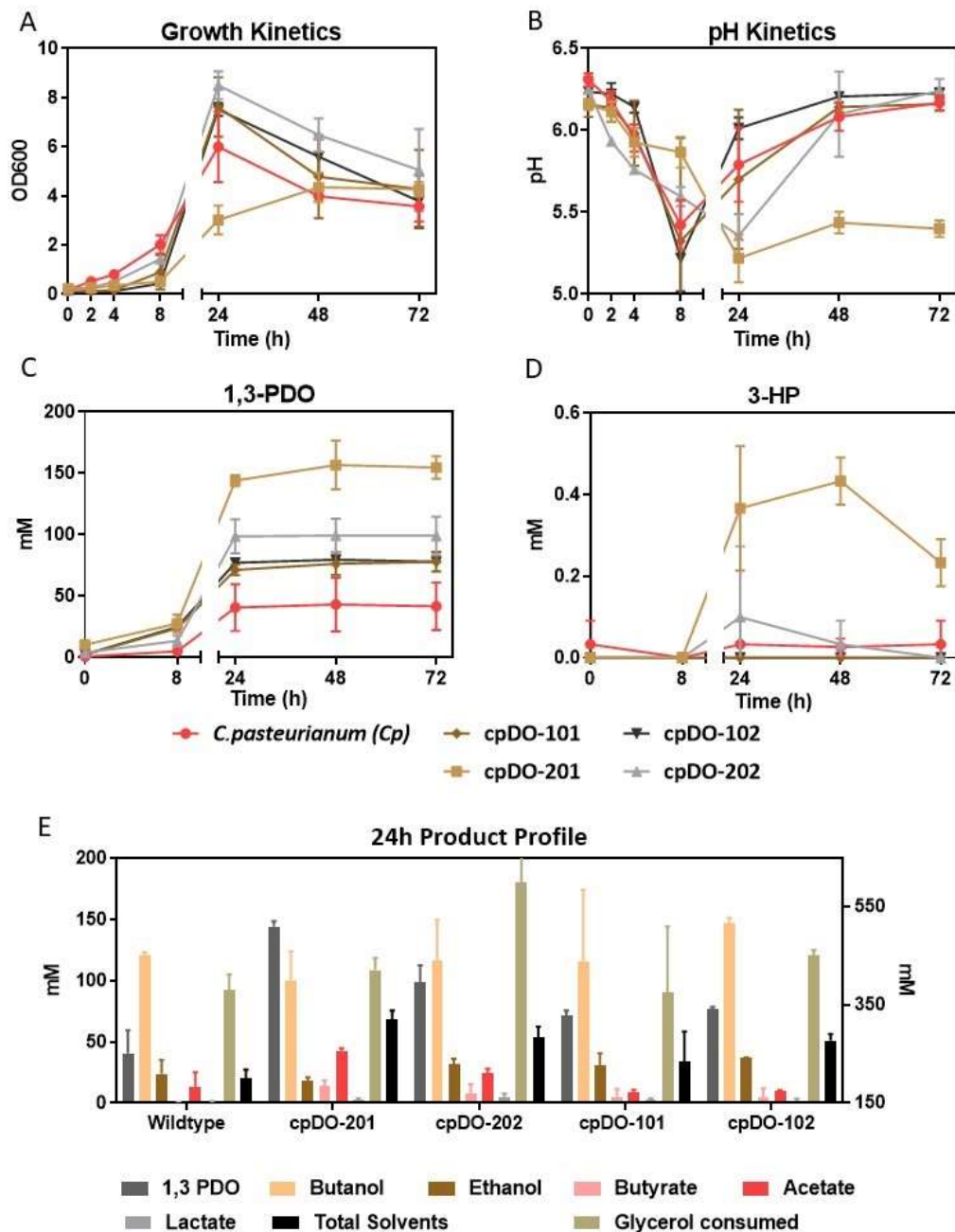


Figure 4.4 | Characterising the CoA-dependent Pdu 3-HP pathway in *C. pasteurianum*. The Pdu enzymes were cloned in two variants, in operon format (PduO) or with each gene being individually expressed (PduI) and both variants were expressed from a plasmid or integrated into the genome. The growth (A) and pH (B) kinetics for glycerol only fermentation of the four strains and the wildtype. Production kinetics of 1,3-PDO (C) and 3-HP (D). (E) Product profile for glycerol only fermentation in 50 ml of minimal media in Erlenmeyer flasks. Titres of individual products are shown on the left y-axis, total solvents and glycerol consumed are shown on the right y-axis. Error bars represent standard error for three biological and three technical replicates for the Pdu expressing strains and the wildtype, respectively.

cpDO-201 = H4 *thlA2::pduI*
cpDO-201 = H4 *thlA2::pduO*
cpDO-101 = H4 + pDO-01 (Q5_82251_*pduI*)
cpDO-201 = H4 + pDO-02 (Q5_82251_*pduO*)

4.2.3 The CoA-independent (AldH) 3-HP pathway

Two candidates AIDHs were assessed for production of 3-HP using the CoA-independent pathway. The *kgsadh* (*kgs*) from *Azospirillum brasilense* and *gabD4* from *Cupriavidus necator* were ordered codon optimised for *C. pasteurianum*. Both genes were initially integrated at the *thlA2* locus in the wildtype but did not lead to 3-HP formation (data not shown). Plasmid based expression of each AIDH was tested next with the strong ferredoxin promoter from *Clostridium kluyveri* (P_{fdx}) driving expression of the AIDHs. Both *kgs* and *gabD4* were cloned into the plasmids Q5_pMTL_82251 and pMTL_85151, generating plasmids pDO-03, pDO-04, pDO-05 and pDO-06. Transformation of plasmids pDO-03-06 into wildtype *C. pasteurianum* generated strains cpDO-103-106, respectively. These strains were evaluated for co-production of 3-HP and 1,3-PDO.

Plasmid based expression of *kgs* and *gabD4* resulted in slower growth rates and lower biomass formation than the wildtype (Fig. 4.5A). Production of 3-HP occurred primarily between 8 h and 24 h. Higher amounts of 3-HP were produced when *kgs* and *gabD4* were expressed on plasmid Q5_pMTL_82251 than pMTL_85151. Expression of *kgs* and *gabD4* from Q5_pMTL_82251 resulted in 1.1 mM and 1.5 mM 3-HP after 24 h, respectively (Fig. 4.5B). Unlike 3-HP, production of 1,3-PDO continued until 48 h in strains cpDO-107 and cpDO-108, which produced 111 mM and 91 mM 1,3-PDO (Fig. 4.5C).

To verify *in vivo* activity of both AIDHs, crude cell lysates were prepared from samples taken after 24 h of strains cpDO-105 and cpDO-106 as they had produced the highest amounts of 3-HP. With both substrates, a higher activity was observed for GabD4; these results correlated well with the fermentation data (Fig. 4.5D). Higher activity was observed with both enzymes when expressed from plasmid pMTL_Q5_82251; thus, this plasmid became the preferred plasmid for expression of pathways. No activity was detected when using crude cell lysates prepared from wildtype cultures.

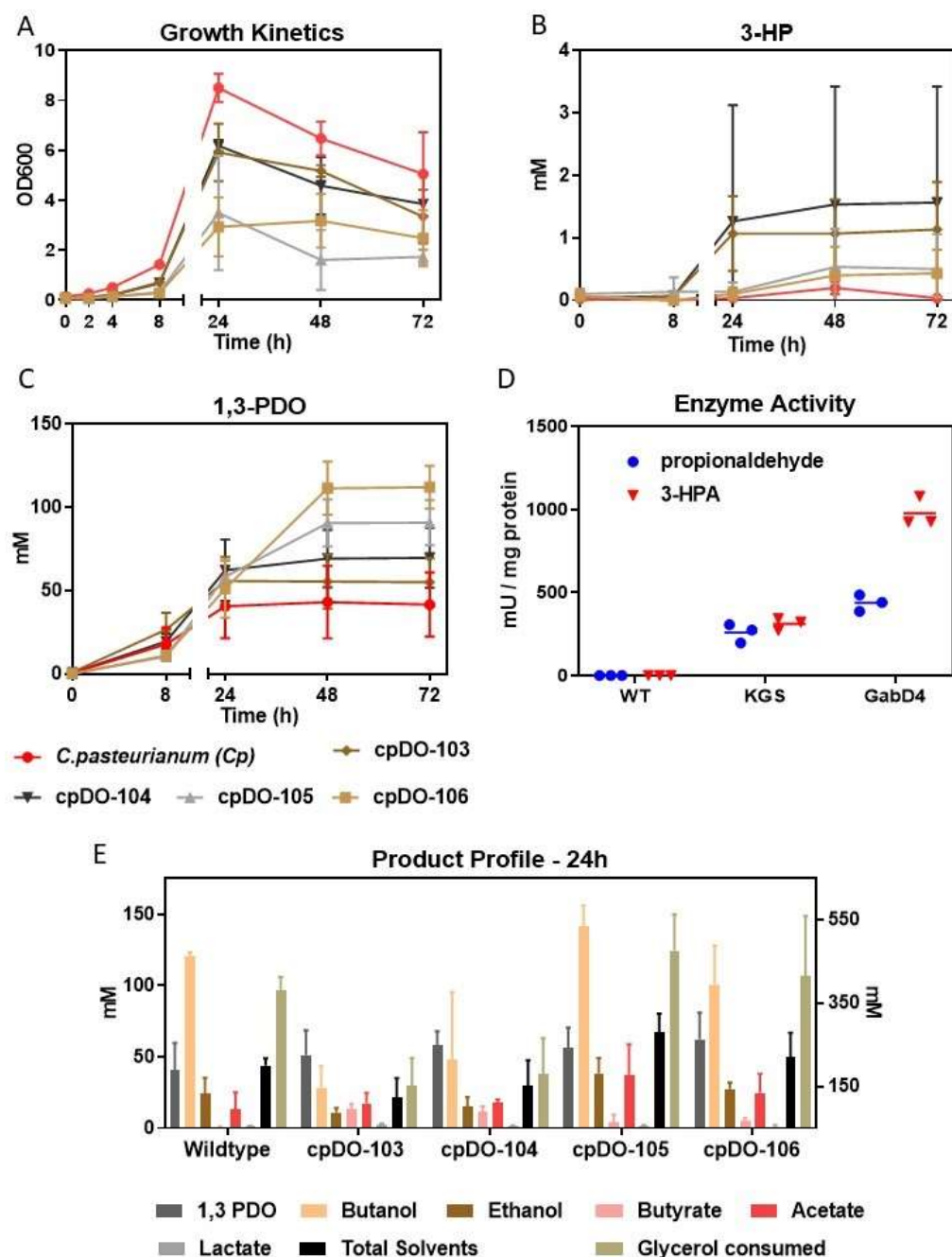


Figure 4.5 | Characterising the CoA-independent pathway in *C. pasteurianum*. Fermentation kinetics were taken for growth as measured by OD₆₀₀ (**A**), production of 3-HP (**B**) and production of 1,3-PDO (**C**) over 72 h. (**D**) Enzyme activity was measured by watching reduction of NAD⁺ to NADH at 340_{nm} with substrates propionaldehyde and 3-HPA. (**E**) Product profile of fermentation after 24 h. Error bars represent standards error for three biological and three technical replicates for production and wildtype strains, respectively.

cpDO-103 = H4 + pDO-03 (pMTL_Q5_82251_ *P_{fdx_kgs}*)

cpDO-104 = H4 + pDO-04 (pMTL_Q5_82251_ *P_{fdx_gabD4}*)

cpDO-105 = H4 + pDO-05 (pMTL_85151_ *P_{fdx_kgs}*)

cpDO-106 = H4 + pDO-06 (pMTL_85151_ *P_{fdx_gabD4}*)

Like expression of the Pdu enzymes in *C. pasteurianum*, expression of *kgs* and *gabD4* resulted in changes to the product profile of the fermentation. Although overall biomass decreased, glycerol consumption and total solvent produced were higher relative to the wildtype in strains cpDO-105 and cpDO-106. Strains cpDO-105 and cpDO-106 generated more acetate than the wildtype while strains cpDO-107 and cpDO-108 generated much more butyrate than the wildtype. A 50% decrease in butanol formation relative to the wildtype was also observed in cpDO-107 and cpDO-108 (Fig. 4.5E).

Carbon mass balancing of the fermentation was calculated with values collected at the end of the fermentation, defined as the point at which formation of new products ceased. For this reason, the carbon mass balancing tells a different story to the product profile shown in Fig. 4.5E after 24 h. For example, solvent production (ethanol and butanol) in strains cpDO-103 and cpDO-104 is less than the wildtype after 24 h but is comparable to that produced by the wildtype after 48 h. Similarly, production of 1,3-PDO in strains cpDO-105 and cpDO-106 is comparable to the wildtype after 24 h, but much higher after 48 h. Interestingly, strains cpDO-105 and cpDO-106 were able to generate an overall higher product yield, as the total products increased significantly while consumption of glycerol increase only marginally relative to the wildtype (Table 4.3).

Table 4.3 | Carbon mass balance of fermentations for strains expressing the CoA-independent (AIDH) 3-HP pathway. Acids produced are acetate, lactate and butyrate. Solvents produced are ethanol and butanol. Total products are the sum of acids, solvents, 1,3-PDO and 3-HP. The time at which solvent and acid production ceased was taken as the fermentation end point. *Cp* = wildtype *C. pasteurianum*. Plasmid harboured by each strain: cpDO-106 = pMTL_85151_ *kgs*, cpDO-105 = pMTL_85151_ *gabD4*, cpDO-103 = Q5_pMTL_82251_ *kgs*, cpDO-104 = Q5_pMTL_82251_ *gabD4*.

Strain (48 h)	Glycerol Consumed (moles)	Total Products (moles)	Product Yield [moles / 100 moles glycerol]				Carbon Recovery [%]
			1,3-PDO	3-HP	Acids	Solvents	
<i>Cp</i>	0.37	0.19	11.4	0.0	4.1	36.3	85
cpDO-106	0.40	0.29	28.0	0.1	7.9	37.2	105
cpDO-105	0.40	0.28	22.5	0.1	7.5	39.0	103
cpDO-103	0.50	0.29	11.4	0.2	6.2	35.4	83
cpDO-104	0.46	0.28	15.6	0.4	4.6	33.1	80

4.2.4 Increasing carbon flux towards reductive branch of metabolism increases 3-HP and 1,3-PDO titres

Having demonstrated production of 3-HP with both the Pdu (CoA-dependent) and ALDH (CoA-independent) pathways, the next step was to improve these initial results. Both the CoA-dependent and CoA-independent pathways require the upstream metabolite 3-HPA, generated by the dehydration of glycerol by DhaB (Fig. 4.1). It was hypothesized that pushing carbon flux towards the reductive branch by increasing 3-HPA would result in increased production of 3-HP and 1,3-PDO. Another species of *Clostridium* that is also able to ferment glycerol is *C. butyricum*, which contains an isozyme of glycerol dehydratase (DhaB). This isozyme has previously been characterised and shown to be B₁₂-independent and is referred to as DhaB_{1B2}. The *dhaB_{1B2}* from *C. butyricum* was cloned into the plasmid Q5_pMTL_82251 with expression driven by an inducible promoter. As the fermentative substrate was glycerol, a glycerol inducible promoter was ideal but was not available. Hypothesizing that genes required for catabolism of a secondary carbon source would be inducible, the native promoter of *dhaB* was evaluated.

The sequence directly upstream of *dhaB*, the first gene in the reductive glycerol operon, was evaluated using Softberry, an online tool for analysis of bacterial promoter sequences. Using this as a guide, primers were designed to amplify 122 bp and 215 bp sequences directly upstream of *dhaB* and were designated P_{dhaBS} and P_{dhaBL}, respectively. To determine relative promoter strength, the *catP* gene was cloned downstream of the P_{fdx}, P_{dhaBS} and P_{dhaBL} promoters into the plasmid Q5_pMTL_82251. A promoter-less *catP* was used as the negative control. The resulting reporter vectors were transformed into *C. pasteurianum* and grown on either glucose or glycerol only to assess CAT activity. The CAT activity of P_{dhaBL} was 133,350 U/mg in glycerol, representing a 60-fold level of induction relative to glucose and providing stronger relative expression than P_{fdx}. The CAT activity of P_{dhaBS} was 40,500 u/mg in glycerol, representing a 2.5-fold level of induction relative to glucose (Fig. 4.6A). This is the first characterised inducible promoter system in *C. pasteurianum*; a strong level of induction of was observed as well as a stronger level of absolute expression relative to the strong P_{fdx} promoter. This provides future researchers of *C. pasteurianum* with a useful tool for synthetic biology applications.

To evaluate overexpression of glycerol dehydratase, *dhaB₁B₂* was cloned into the plasmid Q5_pMTL_82251 under the control of the newly characterised inducible glycerol promoter *P_{dhaBL}* generating plasmid pDO-11, which was transformed into *C. pasteurianum* generating strain cpDO-111. Overexpression of *dhaB₁B₂* resulted in similar fermentation growth kinetics to the wildtype although less overall biomass formation was formed (Fig. 4.6B, 4.6C). A substantial increase in production of 1,3-PDO was observed, more than 90 mM of 1,3-PDO was formed after 24 h, compared to 40 mM in the wildtype, which increased further to 114 mM after 48 h (Fig. 4.6D). The fermentation product profile of strain cpDO-111 is shown in Fig. 4.7E for ease of comparison to the wildtype.

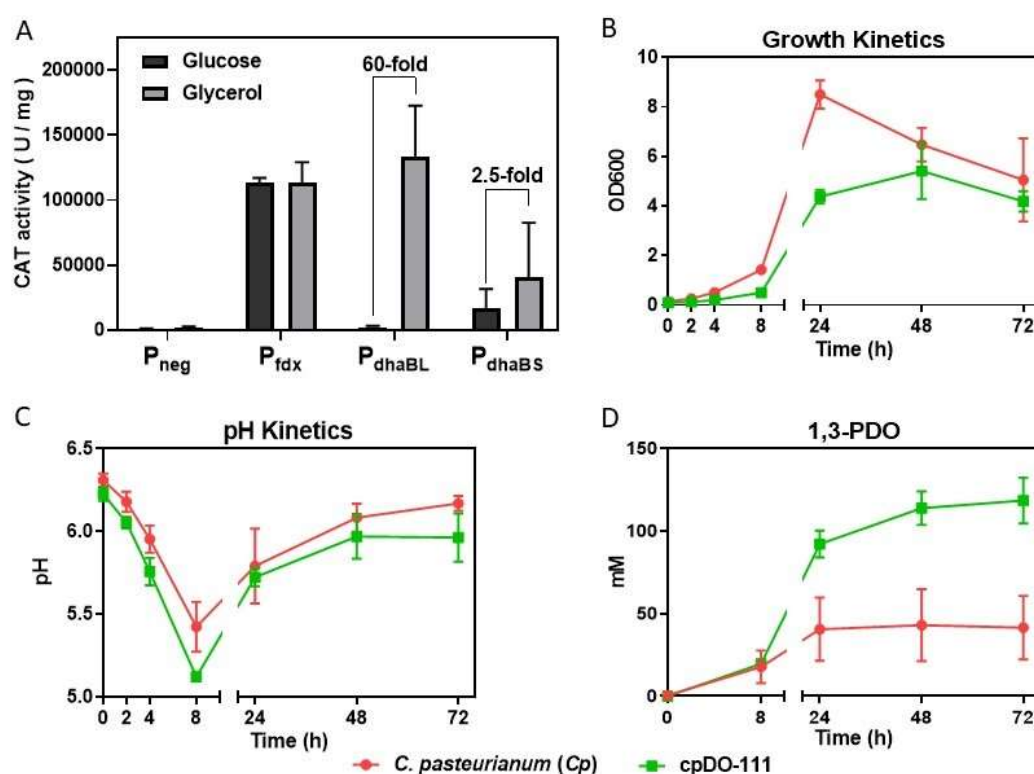


Figure 4.6 | Characterisation of the native inducible glycerol promoter and overexpression of heterologous *dhaB₁B₂*. **(A)** Promoter of the reductive glycerol operon was characterised using the CAT assay and assessed under glucose or glycerol only conditions. Two promoter sequences *P_{dhaBL}* and *P_{dhaBS}* were analysed. **(B-D)** The *dhaB₁B₂* from *C. butyricum* was cloned into plasmid Q5_pMTL_82251 with expression driven by the *P_{dhaBL}* promoter (pDO-11). This plasmid was transformed into wildtype *C. pasteurianum* (strain cpDO-111) to evaluate the fermentation phenotype and production of 1,3-PDO. Error bars represent standard error of three biological and technical replicates for production and control strains, respectively. cpDO-111 = H4 + pDO-11 (Q5_82251_*P_{dhaBL}dhaB₁B₂*)

Overexpression of *dhaB₁B₂* resulted in increased carbon flux towards the reductive branch of glycerol metabolism as evidenced by the increase in formation of 1,3-PDO. The best results achieved in terms of production of 3-HP thus far had been via the CoA-independent pathway. Next, *dhaB₁B₂* was co-expressed with *kgs* or *gabD4* by co-transforming plasmid pDO-11 with pDO-05 or pDO-05 into *C. pasteurianum* generating strains cpDO-115 and cpDO-116, respectively. Although better 3-HP results had been achieved with plasmids pDO-03 pDO-04, they shared the same backbone (Q5_pMTL_82251) as plasmid pDO-11 and thus could not be used together.

Strains cpDO-115 and cpDO-116 had severely impaired growth rates and biomass formation, presumably due to the metabolic burden associated with harbouring two plasmids. (Fig. 4.7A) (197). This different growth pattern is also reflected in the pH curve; it's possible that if the fermentation had been continued, the pH would have increased as it does in the wildtype although this was not tested (Fig. 4.7B). Co-expression of *dhaB₁B₂* with either AIDH resulted in 1,3-PDO formation increasing to 123 mM after 72 h, compared to 118 mM when *dhaB₁B₂* was expressed in isolation (Fig. 4.7C vs Fig. 4.6D). The decreased rate of 1,3-PDO formation may have also been due to the slow growth rate. Co-expression of *dhaB₁B₂* with *kgs* (cpDO-115) resulted in 3 mM of 3-HP after 72 h, an increase of 2.5-fold compared to when *kgs* was expressed in isolation (cpDO-103). The best combination was co-expression of *dhaB₁B₂* with *gabD4* (cpDO-116), which resulted in 5.1 mM of 3-HP after 72 h, an increase of 3.4-fold relative to expression of *gabD4* in isolation (cpDO-104) (Fig. 4.7D).

Interestingly, although glycerol consumed decreased slightly in strains cpDO-115 and cpDO-116 relative to the wildtype, total products increased. Co-expression of *dhaB₁B₂* and the CoA-independent 3-HP pathway increased acid production and decreased butanol; titres of acetate, butyrate and lactate increased relative to the wildtype while butanol titres decreased (Fig. 4.7E).

The strategy to express *dhaB₁B₂* and the AIDHs on two separate plasmids, although effective for increasing titres of 3-HP, resulted in a detrimental effect on growth and overall slower fermentation. Formation of 3-HP did not occur until after 48 h, compared to 24 h in the fermentations where only the AIDHs were expressed (Fig. 4.7D vs Fig. 4.5B). In attempt to improve the growth rate and overall speed of the fermentation, *dhaB₁B₂* and the CoA-dependent and CoA-independent 3-HP pathways were first cloned onto a single plasmid.

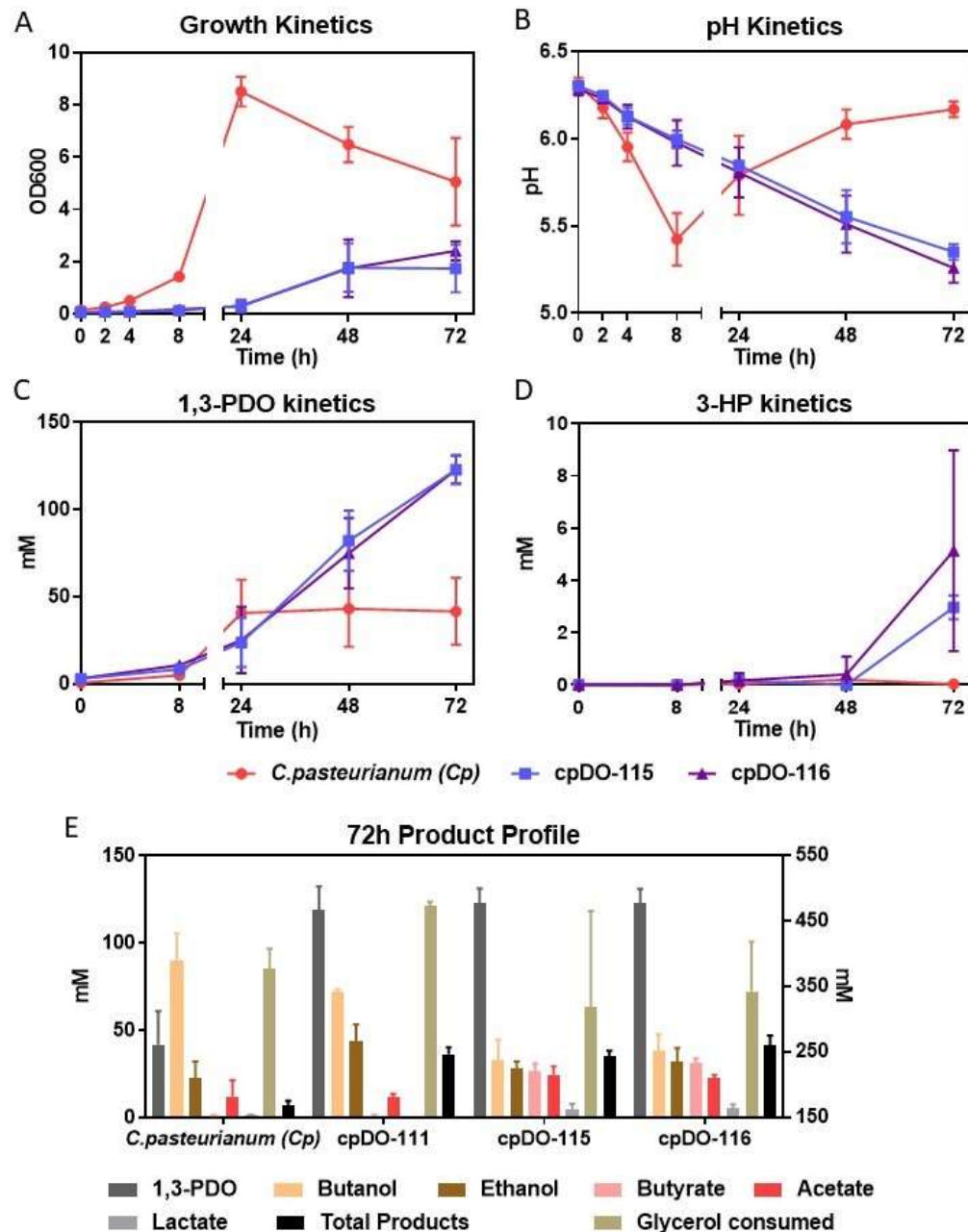


Figure 4.7 | Evaluating co-production of 3-HP and 1,3-PDO by overexpressing *dhaB₁B₂* with *kgs* or *gabD4*. Fermentation phenotype as measured by growth (A) and pH (B) kinetics. Production kinetics for 3-HP (C) and 1,3-PDO (D) formation. (E) Product profile of fermentation is shown for 72 h timepoint as product formation continued until 72 h. Individual products are shown on the left y-axis, glycerol consumed, and total products are shown on the right y-axis. Error bars represent standard error of three biological and technical replicates for production and control strains, respectively.

cpDO-115 = H4 + pDO-11 (Q5_82251_ *P_{dhaBL}dhaB₁B₂*) + pDO-05 (85151_ *P_{fdx}kgs*)

cpDO-116 = H4 + pDO-11 (Q5_82251_ *P_{dhaBL}dhaB₁B₂*) + pDO-06 (85151_ *P_{fdx}gabD4*)

4.2.5 Expression of *DhaB₁B₂* and 3-HP pathways from a single plasmid increases biomass formation but does not increase 3-HP titres

The GabD4 used in this work has been the wildtype form of the enzyme. The same authors who reported its effective use for production of 3-HP in *E. coli* also completed some mutational studies and identified two amino acid substitutions that enhanced catalytic efficiency and substrate dependent activity (117). Substitution of glutamic acid (E) for glutamine (Q) at positions 209 and 269 each increased the enzyme activity of GabD4, however the highest activity exhibited was observed when both substitutions were made together. Using SDM, two variants of *gabD4* were created: *gabD4* with substitution E209Q (*gabD4i*) and *gabD4* with both substitutions E209_269Q (*gabD4ii*). Wildtype *gabD4* as well as these two variants *gabD4i* and *gabD4ii* were cloned into a single transcript with *dhaB₁B₂* driven by the inducible glycerol promoter resulting in plasmids pDO-22, pDO-23, and pDO-24, respectively.

The Pdu pathway had until now had not been co-expressed with *dhaB₁B₂*. The *pduO* variant was cloned in an operon structure with *dhaB₁B₂* under the control of the inducible glycerol promoter generating plasmid pDO-21. Although this plasmid was quite large (>12 kbp) and was expected to create some metabolic burden, given that the Pdu pathway results in 1:1 formation of 3-HP and ATP, it was hypothesized that this pathway would result in some growth advantage. Plasmids pDO-21, pDO-22, pDO-23 and pDO-24 were transformed into *C. pasteurianum* generating strains cpDO-121, cpDO-122, cpDO-123 and cpDO-124, respectively.

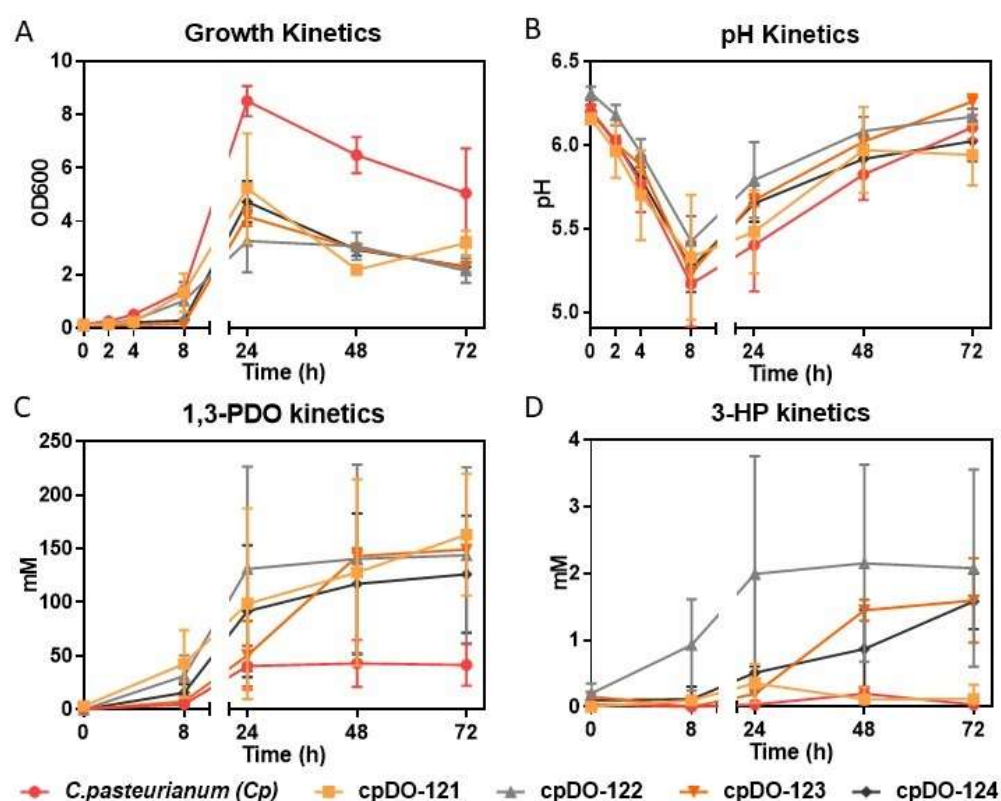


Figure 4.8 | Reducing metabolic burden by expressing both 3-HP pathways from a single plasmid and testing GabD4 variants. **(A-B)** Fermentation phenotype of strains expressing the entire 3-HP pathway as determined by growth and pH kinetics. **(C-D)** Production kinetics for 3-HP and 1,3-PDO formation. Error bars represent standard error for three biological and three technical replicates for production and control strains, respectively.

cpDO-121 = H4 + pDO-21 (pMTL_Q5_82251_ $P_{dhaBL_dhaB_1B_2_pduO}$)

cpDO-122 = H4 + pDO-21 (pMTL_Q5_82251_ $P_{dhaBL_dhaB_1B_2_gabD4}$)

cpDO-123 = H4 + pDO-21 (pMTL_Q5_82251_ $P_{dhaBL_dhaB_1B_2_gabD4i}$)

cpDO-124 = H4 + pDO-21 (pMTL_Q5_82251_ $P_{dhaBL_dhaB_1B_2_gabD4ii}$)

Co-expression of the entire 3-HP pathway on a single plasmid relieved the metabolic burden of harbouring two plasmids as evidenced by the increased growth rates and overall biomass formation relative to the strains harbouring two separate plasmids (Fig. 4.8A). The pH curve also followed that of the wildtype more closely (Fig. 4.8B). Interestingly, an increase in the production of 1,3-PDO was observed, although with large variation between biological clones. The majority of 1,3-PDO was formed in the first 24 h with some clones producing in excess of 200 mM (Fig. 4.8C). Unfortunately, this strategy did not result in increased titres of 3-HP, although 3-HP formation did occur earlier in the fermentation (cpDO-122) compared to the dual plasmid fermentation where 3-HP formation occurred after 48 h. Co-expression of

dhaB₁B₂ and *pduO* (cpDO-121) produced less 3-HP than when the Pdu pathway was expressed on its own. Furthermore, the variants of GabD4 with reported improved catalytic properties, produced less 3-HP than the wildtype variant; this was also an unexpected result (Fig. 4.8D).

To confirm activity of the GabD4 enzymes and if the variants were indeed superior to the wildtype *in vivo*, 24 h cultures were collected, and crude cell lysates prepared. Using propionaldehyde as the substrate, activity of both GabD4i and GabD4ii variants was higher than the wildtype GabD4 (Fig. 4.9). Oddly, the activity of the single substitution variant (GabD4i) was higher than the variant with two substitutions (GabD4ii), which was in contrast to what had been reported in the literature (117). It is possible that the increased activity reported for the variant GabD4ii was specific for the substrate 3-HPA and did not translate to increased activity with propionaldehyde as the substrate. In either case, the fermentation data did not correlate with the enzyme activities; higher 3-HP was produced with wildtype GabD4, but higher enzymatic activity was observed with both GabD4i and GabD4ii variants.

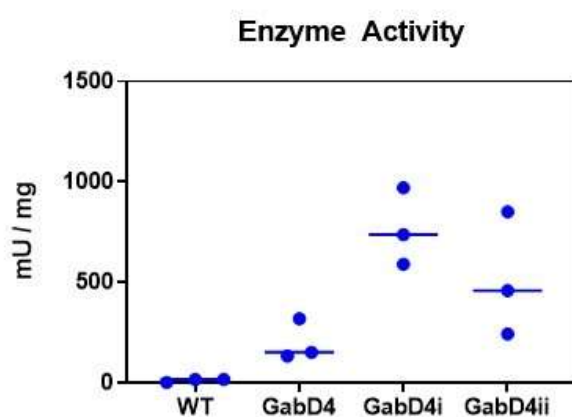


Figure 4.9 | Characterising enzyme activity of GabD4 variants. Crude cell lysates were used to measure enzyme activity by monitoring reduction of NAD⁺ to NADH at 340_{nm} with propionaldehyde as the substrate. The substrate 3-HPA was not commercially available as it is extremely unstable and could not be sourced at the time of this experiment. Cell lysates were prepared from wildtype *C. pasteurianum* and used as the control. The GabD4i variant has the amino acid substitution: E209Q. The GabD4ii variant has two substitutions: E209_269Q.

4.2.6 Metabolic engineering – deletion of *dhaD* and *dhaK* prevents growth on glycerol

In the previous sections, the production of 3-HP was established by expression of the CoA-dependent and CoA-independent 3-HP pathways. Titres of 3-HP were improved by expressing *dhaB₁B₂* with the inducible glycerol promoter in combination with the CoA-independent 3-HP pathway. In attempt to increase titres further while decreasing metabolic burden, both 3-HP pathways and *dhaB₁B₂* were expressed from a single plasmid but this resulted in less 3-HP than when expressing *dhaB₁B₂* and the CoA-independent 3-HP pathway on separate plasmids. Use of the GabD4 variants also did not result in higher amounts of 3-HP. The next step was to explore the increasing the titres and yield of the products through metabolic engineering; this entailed shifting carbon flux towards the reductive branch of glycerol metabolism by reducing carbon flux towards the oxidative branch (Fig. 4.1).

Fermentation of glycerol by *C. pasteurianum* results in the formation of reduced product 1,3-PDO and oxidative products acetate, butyrate, lactate, ethanol, and butanol (Table 4.1). In the wildtype, oxidative products represent $\approx 75\%$ of the total products formed, with 1,3-PDO representing $\approx 25\%$ (Fig. 4.4E, Table 4.2). In strains expressing the CoA-independent 3-HP pathway, product yield of 3-HP and 1,3-PDO increased to approximately 55% of total products (Fig. 4.7E). The enzymes DhaD and DhaK are responsible for catalysing the first two steps required for the oxidation of glycerol and their removal would abolish the ability for *C. pasteurianum* to oxidise and grow on glycerol (Fig. 4.1). The genes *dhaD* and *dhaK*, are found as an operon in the genome of *C. pasteurianum*. A KO cassette was created by cloning regions of homology approximately 500 bp upstream and downstream of the start and stop codons. These two DNA fragments were joined using SOE PCR and cloned into the KO vector pMTL_KS15, which was then methylated and transformed into H4 Δ *pyrE*. Using FOA as a counter selection marker, the mutant Δ *dhaDK* Δ *pyrE* was isolated and confirmed by colony PCR (Fig. 4.10). This mutant had the truncated *pyrE* corrected by using plasmid pMTL_AGH12 and selecting for growth on minimal media without uracil supplemented, generating the mutant Δ *dhaDK*. A strain with chromosomal complementation of *dhaD* and *dhaK* was also created by cloning the same genes including the native promoter into the plasmid pMTL_KS12, generating pMTL_KS12_*dhaDK*. This plasmid allows for rapid integration of the cargo by selection for growth on CBMS agar plates lacking uracil and was previously reported (78).

Transformation of pMTL_KS12_ *dhaDK* into $\Delta dhaDK \Delta pyrE$ followed by selection for integration resulted in the complemented strain $\Delta dhaDK pyrE::dhaDK$, which was evaluated for its growth and fermentation phenotype.

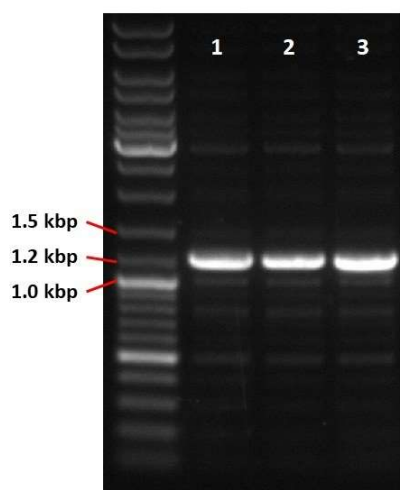


Figure 4.10 | Confirming in-frame deletion of *dhaD* and *dhaK* in H4 $\Delta pyrE$ (H4P5). Screening single colonies for double crossover event and deletion of *dhaD* and *dhaK* following introduction of pMTL_KS12_ *dhaDK* and plating on CBMS agar supplemented with FOA. Single colonies were screened using colony PCR with primers K2 and K4. Expected band size if knockout succeeded = 1.23 kbp. Expected band size if no deletion has occurred = 4.13 kbp.

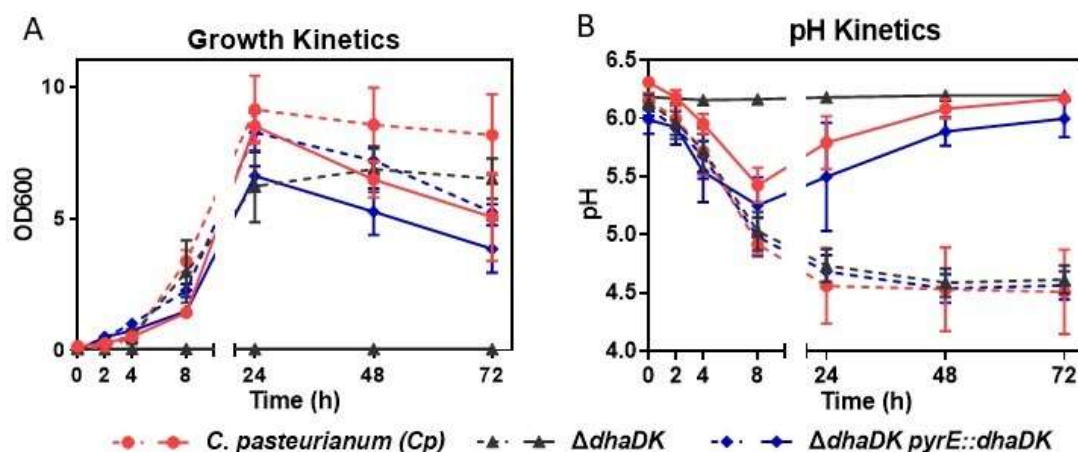


Figure 4.11 | Characterisation of $\Delta dhaDK$ and its complement $\Delta dhaDK pyrE::dhaDK$. An in-frame deletion of *dhaD* and *dhaK* was created using a KO cassette with FOA as a counter-selection marker. Chromosomal complementation of the genes *dhaD* and *dhaK* was then completed by integration at the *pyrE* locus using activation of a selectable marker. Wildtype *C. pasteurianum*, $\Delta dhaDK$ and $\Delta dhaDK pyrE::dhaDK$ were then grown on either glucose (dotted lines) or glycerol (solid lines) and the fermentation phenotype characterised by growth **(A)** kinetics and pH **(B)** kinetics. Error bars represent standard error for two and three replicates for wildtype and mutant strains, respectively.

The mutant $\Delta dhaDK$ was able to grow on glucose, but not solely on glycerol. Chromosomal complementation of *dhaD* and *dhaK* restored the ability for growth solely on glycerol, although overall biomass formation was lower than that of the wildtype (Fig. 4.11A). Fermentation of glucose or glycerol results in different pH curves; growth on glucose results in acidification of the media with no subsequent increase in pH while growth on glycerol results in a decrease in pH until approximately 8 – 12 h followed by an increase back to a pH of 6.0 over the next 24 h. The pH curve of $\Delta dhaDK$ *pyre::dhaDK* had the same pattern as the wildtype (Fig. 4.11B).

The next step was to introduce the 3-HP pathways into $\Delta dhaDK$; as growth solely on glycerol was not possible, glucose was added to provide carbon and energy for growth and biomass formation. To determine a minimal amount of glucose to add, growth curves with decreasing amounts of glucose were completed to evaluate the effect on growth and biomass formation. Almost no difference was observed between using 5% or 6% glucose and a small decrease was observed when glucose was 2% (Fig. 4.12). A large decrease in biomass formation was observed when the glucose amount was reduced to 1.0%, 0.5% or 0.25%. Three factors were considered when deciding which substrate mix to use: (1) a larger amount of glycerol was preferred as it was the substrate being converted to product. (2) Sufficient glucose was required for good biomass formation. (3) The total amount of carbon added to the fermentation needed to add to 6% to allow comparison to other fermentations. Using these factors as a guide, a substrate mix consisting of 2% glucose and 4% glycerol was tested first.

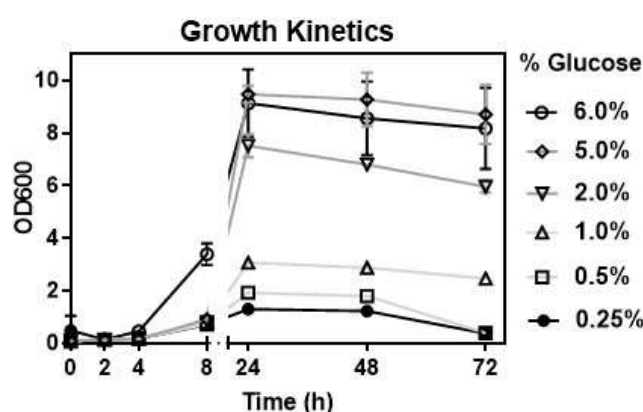


Figure 4.12 | Characterising the growth phenotype of *C. pasteurianum* with varied concentrations of glucose. To evaluate the effect of lower amounts of carbon on growth and biomass formation, decreasing concentrations of glucose were used. One percent of glucose (carbon) is equal to 10 g/L. Error bars represent standard error of three technical replicates.

4.2.7 Fermentation of glucose-glycerol mixtures with the mutant $\Delta dhaD_K$ expressing the 3-HP pathways

The previously assembled plasmids for the expression of *pduO* (pDO-02), *kgs* (pDO-03) and *gabD4* (pDO-04) were again used in this dual substrate fermentation strategy. All three plasmids were transformed into $\Delta dhaDK$. Precultures were grown in 6% glucose and then inoculated into the main cultures (50 ml) containing 2% glucose and 4% glycerol. In these conditions, the wildtype generated more biomass than when grown solely on glucose or glycerol (Fig. 4.13A vs Fig. 4.12). Strains expressing the 3-HP pathways had decreased growth rates although biomass formation was still strong; only cpDO-302 had a long lag phase before growth began (Fig. 4.13A). The pH curve of the wildtype was also the typical curve seen when grown solely on glycerol, a decrease in the first 8-24 h followed by an increase over the next 24 h (Fig. 4.13B). This was in contrast to the pH curves of $\Delta dhaDK$ and the 3-HP expressing strains where the pH dropped in the first 24 h and remained below 5.0, as is typically observed in glucose only fermentation (Fig. 4.13B). Unfortunately, none of the 3-HP pathway expressing strains generated any 3-HP (data not shown). Interestingly, $\Delta dhaDK$ and the 3-HP pathway expressing strains had *negative* glycerol consumption; they generated small amounts (5-15 mM) of glycerol (Fig. 4.13C). Glycerol formation is observed in yeast as means of consuming reducing (NADH) equivalents and is a possible explanation in this case (198). Production of 1,3-PDO was disappointingly low; less was produced than when the wildtype is grown purely on glycerol (≈ 40 mM) (Fig. 4.13D). Formation of 1,3-PDO is the preferred means of recycling reducing equivalents when *C. pasteurianum* is grown on glycerol. A net increase in the amount of glycerol coupled with low production of 1,3-PDO suggests that the reductive branch of glycerol metabolism may not have been expressed, preventing formation of both 1,3-PDO and 3-HP. A possible explanation is that 2% glucose was sufficient carbon for substantial growth to occur and thus genes related to glycerol metabolism were never activated due to catabolite repression. It was hypothesized that a lower percentage of glucose might necessitate activation of genes related to glycerol metabolism and circumvent catabolite repression. Thus, the experiment was repeated using a substrate mixture consisting of 0.25% glucose and 5.75% glycerol.

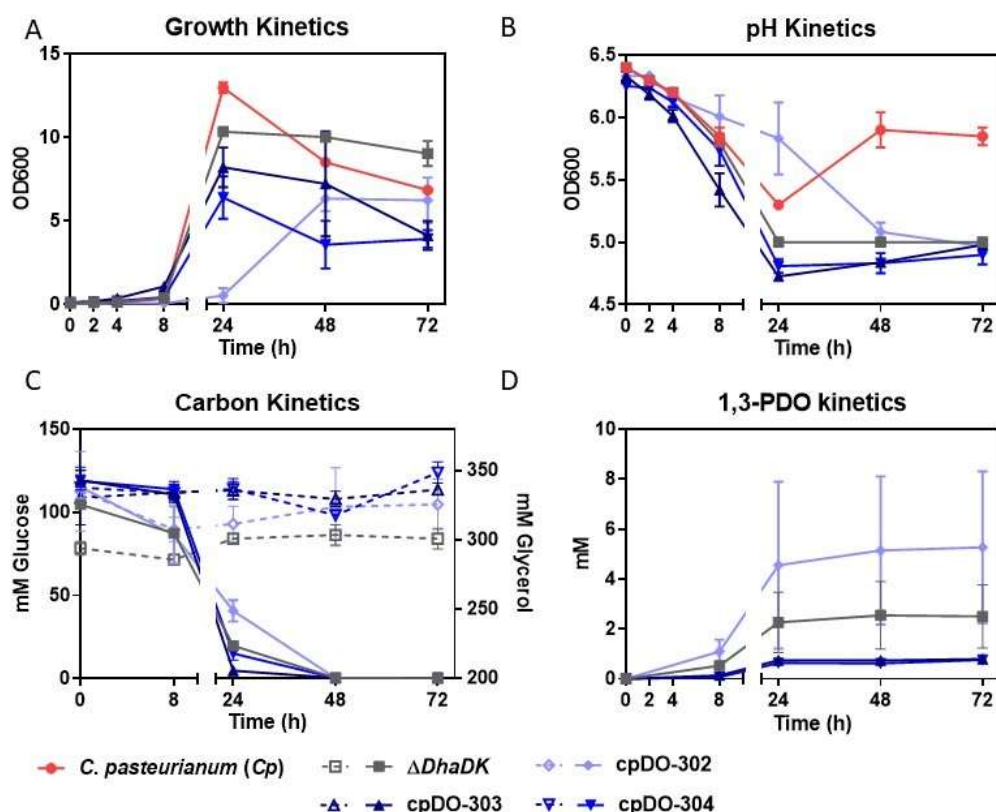


Figure 4.13 | Introducing the CoA-dependent and CoA-independent pathways into $\Delta dhaDK$. As $\Delta dhaDK$ cannot grow solely on glycerol, a carbon mixture consisting of 2% glucose and 4% glycerol was used for the fermentation. Wildtype *C. pasteurianum* and $\Delta dhaDK$ and 3-HP pathway expressing strains were characterised by measuring growth (A) kinetics and pH (B) curve. (C) The consumption kinetics of glucose (solid lines) and glycerol (dashed lines) are shown. (D) Production kinetics of 1,3-PDO. Error bars represent standard error for three biological and two technical replicates for production and control strains respectively.

cpDO-302 = $\Delta dhaDK$ + pDO-02 (pMTL_Q5_82251_pduO)

cpDO-303 = $\Delta dhaDK$ + pDO-03 (pMTL_Q5_82251_kgs)

cpDO-304 = $\Delta dhaDK$ + pDO-04 (pMTL_Q5_82251_gabD4)

Biomass formation was severely limited when only 0.25% glucose was provided, but an OD₆₀₀ reading above one was attained and was deemed sufficient for growth (Fig. 4.12). The fermentation was then repeated with strains cpDO-303 and cpDO-304; strain cpDO-302 was omitted as better co-production had previously been achieved with the CoA-independent pathway. To increase carbon flux towards the 3-HP and 1,3-PDO pathways, *dhaB₁B₂* was expressed in addition to *kgs* and *gabD4*. Plasmid pDO-11 was co-transformed with plasmids pDO-03 and pDO-04 into $\Delta dhaDK$ generating strains cpDO-315 and cpDO-316. The controls, and the four expression strains were then grown in 0.25% glucose and 5.75% glycerol to evaluate for co-production of 1,3-PDO and 3-HP.

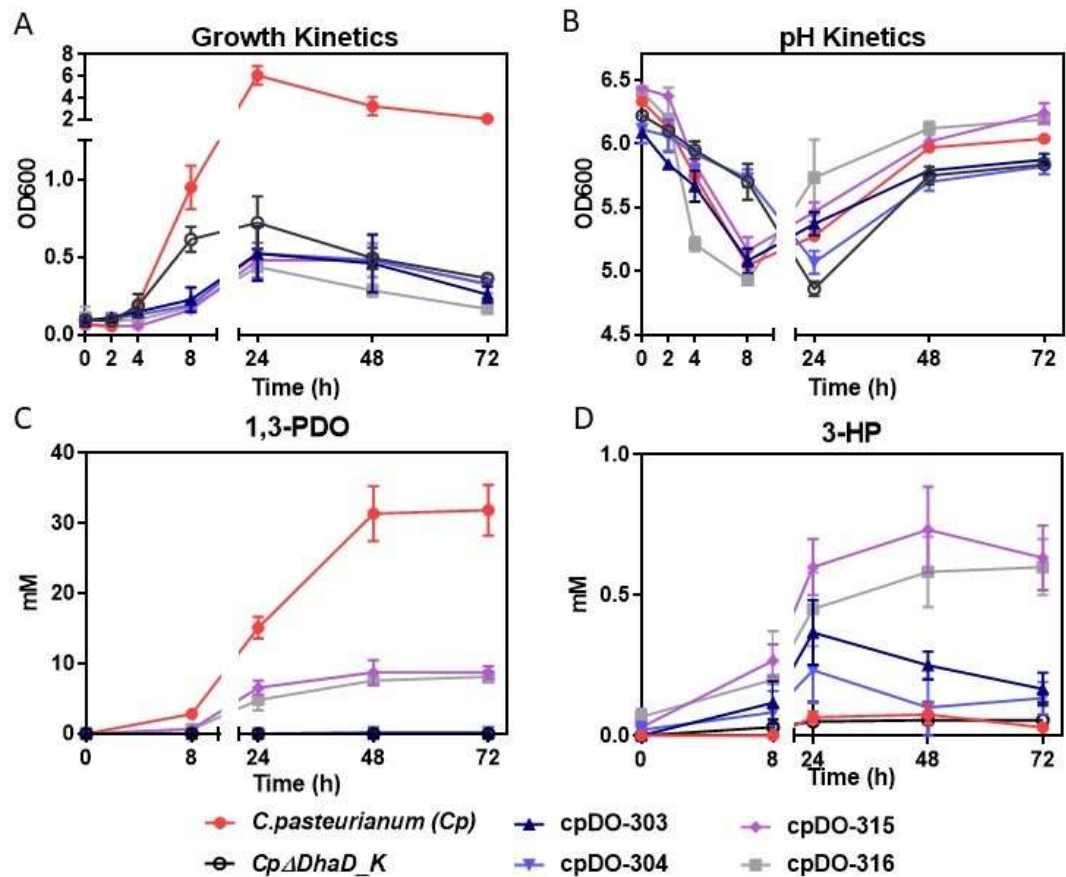


Figure 4.14 | Fermentation of 0.25% glucose and 5.75% glycerol using $\Delta dhaDK$ expressing solely the CoA-independent pathway or in combination with $dhaB_1B_2$. Fermentation phenotype was characterised by measuring growth (**A**) and pH (**B**) kinetics. Production kinetics for 1,3-PDO (**C**) and 3-HP (**D**). Error bars represent standard error for three biological and two technical replicates for production and control strains respectively.

cpDO-303 = $\Delta dhaDK$ + pDO-03 (pMTL_Q5_82251_ *kgs*)

cpDO-304 = $\Delta dhaDK$ + pDO-04 (pMTL_Q5_82251_ *gabD4*)

cpDO-315 = $\Delta dhaDK$ + pDO-11 (Q5_82251_ $P_{dhaBL}dhaB_1B_2$) + pDO-05 (85151_ $P_{fdx}kgs$)

cpDO-316 = $\Delta dhaDK$ + pDO-11 (Q5_82251_ $P_{dhaBL}dhaB_1B_2$)

+ pDO-06(85151_ $P_{fdx}gabD4$)

The metabolic burden associated with expressing two plasmids and the limited glucose available was evident by the slow growth and low biomass formation (Fig. 4.14A). It is unclear why the pH curve of recombinant strains followed that of the wildtype, given that glycerol could not be oxidised. Approximately 8 mM of 1,3-PDO was produced when $dhaB_1B_2$ was co-expressed with *kgs* or *gabD4* compared to 1 mM when the ALDHs were expressed in isolation (Fig. 4.14C vs 4.13D). Given that some 1,3-PDO formation was observed suggests that DhaT was expressed and active. The lack of 1,3-PDO in the strains not expressing $dhaB_1B_2$ suggests that the native DhaB, which is B₁₂-dependent, was not active. Expression of the native *dhaB* is suspected as it is the

first gene in the reductive glycerol operon compared to *dhaT* which is the last gene in the operon. This suggests that perhaps that genes related to vitamin-B₁₂ biosynthesis were not sufficiently expressed to confer activity to the native DhaB and thus, co-expression of the B₁₂-independent *dhaB₁B₂* resulted in production of 1,3-PDO. This is further supported by the increase in 3-HP production in the same strains where *dhaB₁B₂* was co-expressed (Fig. 4.14D).

Some more tinkering with the ratio of glucose to glycerol may have resulted in improved production of 1,3-PDO and 3-HP. For example, using 0.5%, 1.0% or 1.5% glucose with 5.5%, 5.0% or 4.5% glycerol, respectively, may have resulted in better growth, better expression of genes related to glycerol metabolism and thus better conversion of glycerol to products. However, it was hypothesized that catabolite repression would limit the effectiveness of this strategy, and thus this strategy would require further genetic modifications to $\Delta dhaDK$. Given that there are two catabolite repressor proteins (*ccpA1* and *ccpA2*) that were located at different loci, this strategy was abandoned and an alternative strategy was attempted next.

4.2.8 Conversion of glycerol to 3-HP and 1,3-PDO using resting cells of *ΔdhaDK* expressing the Pdu pathway

Up to this point, all experiments for the co-production of 3-HP and 1,3-PDO were completed with growing cultures of recombinant strains. An alternative strategy that has been reported in the literature is the use of resting cells. Resting cells are those cells that are still biochemically active but are no longer growing. In this strategy, growing cultures are harvested by centrifugation, washed with buffer to remove fermentation products and other metabolites and then resuspended in a second buffer containing the desired substrate. This strategy has been used successfully to convert glycerol into 3-HP and 1,3-PDO using resting cells of *L. reuteri*, *K. pneumoniae*, both of which natively express the Pdu 3-HP pathway as well as recombinant resting cells of *E. coli* expressing the Pdu pathway (199–202).

The above reports differ in both the strains used in the bioconversion and in methods used to prepare the resting cells. Thus, a method had to be adapted for *C. pasteurianum* based on what was reported. In general, the methods described in the above works consisted of an initial step where biomass was accumulated, followed by harvesting of the cells, in some cases the cells were washed with 100 mM phosphate buffer pH 7.0, followed by resuspension in a final solution containing glycerol or 3-HPA. The nature of the final solution in which the resting cells were resuspended was inconsistent between the reports. For bioconversion using resting cells of *E. coli*, a simple solution of 100 mM phosphate buffer (pH 7.0) was used, with 3-HPA added directly into the solution (201). In contrast, for bioconversion of glycerol to 3-HP and 1,3-PDO using *L. reuteri* and *K. pneumoniae*, the MRS and M9 media supplemented with glycerol were used (200,202). The MRS and M9 medias are minimal growth medias and thus it is unclear whether growth occurred in the bioconversion step even if resting cells are reported to have been used.

The protocol used here in the preparation of resting cells of *C. pasteurianum* consisted of growing the precultures in minimal media containing a mixture of glucose (0.5%) and glycerol (5.5%) for biomass formation, harvesting cells in mid exponential (OD_{600} 1.0 – 1.5) by centrifugation (10 min at 1500 x g) followed by washing twice with ice cold 100 mM phosphate buffer (pH 7.0), and then resuspension in the bioconversion solution at a final OD_{600} of 2.5 in a volume of 20 ml. The bioconversion solution consisted of minimal media supplemented with 6% glycerol (w/v).

The CoA-dependent 3-HP pathway, consisting of the Pdu enzymes, provides an additional source of energy as 3-HP and ATP in a 1:1 ratio. Co-production of 1,3-PDO and 3-HP is redox balanced and thus full conversion of glycerol to products is theoretically possible (Fig. 4.1). Furthermore, it has also been reported that oxidative products acetate and lactate have inhibitory effects on production of 3-HP by the Pdu pathway (203). Taken together, washing of the cells to remove acetate and lactate accumulated during the biomass production phase, followed by use as resting cells in the mutant $\Delta dhaDK$ would potentially allow for full bioconversion of glycerol to 3-HP and 1,3-PDO. To test this, plasmids containing the PduO pathway (pDO-02) and DhaB₁B₂ with PduO (pDO-21) were transformed into $\Delta dhaDK$ generating strains cpDO-302 and cpDO-321, respectively. These strains were evaluated using the conditions described above.

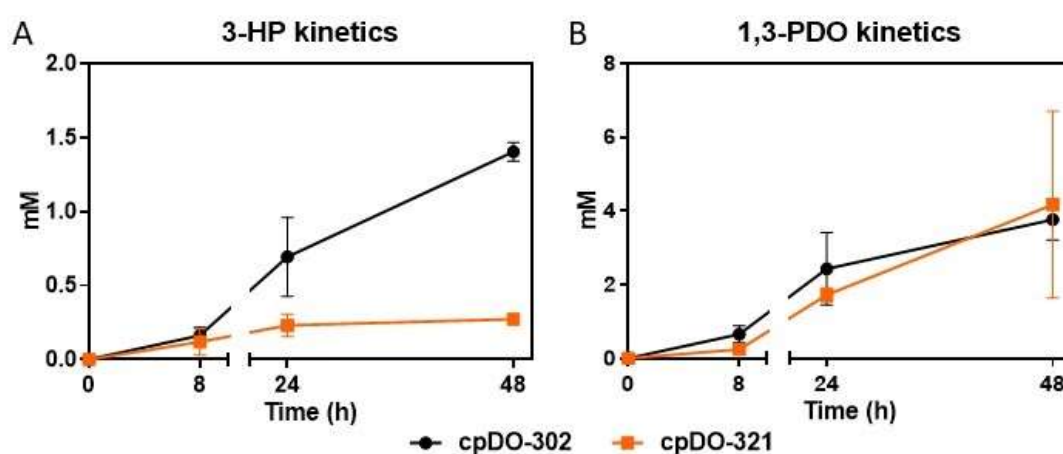


Figure 4.15 | Co-production of 1,3-PDO and 3-HP using resting cells of $\Delta dhaDK$ expressing the Pdu pathway. Cells were grown in a preculture media consisting of 0.5% glucose and 5.5% glycerol, harvested during mid exponential growth, washed with phosphate buffer and resuspended in minimal media supplemented with 6% glycerol. Error bars represent standard error for three biological replicates.

cpDO-302 = $\Delta dhaDK$ + pDO-02 (pMTL_Q5_82251_pduO)

cpDO-321 = $\Delta dhaDK$ + pDO-21 (Q5_82251_P_{dhaBL}_dhaB₁B₂_pduO)

Introduction of the PduO pathway into $\Delta dhaDK$ resulted in 0.5 mM 3-HP after 24 h and approximately 1.5 mM 3-HP after 48 h. Oddly, co-expression of dhaB₁B₂ resulted in decreased titres of 3-HP as only 0.3 mM 3-HP was produced after 48 h. (Fig. 4.15A). Production of 1,3-PDO was unaffected by co-expression of dhaB₁B₂ (cpDO-321) with pduO, as both recombinant strains resulted in approximately 2 mM and 4 mM 1,3-PDO after 24 h and 48 h, respectively (Fig. 4.15B). The use of resting cells resulted in a higher product yield of 3-HP and 1,3-PDO as no oxidative products (acetate,

butanol, ethanol and butyrate) were formed (data not shown). However, in absolute terms, production of 3-HP and 1,3-PDO was quite low; titres were much lower than those achieved when using growing cells. It is unclear why the use of resting cells of *C. pasteurianum* was not effective at converting glycerol to 3-HP and 1,3-PDO, as this had been achieved with native and recombinant strains expressing the Pdu pathway. When a similar approach was taken by Kumar *et al.* high product yields for products 3-HP and 1,3-PDO were attained (87% and 71%) with *K. pneumoniae* in shake flask and fed-batch fermentations (202). Sabet-Azad *et al.* report product yield of 100% when using resting cells of *E. coli* expressing the Pdu pathway, however they supplement 3-HPA directly into the media and used very low amounts of substrate (<10 mM). The supplementation of 3-HPA directly into the media circumvents one of the main steps in conversion of glycerol to 3-HP and thus makes the work somewhat irrelevant in the eyes of this author (201). It is unclear why the resting cells experiment here did not produce a better result as previous works had shown success. What is clear however, is that some unknown factor was preventing efficient bioconversion. Various attempts were made to increase the product yield by expressing the 3-HP pathways in $\Delta dhadK$ and manipulating the fermentation conditions but resulted ineffective. A metabolic engineering approach was again pursued to increase product yield but this time focusing on different targets within the metabolism.

4.2.9 Metabolic engineering – deletion of active thiolase abolishes production of C4 products butyrate and butanol

In the previous sections, metabolic engineering was applied to increase the yield of products 3-HP and 1,3-PDO; deletion of *dhaD* and *dhaK*, the two genes allowing for glycerol oxidation, resulted in the inability to grow solely on glycerol. Although the expression of both the CoA-dependent and CoA-independent 3-HP pathways in $\Delta dhaDK$ resulted in low amounts of 3-HP and 1,3-PDO, these strategies failed to improve on the results attained when the same pathways had been expressed in wildtype *C. pasteurianum*. A second metabolic engineering strategy was tested next by targeting another central carbon node in the metabolism of *C. pasteurianum*.

The oxidative products formed during growth on glycerol can be separated by the number of carbons they contain. The C2 compounds are acetate and ethanol, the C3 compound is lactate and the C4 compounds are butyrate and butanol. The key enzyme that catalyses the first committed step towards formation of the C4 compounds is the thiolase (Thl) enzyme (Fig. 4.1). This enzyme catalyses the Claisen condensation of two acetyl-CoA molecules into acetoacetyl-CoA and is a central node in central carbon metabolism of *C. pasteurianum*. Deletion of this enzyme has not been reported in any Clostridia to date and would be an interesting mutant to isolate as it would result in an altered fermentation phenotype.

The genome of *C. pasteurianum* contains two annotated thiolase genes denoted *thlA1* and *thlA2*, of which the latter is hypothesized to be the active homolog. A knockout cassette was created by cloning regions of homology approximately 500 bp upstream and downstream of the start and stop codons, respectively. These regions of homology were joined using SOE PCR and cloned into the KO cassette pMTL_KS15 which was methylated and transformed into H4 $\Delta pyrE$. Using FOA as a counter selection marker a $\Delta thlA2 \Delta pyrE$ mutant was isolated and confirmed using colony PCR (Fig. 4.16A). This mutant contained a truncated version of *pyrE* which was repaired by transforming pMTL_AGH12 and selecting for auxotrophic growth on CBMS agar without uracil supplemented generating the mutant $\Delta thlA2$ (Fig. 4.16C). Chromosomal complementation of *thlA2* was achieved by cloning *thlA2* with its native promoter into the *pyrE* integration plasmid pMTL_KS12 followed by transformation into $\Delta thlA2 \Delta pyrE$. Selection for autotrophic growth on CBMS agar without uracil supplemented resulted in the strain $\Delta thlA2 pyrE::thlA2$ (Fig. 4.16B). Before introducing the 3-HP

pathways into $\Delta thlA2$, the strain was characterised by evaluating its growth and fermentation profile on glycerol and glucose.

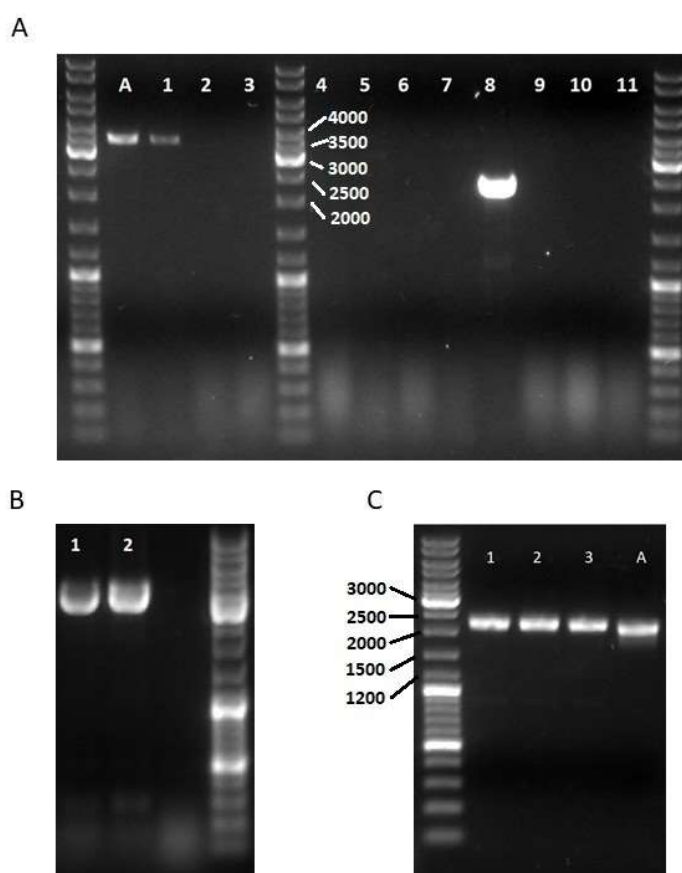


Figure 4.16 | Gels confirming deletion of *thlA2*, chromosomal complementation of *thlA2* and repairing *pyrE* in $\Delta thlA2 \Delta pyrE$. **(A)** Screening single colonies for double crossover event and deletion of *thlA2* using primers 1A and D8 and colony PCR. Wildtype band size = 3.56 kbp, expected band size if knockout = 2.39 kbp. Lane A is positive control PCR using genomic DNA as template, lanes 1-11 were colonies screened. **(B)** The *thlA2* gene and native promoter were chromosomally integrated at the *pyrE* locus. Integration of cargo was confirmed by colony PCR using primers 2R and 9Qb. Lanes 1 and 2 are two colonies that were growing on CBMS agar without uracil confirming restoration of uracil autotrophy. Expected band size = 3.54 kbp. **(C)** The truncated *pyrE* in $\Delta thlA2 \Delta pyrE$ was repaired and colonies were screened by colony PCR using primers 2R and 9Qb. Lanes 1 – 3 three clones observed to be growing on CBMS agar without uracil. Lane A is a positive control PCR using genomic DNA isolated from H4 $\Delta pyrE$. Expected band size for restored *pyrE* = 2.2 kbp. Expected band size for H4 $\Delta pyrE$ = 1.95 kbp.

Deletion of *thlA2* resulted in a slower growth rate and lower overall biomass formation than the wildtype (Fig. 4.17A). This was expected as abolishing butyrate production results in less ATP formation; acetate formation and glycolysis become the only sources of ATP (Fig. 4.1, Table 4.1). The pH curve of $\Delta thlA2$ followed a similar

pattern as the wildtype albeit slower, presumably due to the slower growth rate (Fig. 4.17B). Chromosomal complementation of *thlA2* did not fully restore biomass formation and growth rate although glycerol consumption and 1,3-PDO formation were similar to that of the wildtype (Fig. 4.17C, 4.17D). Interestingly, although less glycerol was consumed by $\Delta thlA2$, formation of 1,3-PDO increased more than 2-fold relative to the wildtype (Fig. 4.17D). This is was an expected result as formation of butanol is abolished and thus formation of 1,3-PDO becomes the only product that results in net consumption of NADH (Table 4.1). Acetate formation was elevated in $\Delta thlA2$ relative to both the wildtype and the complementation mutant. This is expected as acetate formation becomes the main non-glycolytic source of ATP formation. Ethanol formation was increased relative to both the wildtype and complementation strains, which was unexpected as it does not result in net NADH consumption during glycerol fermentation (Table 4.1). Complementation of *thlA2* resulted in restoration in the ability to form C4 products butyrate and butanol (Fig. 4.17E). Only very low amounts of butyrate were observed as it is hypothesized that it is re-assimilated as is the case with *C. acetobutylicum*. The re-assimilation pathway has not been experimentally validated in *C. pasteurianum*. Although not directly relevant to the work contained in this chapter, because $\Delta thlA2$ is an interesting mutant, a glucose fermentation was also completed to observe the fermentation phenotype and to report the results.

Both wildtype and $\Delta thlA2$ strains were grown in minimal media supplemented with 6% (w/v) glucose as had been done with glycerol only fermentations. No C4 products were formed when fermenting glucose, confirming that *thlA2* was the active thiolase in the genome (Fig. 4.18). Again, abolishment of C4 products resulted in increased formation of acetate as it becomes the only product that results in ATP formation in $\Delta thlA2$. Formation of ethanol was also elevated with even higher titres than observed during glycerol fermentation. This was expected as formation of 1,3-PDO is not possible when growing on glucose and thus net consumption of reducing equivalents can only occur during the production of ethanol. Interestingly, when fermenting glucose, the wildtype generates acetone, while $\Delta thlA2$ does not, suggesting that acetone formation may be linked to C4 product formation (Fig. 4.18). Thus, $\Delta thlA2$ may be a good candidate strain in which to study the mechanism of acid re-assimilation in *C. pasteurianum* as it has not yet been elucidated and may be affected by butyrate as is the case with *C. acetobutylicum* (204).

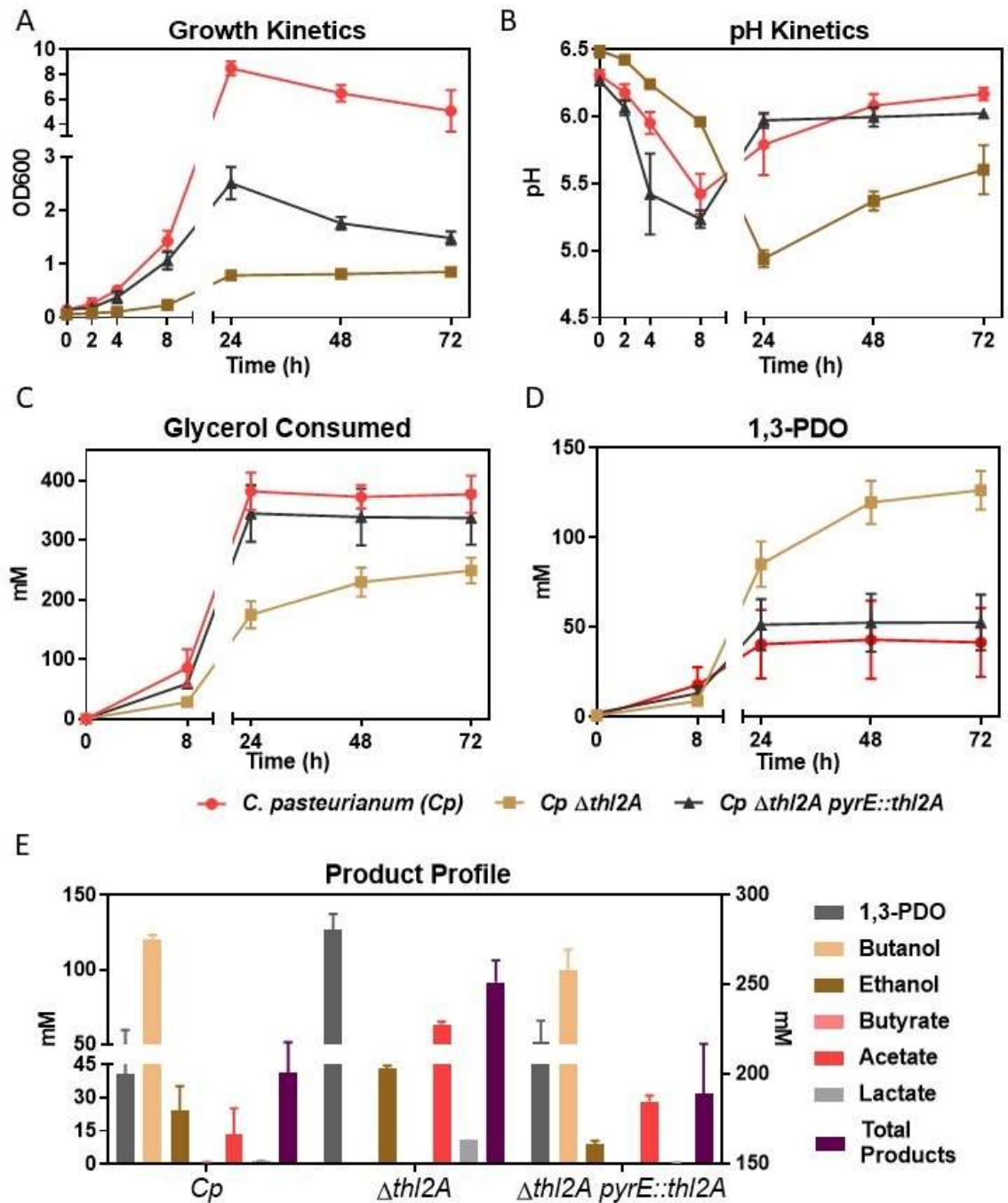


Figure 4.17 | Confirming the role of Thl in host metabolism. To evaluate the effects of deleting the active *thlA2* gene, a strain with an in-frame deletion was created as well as a strain with chromosomal complementation of *thlA2*. The mutant and complemented strains were characterised by growing in Biebl minimal media supplemented with 6% glycerol. **(A)** Growth kinetics, **(B)** pH kinetics, **(C)** glycerol consumed, kinetics of **(D)** 1,3-PDO formation and **(E)** 24 h solvent profile. Product titres shown in **(E)** are end-point data points to allow for better comparison between strains. Error bars represent three technical replicates for wildtype and $\Delta thlA2$ and three biological replicates for complementation strain.

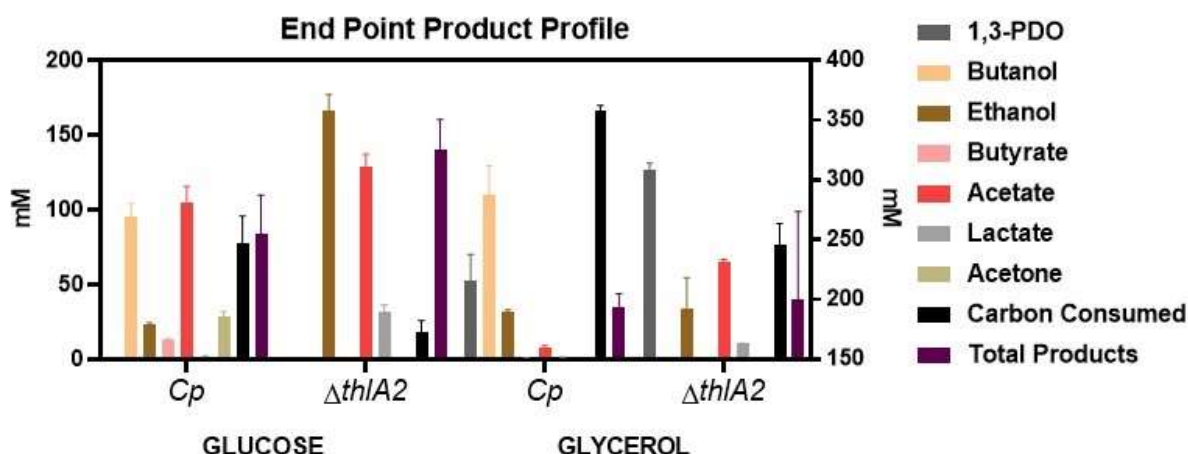


Figure 4.18 | Comparing the glucose and glycerol fermentation product profile of $\Delta thlA2$. Glucose or glycerol were added to the media to a final concentration of 6% (w/v) in a volume of 50 ml of minimal media. Values of products shown were calculated at the fermentation endpoint, meaning when product formation ceased. Individual products are shown on the left y-axis and carbon consumed and total products are shown on the right y-axis. Note that one mole of glucose has 6 carbons while one mole of glycerol has 3 carbons. Error bars represent standard error for two technical replicates. *Cp* = wildtype *C. pasteurianum*.

Lastly, product yield is calculated by dividing total products formed by the carbon consumed and thus can be increased by either increasing the amount of product formed or decreasing the carbon consumed. Since less carbon was consumed by $\Delta thlA2$ relative to the wildtype and product formation increased or was unchanged when growing on glycerol or glucose, respectively, total product yield increased. This is an attractive fermentation phenotype of $\Delta thlA2$ if the target product is not butanol as the fermentation is more efficient; a higher proportion of feedstock (glycerol) is converted into product. Within the context of this chapter, deletion of *thlA2* resulted in increased product yield of 1,3-PDO; production of 1,3-PDO increased and glycerol consumption decreased due to abolishment of C4 products butyrate and butanol. Next, the 3-HP pathways were introduced into $\Delta thlA2$ to evaluate co-production of 3-HP and 1,3-PDO.

4.2.10 Plasmid based expression of Pdu 3-HP pathway in $\Delta thIA2$

Deletion of *thIA2* resulted in a slowed growth rate due to the inability to form ATP through substrate level phosphorylation (SLP) via butyrate. The Pdu pathway results in production of 3-HP and ATP in a 1:1 ratio and was hypothesized to provide a growth benefit if introduced into $\Delta thIA2$ (Fig. 4.1). Expression of both Pdu variant constructs was tested first without co-expressing *dhaB₁B₂* by transforming plasmids pDO-01 and pDO-02 into $\Delta thIA2$ generating strains cpDO-401 and cpDO-402, respectively. Recall, expressing *dhaB₁B₂* in wildtype *C. pasteurianum* resulted in increased 1,3-PDO formation. To evaluate if expressing *dhaB₁B₂* in $\Delta thIA2$ would again cause the same phenotype, plasmid pDO-11 was also transformed into $\Delta thIA2$ resulting in strain cpDO-411. These three strains were evaluated for their fermentation phenotype.

Expression of the Pdu 3-HP pathway in $\Delta thIA2$ resulted in improved growth and biomass formation relative to $\Delta thIA2$. The PduI variant resulted in better growth than the PduO variant and this was also reflected in the pH curve following that of the wildtype more closely (Fig. 4.19A, 4.19B). Titres of 1,3-PDO increased dramatically to 200 mM after 48 h compared to 125 mM after 72 h in $\Delta thIA2$ (Fig. 4.19C). Expression of the PduI and PduO variants of the CoA-independent 3-HP pathway in $\Delta thIA2$ resulted in production of 1.7 mM and 0.9 mM 3-HP after 24 h and 72 h, respectively (Fig. 4.19D). This is an improvement on the titres of 3-HP observed when the Pdu variants were integrated in wildtype *C. pasteurianum*, which resulted in less than 0.5 mM 3-HP produced (Fig. 4.4D). Interestingly, expression of *dhaB₁B₂* in $\Delta thIA2$ did not result in a further increase in formation of 1,3-PDO as was observed when expressed in the wildtype (Fig. 4.19C vs. 4.6D). This was as an unexpected result but suggests that increased expression of glycerol dehydratase (DhaB₁B₂) did not increase carbon flux towards the reductive branch of glycerol metabolism in $\Delta thIA2$. This may have been due to the large flux towards 1,3-PDO formation already observed in $\Delta thIA2$ although this is unknown. It should be noted that although biomass formation ceased after 24 h in the production strains, product formation continued until 48 h and even 72 h. The next step was to test the CoA-independent 3-HP pathway in $\Delta thIA2$.

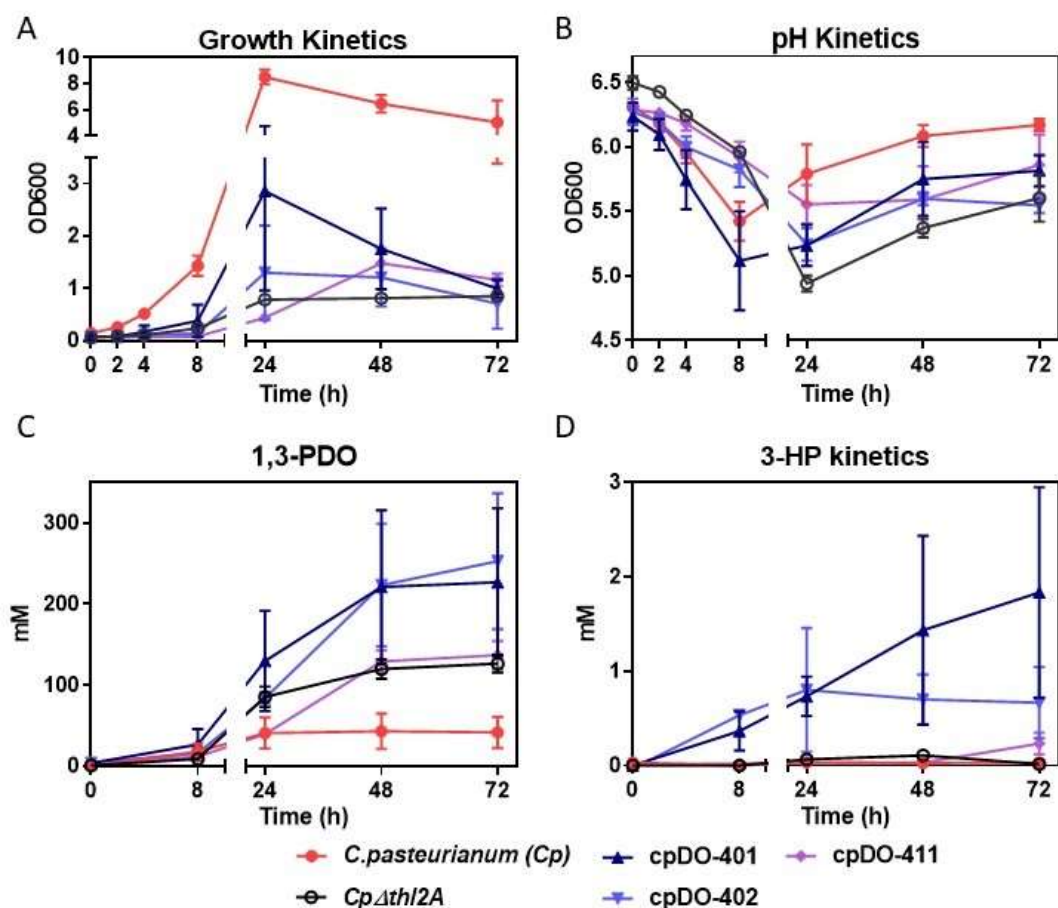


Figure 4.19 | Expression of *dhaB₁B₂* and the Pdu 3-HP pathway in $\Delta thlA2$. Plasmids pDO-01 and pDO-02, harbouring the *pduI* and *pduO* CoA-dependent Pdu pathway were introduced into $\Delta thlA2$ generating strains cpDO-401 and cpDO-402. Overexpression of *dhaB₁B₂* was also tested by introducing plasmid pDO-11 into $\Delta thlA2$ generating strain cpDO-411. Fermentation (A) growth and (B) pH kinetics are shown. (C) Production kinetics of 1,3-PDO and (D) production kinetics of 3-HP. Error bars represent standard error for three technical and biological replicates for control and production strains.

cpDO-401 = $\Delta thlA2$ + pDO-01 (pMTL_Q5_82251_ *pduI*)

cpDO-402 = $\Delta thlA2$ + pDO-02 (pMTL_Q5_82251_ *pduO*)

cpDO-411 = $\Delta thlA2$ + pDO-11 (Q5_82251_ *P_{dhaBL}* *dhaB₁B₂*)

4.2.11 Integration of 3-HP constructs in $\Delta thlA2$ – batch fermentations

Co-expression of *dhaB₁B₂* with the CoA-independent 3-HP pathway resulted in increased production of 3-HP in the wildtype. To test if the same would occur in $\Delta thlA2$, *dhaB₁B₂* was cloned into an operon with *pduO* or *gabD4* under the control of the inducible glycerol promoter. These two constructs were integrated into the *pyrE* locus using activation of a selectable marker (by repair of truncated *pyrE*). To do this, the constructs were cloned into the *pyrE* repair plasmid pMTL_KS12 and transformed into $\Delta thlA2 \Delta pyrE$. Successful integration was selected by streaking clones carrying the integration vectors onto CBMS agar without uracil and isolating growing colonies; integration was confirmed by colony PCR and Sanger sequencing. Two advantages of integrating the 3-HP pathways was that no antibiotic is required in the media and strains typically grow faster, meaning the fermentation times are less. Integration of *dhaB₁B₂* with *pduO* or *gabD4* at the *pyrE* locus in the strain $\Delta thlA2 \Delta pyrE$ resulted in strains cpDO-421 and cpDO-422, respectively. These strains were then tested for co-production of 3-HP and 1,3-PDO in batch fermentations.

Integration strains (cpDO-421 and cpDO-422) demonstrated strong biomass formation as well as rapid acidification of the media (Fig. 4.20A and 4.20B). Unfortunately, integration of the 3-HP pathways resulted in no 3-HP production, presumably due to lower expression of the pathways due to integration (data not shown). Combined expression of *dhaB₁B₂* with *pduO* or *gabD4* resulted in an increase to 1,3-PDO formation; 285 mM and 296 mM of 1,3-PDO was produced after 24 h and 48 h by cpDO-421 and cpDO-422, respectively (Fig. 4.20C). Production of acetate was lower in both cpDO-421 and cpDO-422 relative to $\Delta thlA2$ even though the growth rate and biomass formation were higher; this is odd as formation of acetate generates ATP and thus it would be expected that more acetate would have been generated than in $\Delta thlA2$. Production of ethanol was drastically higher in cpDO-421 and cpDO-422, reaching 198 mM and 252 mM after 24 h, compared to 43 mM in $\Delta thlA2$ and 24 mM in the wildtype (Fig. 4.20D). Although integration of the 3-HP pathways into $\Delta thlA2$ improved titres of 1,3-PDO relative to plasmid-based expression, no 3-HP was produced. The next step was to investigate if process conditions could influence product formation, primarily 3-HP formation. Controlling pH during ABE fermentation is a strategy that has been shown to favour formation of certain solvents and has been shown to influence formation of 1,3-PDO, ethanol and butanol in *C. pasteurianum* (205).

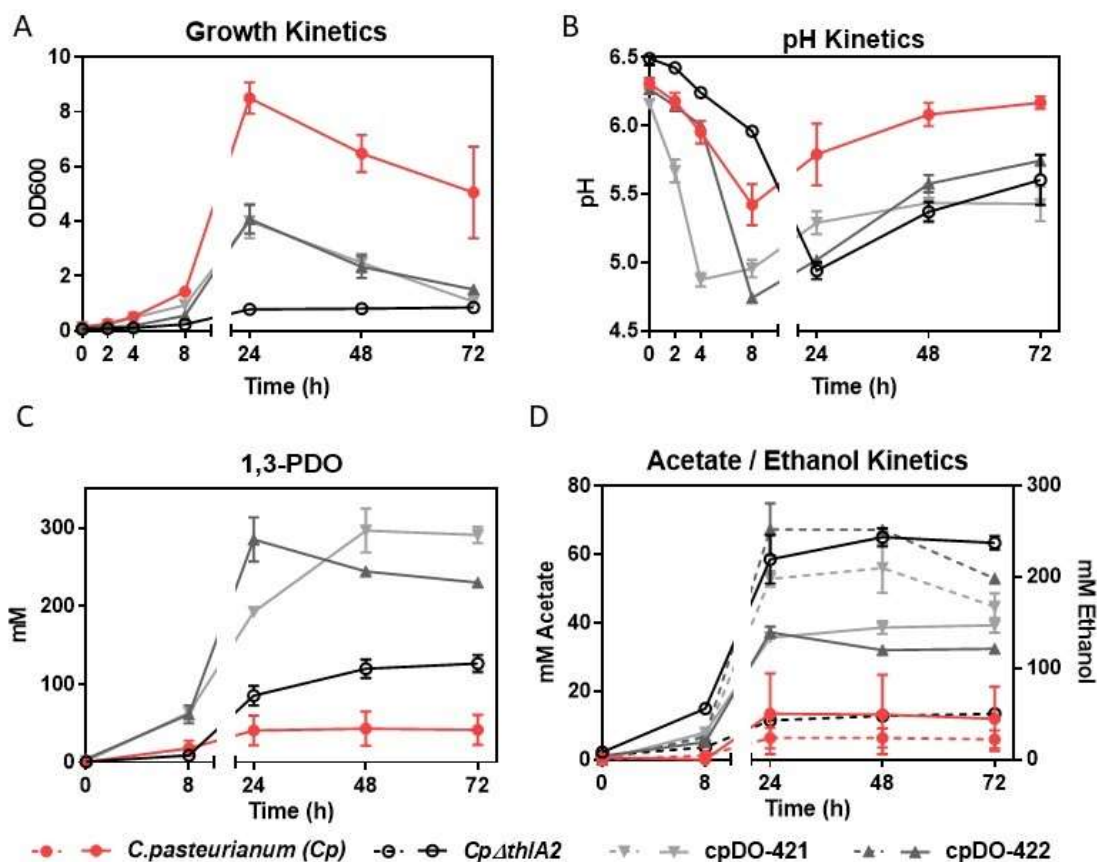


Figure 4.20 | Integration of CoA-dependent and CoA-independent 3-HP pathways in Δ *thlA2*. The *pduO* variant and *gabD4* were cloned into an operon construct with *dhaB₁B₂* and integrated at the *pyrE* locus of Δ *thlA2* and their fermentation phenotype assessed. Fermentation kinetics of (A) growth and (B) pH kinetics are shown. (C) production kinetics of 1,3-PDO. (D) Production kinetics for acetate (solid lines) and ethanol (dashed lines) shown on the left and right y-axis, respectively.
 cpDO-421 = Δ *thlA2 pyrE::P_{dhaBL}_dhaB₁B₂_pduO*
 cpDO-422 = Δ *thlA2 pyrE::P_{dhaBL}_dhaB₁B₂_gabD4*

4.2.12 Process optimisation – using static pH fermentations to control product formation

ABE fermentations are characterised by a rapid acidification of the media due to acid production followed by an increase of the pH of the media as acids are re-assimilated and converted to solvents (16). This characteristic of ABE fermenting organisms can be manipulated to preferentially accumulate acids in the media. This is done by artificially keeping the pH at or close to neutral and thus preventing acidification of the media allowing continued production of acids (91). It was hypothesized that since 3-HP is an acid ($pK_a = 4.87$ compared to acetate $pK_a = 4.75$), formation of this compound may be influenced by the pH of the fermentation in similar fashion to ABE fermenting Clostridia. This had also been demonstrated previously in *E. coli*, where a pH above 6.0 favoured production of 3-HP (139). Thus, fermentations were completed under static pH conditions to investigate the effect of pH on product formation.

Fermentations were carried out with the pH held at three different pH: 6.0, 5.5 and 5.0. A starting pH of 6.2-6.3 was targeted and the fermentation progressed until the target pH was reached, at which point it was artificially held at the target pH by automatic addition of 2M HCL or 4M KOH. Experiments were conducted in 0.5 L fermenters with a working volume of 0.35 L. Strains cpDO-421 and cpDO-422 were used as both 3-HP pathways were integrated meaning the strains were genetically stable and no selection was required; $\Delta thlA2$ was used as the control.

The target pH of 6.0, 5.5 and 5.0 were reached 2 h, 4 h and approximately 6 h into the fermentation. Under all conditions tested, strong biomass formation was observed as a higher OD_{600} was achieved than in batch fermentation, presumably due to the mixing. Biomass formation was highest at pH 5.5 for cpDO-422 and the wildtype, while biomass formation was favoured slightly at pH 5.0 for cpDO-421 (Fig. 4.21A). It is unclear why biomass formation was so low at pH 6.0, as Johnson and Rehmann found that the strongest biomass formation occurred at pH 5.9 and decreased with decreasing pH (205).

Under the conditions tested, pH dependent 3-HP formation was observed; almost no 3-HP produced at pH 5.0 and the most 3-HP was produced at pH 6.0. As had been observed with previous fermentations, the CoA-independent pathway was superior to the CoA-dependent 3-HP pathway for production of 3-HP. Strain cpDO-421,

expressing the PduO pathway produced 2.4 mM and 2.0 mM of 3-HP after 72 h and 48 h at pH's 6.0 and 5.5, respectively. In comparison, strain cpDO-422, expressing GabD4 produced 3.6 mM and 2.2 mM of 3-HP after 24 h at pH 6.0 and 5.5, respectively (Fig. 4.21B).

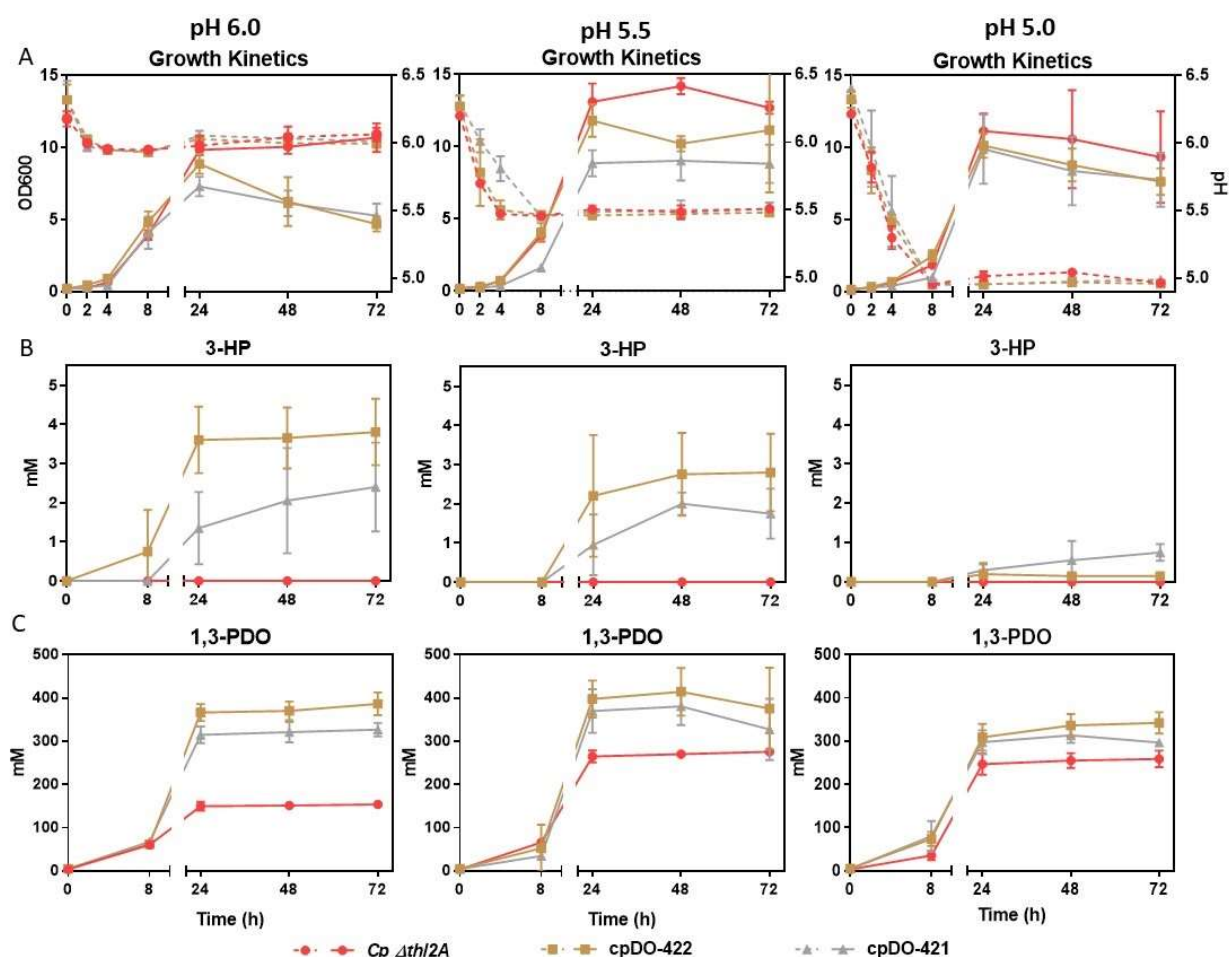


Figure 4.21 | Effects of pH on product formation in $\Delta thlA2$ with 3-HP pathways integrated. Fermentations were carried out in fermenters to allow for online monitoring and control of pH. Biebl minimal media supplemented with 6% glycerol was used and cultures were inoculated targeting a starting OD of 0.1. pH's 6.0, 5.5 and 5.0 were assessed. Fermentation phenotype was characterised by measuring **(A)** growth (solid lines) and pH (dashed lines) kinetics, **(B)** 3-HP production kinetics and **(C)** 1,3-PDO production kinetics. Error bars represent standard error for two technical and two biological replicates for control and production strains, respectively.

Cp = *C. pasteurianum*

cpDO-421 = $\Delta thlA2$ $pyrE::P_{dhaBL_dhaB_1B_2_pduO}$

cpDO-421 = $\Delta thlA2$ $pyrE::P_{dhaBL_dhaB_1B_2_gabD4}$

Production of 1,3-PDO was also shown to be influenced by pH but was not pH dependent. The CoA-independent pathway resulted in slightly higher production of 1,3-PDO than the CoA-dependent pathway at the three pH tested. The highest titres occurred at pH 5.5 with strain cpDO-422 and cpDO-421 producing 398 mM and 370 mM of 1,3-PDO respectively, after 24 h, compared to 265 mM in the control. At pH 6.0, cpDO-422 and cpDO-421 generated 2.4-fold and 2.1-fold more 1,3-PDO than the control, the most in relative terms of all conditions (Fig. 4.21C).

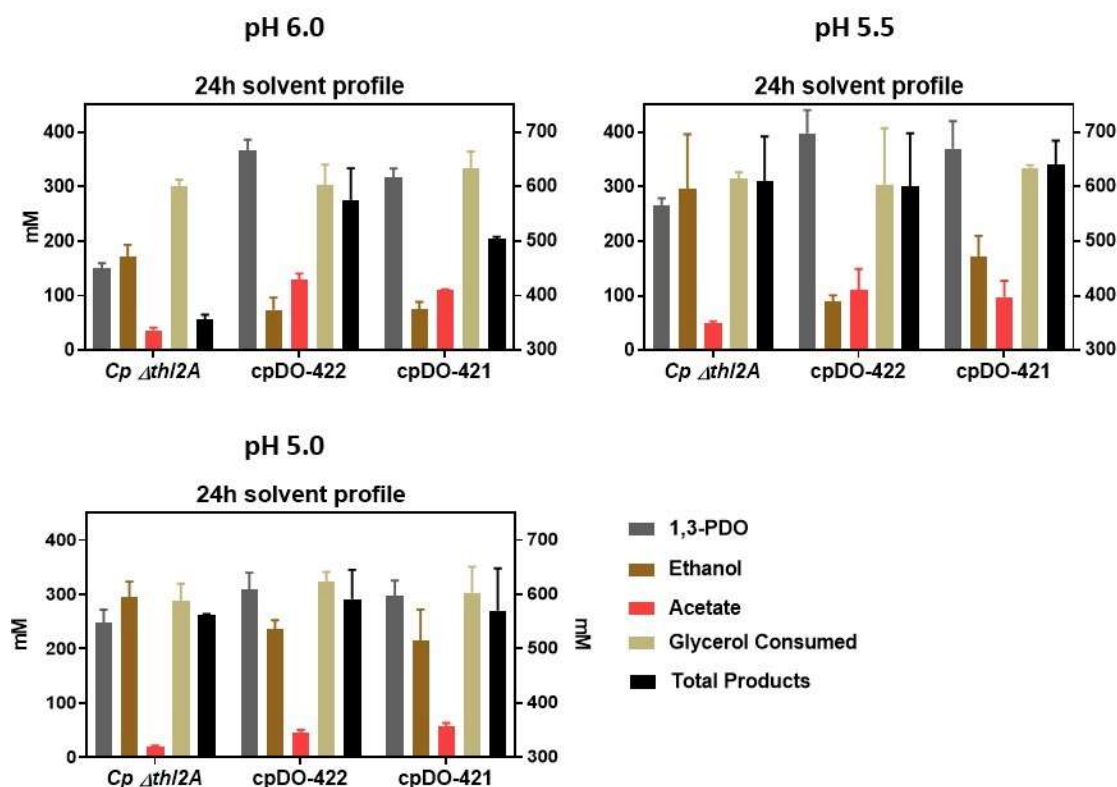


Figure 4.22 | Effects of pH on product profile of $\Delta thlA2$ with 3-HP pathways integrated. Twenty-four-hour solvent profile for cpDO-421, cpDO-421 and $\Delta thlA2$ for fermentation completed under static pH conditions. The data here is from the same fermentation as the data shown in Figure 18. Individual products are shown on the left y-axis, glycerol consumed, and total products formed are shown on the right y-axis. Error bars represent standard error for two technical and two biological replicates for control and production strains, respectively.

Cp = *C. pasteurianum*

cpDO-421 = $\Delta thlA2$ *pyrE*:: *P_{dhaBL}_dhaB₁B₂_pduO*

cpDO-421 = $\Delta thlA2$ *pyrE*:: *P_{dhaBL}_DhaB₁B₂_GabD4*

Although the focus of this experiment was to evaluate the effect on co-production of 3-HP and 1,3-PDO, some effects were observed to acetate and ethanol production and total products formed. Acetate production was favoured at pH 5.5 and 6.0 for control and production. Oddly, although the control ($\Delta thlA2$) generated more

biomass than either production strain, acetate production was lower rather than higher than in production strains. Ethanol formation was also higher in $\Delta thlA2$, even though ethanol results in no net NADH consumption as is the case with production of 1,3-PDO; one would expect that higher biomass formation would result in an increase in 1,3-PDO to maintain redox balance (Table 4.1). Thus, it is unclear how $\Delta thlA2$ generated higher amounts of biomass with lower production of 1,3-PDO. At pH 5.5, total products were almost equivalent to glycerol consumed, meaning product yield was approaching 100% (Fig. 4.22). It is odd that both biomass formation and product yield were both highest at pH 5.5 as it is impossible for complete conversion of glycerol to products occur due to conservation of mass. In general glycerol was completely consumed under the conditions tested, presumably due to improved growth in the bioreactors. Although a starting concentration of 6% glycerol was used, the true starting concentration varied due to human error during prep; the starting amount of glycerol was approximately 650 mM.

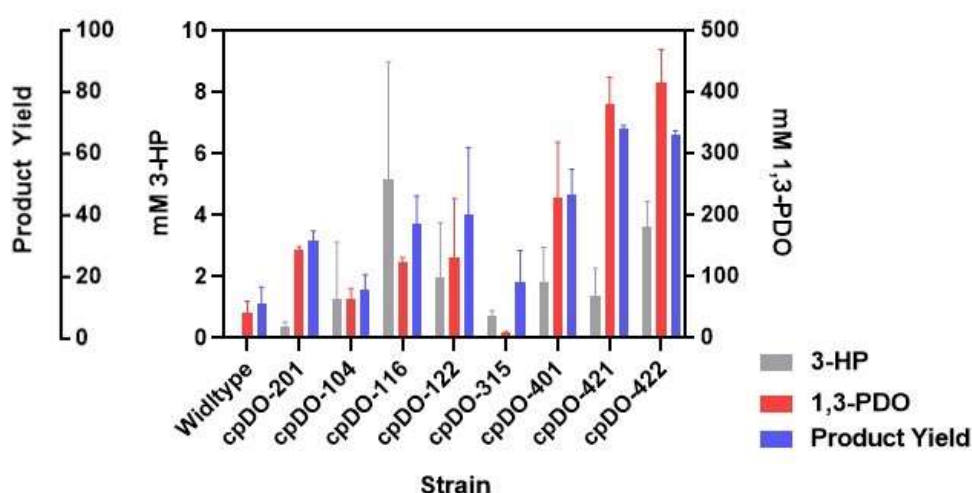


Figure 4.23 | Comparing co-production of 3-HP and 1,3-PDO and product yield between engineered strains and wildtype. A selection of the strains used in this chapter and their respective 3-HP and 1,3-PDO titres and product yield.

In summary, 3-HP, a non-native product was produced in *C. pasteurianum* for the first time. Through a combination of synthetic biology and metabolic engineering approaches, titres of 1,3-PDO were substantially increased over wildtype titres and overall product yield was improved to a respectable 66.6 moles per 100 moles of glycerol. The progression of developed strains generated throughout this chapter and their relative performance are summarised in Figure 4.23. The impacts and significance of the results presented in this chapter are discussed in Chapter 6.

Chapter 5

Whole-cell Biocatalytic Reduction of Ketones to Chiral Alcohols

Project originality and accrediting statement:

The work contained in this Chapter resulted from a collaboration with colleagues from the O'Reilly group in the School of Chemistry whose focus was on enzyme biocatalysis; they are Dr. Harry Eastman and Dr. Andrew Gomm under the supervision of Professor O'Reilly. The three of us, with the support of our supervisors (Professor O'Reilly and Professor Minton), applied for funding to the University of Nottingham Interdisciplinary Centre for Analytical Sciences (UNICAS) and were awarded a small monetary grant to conduct collaborative research. The project results are described below. Although the project written included more enzymes and substrates to test, we were time limited and thus the project was only partially completed. Dr. Gomm aided in the written proposal, Harry provided the plasmid that carried the ADH from *L. kefir* as well as conducted the analytical work and I completed the necessary cloning of the ADH into the expression plasmids, transformed into the strains and cultured the strains according to the conditions being tested. Harry and I were both responsible for experimental design and planning. In my description of the work below, I refer to Harry Eastman, Dr. Gomm and Professor O'Reilly, individually or collectively as 'our collaborators' as required.

5.1 Chapter Introduction

Traditional chemical processes are increasingly being replaced by more environmentally friendly biocatalytic processes as the reactions operate under mild conditions and do not require toxic chemicals. Another motivation for use of biocatalysts over chemical catalysts is the superior regio and stereo-selective catalytic properties, required for the preparation of high value chiral compounds used in the pharmaceutical and specialty chemical industries. Many of these molecules are difficult to manufacture using traditional chemical synthesis, which suffers from poor yields and selectivity. With the onset of modern synthetic biology, the range of applications and the performance of biocatalysts continues to increase.

In both isolated enzyme and whole cell biocatalytic systems, a co-factor such as NADH or NADPH is usually required by the enzyme catalysing the desired reaction. To decrease the cost of expensive cofactor additions, an *in situ* cofactor regeneration system is usually employed. Typically, this involves addition or co-expression of an enzyme that operates in the opposite redox direction thereby regenerating the cofactor, in purified and whole cell systems, respectively. In reality, the mechanism for cofactor regeneration is unimportant as long as it is being regenerated. In Chapter 4, the importance of redox balance was illustrated by the increased formation of the reduced product 1,3-PDO during glycerol fermentation by the mutant $\Delta thlA2$ compared to wildtype *C. pasteurianum*. Presumably, this was due to increased reducing equivalents in the cell due to abolishment of C4 product pathways. It was hypothesised that this metabolic reducing power, in the form of reducing equivalents, could be siphoned for biocatalytic purposes.

The work in Chapter 5 serves as a proof of concept for the use of metabolic reducing power for biocatalysis. The objectives of this chapter are to express an (R)-specific alcohol dehydrogenase from *L. kefir* (LK-ADH) in *C. pasteurianum* and demonstrate that growing *C. pasteurianum* can be used for biocatalysis. Using acetophenone as the substrate, the system was characterised for its ability to convert acetophenone to (R)-1-phenylethanol. Optimisation of the system was carried out by evaluating different biocatalytic conditions before expanding the substrate scope. To confirm the hypothesis that metabolic reducing power can be harnessed for biocatalysis, the biocatalytic performance of the wildtype was compared to the performance of mutant $\Delta thlA2$.

5.2.0 Results

5.2.1 Establishing a *Clostridium* based whole cell system: toxicity, background activity and comparison to the *E. coli* system

In previous studies where different species of *Clostridia* were used for biocatalysis, acetophenone was one of the substrates tested. The concentration of acetophenone used when screening reduction to 1-phenylethanol by cultures of *C. acetobutylicum* and for characterising LK-ADH was 2.5 mM and 10 mM, respectively (167,170,175). However, for industrial applications, it is not uncommon for substrate concentrations to exceed 500 mM (206). Green *et al.* report that when 2.5 mM of acetophenone was used, the growth (as measured by OD) of the culture was reduced to 73% of the control suggesting some inhibitory effects on growth (167). To test the potential toxicity effects of acetophenone, a growth curve was completed using wildtype *C. pasteurianum*; acetophenone was added at the time of inoculation at concentrations of 8 mM and 80 mM and 120 mM and the standard growth parameters OD₆₀₀ and pH were measured.

At a concentration of 8 mM, acetophenone caused a reduced growth rate and a decrease in maximum biomass generated from an OD₆₀₀ of eight to just above four, suggesting that acetophenone was having toxic effects on *C. pasteurianum* (Fig. 5.1A & 5.1B). Cultures of *C. pasteurianum* failed to grow when inoculated with 80 mM and 120 mM of acetophenone suggesting that the maximum amount of acetophenone that *C. pasteurianum* could tolerate at time of inoculation fell between 8 mM and 80 mM.

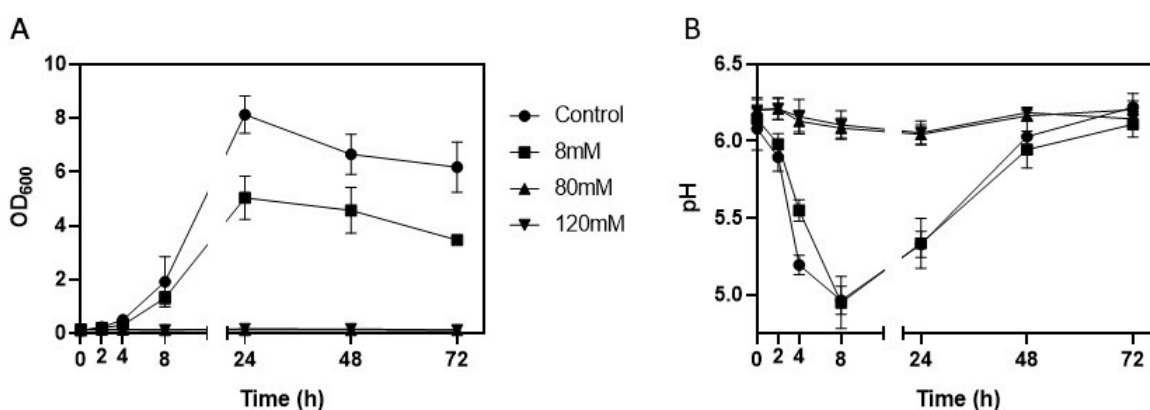


Figure 5.1 | Effects of acetophenone on growing cultures of *C. pasteurianum*. Cultures of wildtype *C. pasteurianum* were inoculated with three different starting concentrations of acetophenone to measure effects on biomass formation (A) and pH (B). Error bars represent standard error for three technical replicates.

The next step was to determine if any native ADHs present in *C. pasteurianum* were able to reduce acetophenone and would compete with LK-ADH. Wildtype cultures of *C. pasteurianum* were again inoculated with a starting concentration of 8 mM acetophenone and time points were taken after 8 h, 24 h, 48 h and 144h for analysis. Neither (R) nor (S)-1-phenylethanol was detected at any time point when using wildtype *C. pasteurianum* suggesting no native ADH was able to reduce acetophenone (Fig. 5.2A). A chiral gas chromatography (GC) column was used to confirm the chirality of any alcohol generated; a GC chromatogram of acetophenone and (R)-1-phenylethanol standards are shown (Fig. 5.2B & 5.2C). This suggested that *C. pasteurianum* did not have any native ADHs that would compete with the heterologous expressed LK-ADH.

To demonstrate the appeal of the Clostridium based system, an improvement relative to *E. coli*-based system had to be demonstrated. The system used by our collaborators was based on the system described by Ivan *et al.* and is a hybrid system; it uses crude cell extracts from lysed cells expressing the ADH of interest (207). This system is simpler than the isolated enzyme systems as it does not require purification of the primary (ADH) enzyme biocatalyst (commercial GDH is readily available) and has been reported by our collaborators in other works due to its effectiveness (208). Our collaborators were able to use this system for complete (> 99%) reduction of acetophenone to (R)-1-phenylethanol by crude cell extracts of *E. coli* as well as establish that LK-ADH could use NADH or NADPH as a cofactor (data not shown). However, this approach is unfavourable due to the requirement for addition of GDH, glucose and the expensive cofactor NADPH. The main appeal of the Clostridium based system is the same as that of all whole cell biocatalyst systems, there is no need for adding exogenous cofactor. To ensure that the Clostridium system was superior to the easier to work with *E. coli* system, the *E. coli* system was tested in the same manner as the Clostridium system. Rather than lyse the cells and add GDH, glucose and NADPH, growing cells of *E. coli* harbouring an expression plasmid containing LK-ADH, were induced and inoculated with acetophenone with time point taken for analysis. Under these conditions, 1% and 3% conversion of acetophenone to (R)-1-phenylethanol was observed after 48 h and 144h, respectively (Fig. 5.2A). To reiterate, the *E. coli* system used by our collaborators used lysed cell extracts and required the addition of GDH and glucose for cofactor regeneration. When using non-lysed whole cells expressing LK-ADH, the *E. coli* system was not able to convert acetophenone to (R)-1-

phenylethanol (data not shown). This makes sense, as GDH is normally co-expressed with the enzyme of interest for effective cofactor regeneration.

Having demonstrated that *C. pasteurianum* did not have any competing ADHs and that the whole cell *E. coli* system did not effectively convert acetophenone to (R)-1-phenylethanol, LK-ADH was next expressed in *C. pasteurianum*. LK-ADH was cloned into plasmids 85151 and Q5_82251 under the control of the previously characterised inducible glycerol promoter generating the expression plasmids 85151_P_{dhaBL}_LK-ADH and Q5_82251_P_{dhaBL}_LK-ADH. These plasmids were transformed into wildtype *C. pasteurianum*, were screened for the presence of each plasmid and used for whole cell reduction of acetophenone to (R)-1-phenylethanol. Growing cultures were inoculated with 8 mM acetophenone and time points were taken after 8 h, 24 h, 48 h and 144 h. Samples were analysed by extraction of the entire solvent layer using ethyl acetate and analysing by gas chromatography (GC).

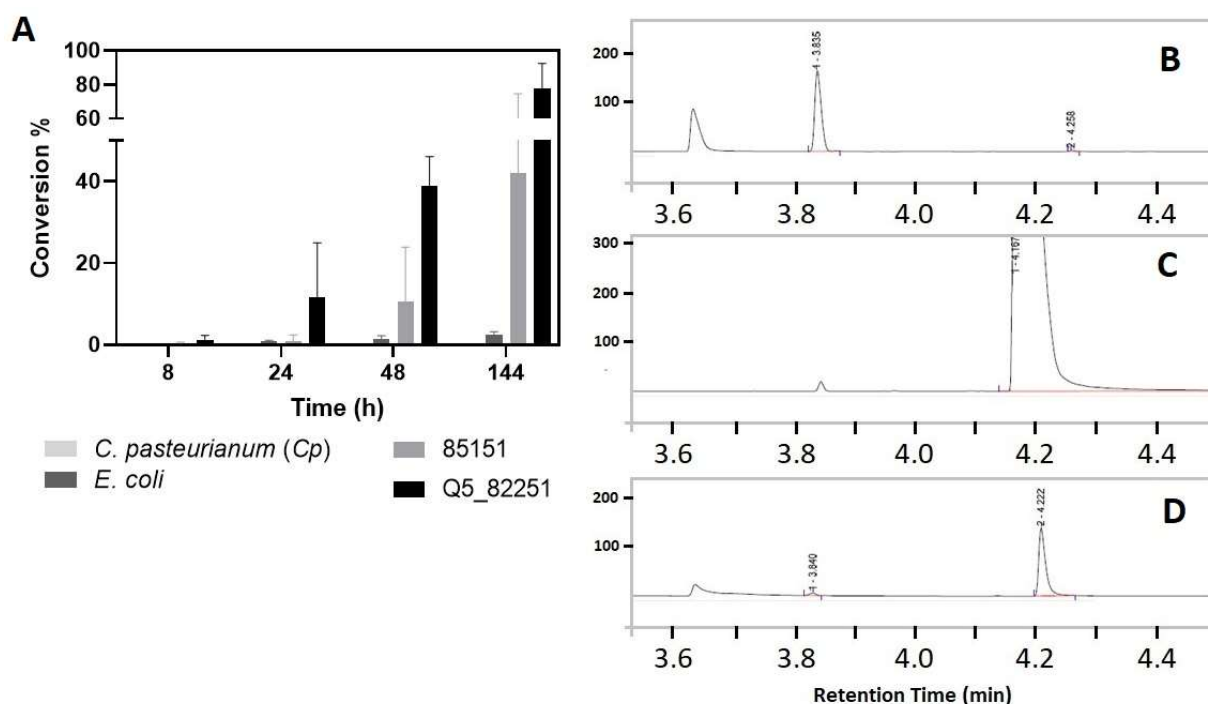


Figure 5.2 | Initial screen for conversion of acetophenone to (R)-1-phenylethanol. **(A)** Time course reduction of acetophenone to (R)-1-phenylethanol using cultures of wildtype *C. pasteurianum*, *E. coli* harbouring LK-ADH and *C. pasteurianum* harbouring either 85151_P_{dhaBL}_LK-ADH or Q5_82251_P_{dhaBL}_LK-ADH. Cultures were inoculated with 8 mM of acetophenone (n = 2). **(B)** Chromatogram for a standard of acetophenone showing a retention time of approximately 3.83 minutes. **(C)** Chromatogram for a standard of (R)-1-phenylethanol showing a retention time of approximately 4.20 minutes. **(D)** Chromatogram of one biological replicate of *C. pasteurianum* harbouring Q5_82251_P_{dhaBL}_LK-ADH analysed after 24 h. Error bars represent standard error for three technical replicates.

Cultures harbouring 85151_P_{dhabL}_LK-ADH converted 1%, 10% and 43% of acetophenone to (R)-1-phenylethanol after 24 h, 48 h and 144h, respectively. Cultures harbouring Q5_82251_P_{dhabL}_LK-ADH converted 10%, 40% and 77% acetophenone to (R)-1-phenylethanol after 24 h, 48 h and 144h, respectively (Fig. 7.22A). An example chromatogram showing conversion of acetophenone to product after 144h by *C. pasteurianum* harbouring Q5_82251_P_{gl}_LK-ADH is shown (Fig. 7.22D). Higher amounts of acetophenone were converted to (R)-1-phenylethanol when expressing LK-ADH from plasmid Q5_82251 than plasmid 85151 and was the plasmid of choice going forward.

5.2.2 Optimising the *Clostridium* system: premixing substrate with ethanol

The results from the initial experiments displayed variance between biological replicates. Qualitatively, it was observed that acetophenone did not readily dissolve into the aqueous layer of the reaction, this was suspected due to the polarity of acetophenone (observation not shown). To evaluate if increased bioavailability of acetophenone would increase the conversion percentage of the biotransformation, premixing of acetophenone with ethanol was explored. Ethanol was chosen as the solvent, as it was natively produced by *C. pasteurianum*. Ethanol and acetophenone (E:A) were mixed in four different ratios (v/v), 1:2, 1:1, 2:1 and 5:1. Four concentrations of acetophenone were tested for conversion to (R)-1-phenylethanol, 10 mM, 20 mM, 30 mM, or 50 mM. Substrate was added at time of inoculation (0h) or after 24 h of growth. Samples were analysed 24 h after the substrate was added and analysed by extraction of the entire solvent layer using ethyl acetate.

When 10 mM of substrate was added at inoculation, the ratio of ethanol to acetophenone did not greatly influence conversion to product, as the four ratios resulted in conversions between 60 – 85%. At 20 mM of substrate, conversion to product was much higher when the ratio of ethanol to acetophenone was 1:1, reaching almost complete conversion. The other ratios resulted in conversions at or below 50%. When substrate was added at time of inoculation, mixing ethanol and acetophenone in a 1:1 ratio yielded the highest and most consistent conversion. Less than 50% conversion of substrate to product was observed at 30 mM acetophenone and no conversion was observed at 50 mM (Fig. 5.3A).

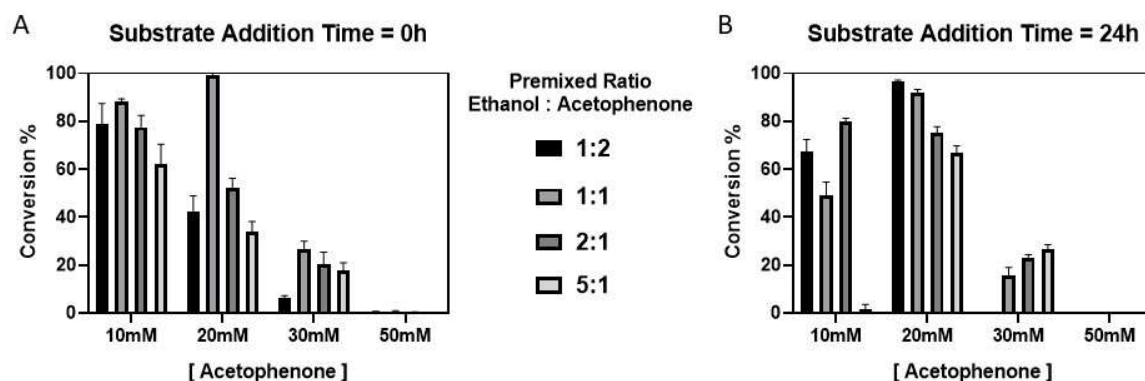


Figure 5.3 | Evaluating effects of premixing ethanol with acetophenone on conversion efficiency. The strain used was wildtype *C. pasteurianum* harbouring Q5_82251_P_{dhabL}_LK-ADH. Ethanol and acetophenone were mixed at four different ratios and added to cultures at time of inoculation (**A**) or after 24 h (**B**) of growth (n = 2). Samples were analysed 24 h after substrate addition. Error bars represent standard error for three technical replicates.

When substrate was added after 24 h of growth, higher conversion was observed with 20 mM acetophenone than 10 mM. When adding 10 mM of acetophenone, the highest conversion was achieved with a ratio of ethanol to acetophenone of 2:1. At 20 mM of acetophenone, the highest conversion was achieved when ethanol and acetophenone were mixed 1:2. In contrast to substrate addition at 0 h where a ratio of 1:1 was optimal, the most consistent results for substrate addition after 24 h were obtained when ethanol and acetophenone were mixed at a ratio of 2:1. Less than 30% conversion of acetophenone was also observed when using 30 mM and no conversion was observed at 50 mM (Fig. 5.3B). Regardless of when substrate was added to the culture or the ratio of ethanol to acetophenone used, a drop in conversion was observed when increasing the concentration of acetophenone from 20 mM to 30 mM, indicating a maximum concentration of acetophenone the system could tolerate. At both addition times (0h and 24 h), >90% conversion of 20 mM acetophenone was achieved when mixed 1:1 with ethanol. As this ratio resulted in the most consistent performance at the highest concentration, it was used as the mixing ratio going forward.

5.2.3 Time of addition affects substrate conversion

Conversion of acetophenone to (R)-1-phenylethanol decreased when the concentration of substrate increased from 20 mM to 30 mM (Fig. 5.3). We next tested whether addition of substrate during mid exponential growth would result in increased conversion at higher concentrations of acetophenone. To compliment this, we first evaluated the effects of substrate addition on growing cultures of *C. pasteurianum* after 8 h and 24 h. Recall, the initial growth curve was completed with addition of acetophenone only at time of inoculation (0 h). To probe whether decreased growth was due to acetophenone or its product (R)-1-phenylethanol, 10 mM and 50 mM of each was mixed 1:1 with ethanol and added to growing cultures.

Addition of 50 mM acetophenone or (R)-1-phenylethanol prohibited growth of *C. pasteurianum* when added at time of inoculation. Addition of 10 mM acetophenone was more detrimental to growth than (R)-1-phenylethanol suggesting that the toxic effects are primarily caused by the substrate and not the product (Fig. 5.4A). In contrast, when both acetophenone and (R)-1-phenylethanol were added after 8 h only the addition of 50 mM (R)-1-phenylethanol caused a decrease in growth while 50 mM of acetophenone had little effect (Fig. 5.4B). When substrates were added after 24 h, no effects on growth were observed (Fig. 5.4C). This may be because biomass production is reduced after 24 h and thus addition of substrate at this point has little effect on further biomass formation. These results demonstrated that addition of substrate had less of an effect on biomass formation if added during mid exponential growth rather than at time of inoculation and almost no effect if added after 24 h.

Whole cell reduction of acetophenone using wildtype *C. pasteurianum* harbouring Q5_82251_Pgl_LK-ADH was next repeated with concentrations of 10 mM, 20 mM, 25 mM, 30 mM and 40 mM acetophenone. The growth experiment showed that *C. pasteurianum* tolerated higher concentration of acetophenone if added during mid exponential (8 h) compared to if added at inoculation (0 h) and thus substrate was added after 8 h. Addition of substrate after 8 h did not affect the concentration of acetophenone that could be reduced to (R)-1-phenylethanol. Approximately 78% and 83% conversion were achieved when 10 mM and 20 mM acetophenone were added after 8 h, respectively (Fig. 5.4D). The conversion of acetophenone at 30 mM was very similar when substrate was added at 0 h and 24 h (approximately 20%) to when added after 8 h (approximately 30%). Furthermore, a linear like decrease in conversion was

observed as the concentration of acetophenone increased from 20 mM to 30 mM and 40 mM. These results suggest that the upper limit or maximum concentration of acetophenone that can be efficiently reduced to (R)-1-phenylethanol when using whole cells of wildtype *C. pasteurianum* expressing LK-ADH is 20 mM.

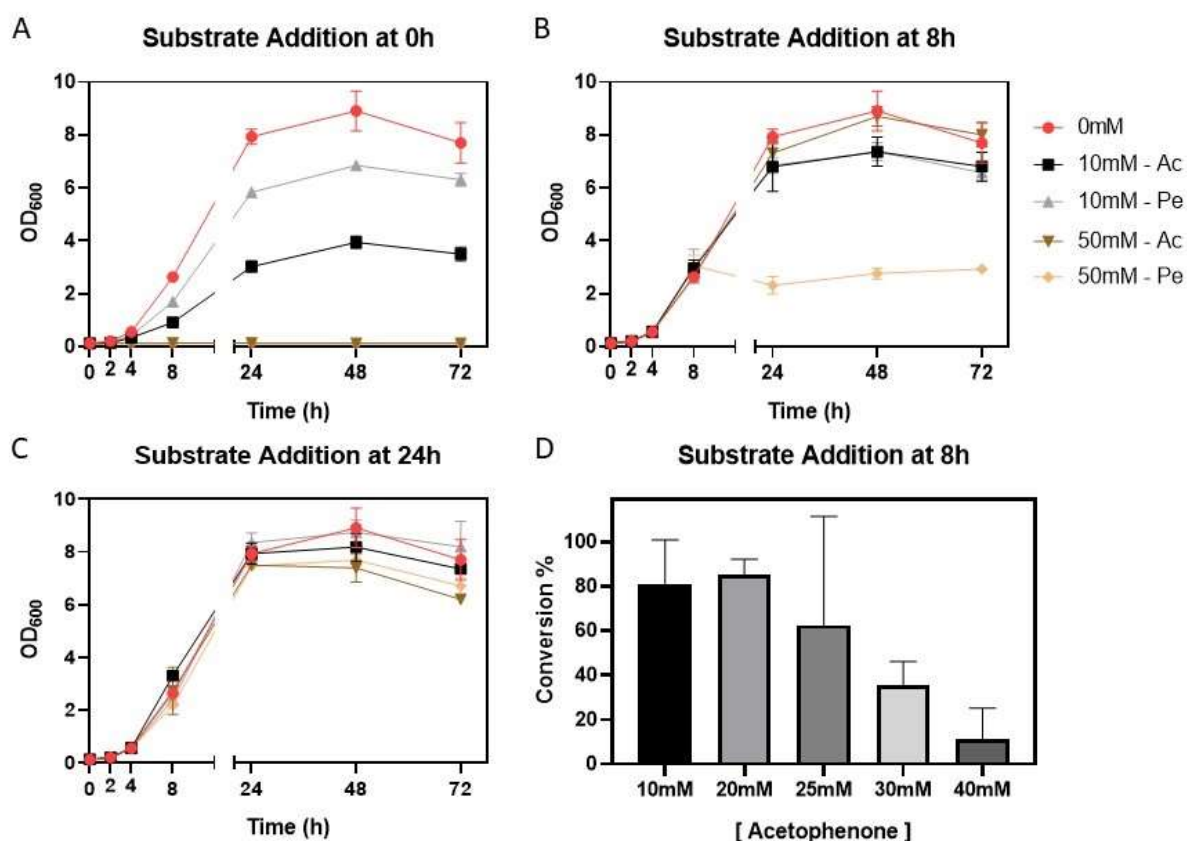


Figure 5.4 | Effects of adding acetophenone or (R)-1-phenylethanol at different time points on growth of wildtype *C. pasteurianum*. Acetophenone or (R)-1-phenylethanol were mixed 1:1 with ethanol and added to growing cultures of wildtype *C. pasteurianum* at time inoculation (**A**), after 8 h (**B**) or after 24 h (**C**). (**D**) Addition of acetophenone (mixed 1:1 with ethanol) after 8 h was tested at increasing concentrations (n = 2). Ac = acetophenone, Pe = (R)-1-phenylethanol. Error bars represent standard error for technical replicates.

5.2.4 Comparing the mutant $\Delta thlA2$ to wildtype *C. pasteurianum* for reduction of acetophenone to (R)-1-phenylethanol

Through optimisation of the system, whole cells of *C. pasteurianum* expressing LK-ADH could reliably reduce 10 mM and 20 mM of acetophenone to (R)-1-phenylethanol achieving > 90% and > 80% conversion when substrate was added after 0 h and 8 h, respectively. The performance of the mutant $\Delta thlA2$ for whole cell reduction of acetophenone to (R)-1-phenylethanol was tested next.

The mutant $\Delta thlA2$ has reduced biomass formation (characterised in Chapter 4) and a reduced growth rate. Addition of acetophenone was shown to be more detrimental for growth and biomass formation in the wildtype if added at time of inoculation (0 h) than if added after 8 h (Fig. 5.4A and 5.4B). Addition after 8 h in the wildtype represents addition during mid-exponential growth, which is an OD₆₀₀ of approximately 0.6 – 1.0. Since the mutant $\Delta thlA2$ has a reduced growth rate compared to the wildtype, it made sense to use OD₆₀₀ instead of time to represent when substrate should be added. Using the mutant $\Delta thlA2$, a growth curve was completed to track the OD₆₀₀ over time. The mutant $\Delta thlA2$ reached an OD₆₀₀ of just below one after 24 h, peaking at an OD₆₀₀ of 1.6 after 40 h (Fig. 5.5A). The growth curve revealed that growth slowed after 24 h suggesting that mid exponential growth occurred slightly earlier between 16 h and 24 h, corresponding to an OD₆₀₀ between 0.4 – 0.9.

Wildtype *C. pasteurianum* was able to convert > 95% of acetophenone at a concentration of 20 mM when added after 8 h, but conversion decreased when the concentration was increased to 25 mM. To test whether the mutant $\Delta thlA2$ could outperform the wildtype for reduction of acetophenone, a concentration of 25 mM was used. Acetophenone was mixed 1:1 with ethanol and added when the growing cultures reached five different ranges of OD₆₀₀ values. When acetophenone was added at OD₆₀₀ values 0.6 – 0.8, 0.8 – 1.0 and 1.0 – 1.2, conversion to product was 90%, 97.5% and 94%, respectively. A decrease in conversion was observed when substrate was added to cultures between an OD₆₀₀ of 1.2 – 1.4 and almost no conversion was observed when substrate was added at an OD₆₀₀ above 1.4 (Fig. 5.5B). These results suggested the optimal OD₆₀₀ to add substrate to cultures of the mutant $\Delta thlA2$ was between 0.6 – 1.2. More importantly, these results showed that the mutant was a more effective biocatalyst than the wildtype strain, as 97.5% conversion was achieved compared to 62.5% in the wildtype at a concentration of 25 mM acetophenone.

To explore the limits of the mutant $\Delta thIA2$ for reduction of acetophenone, increasing concentrations of acetophenone were tested. Acetophenone was mixed 1:1 ethanol at concentrations of 20 mM, 30 mM, 40 mM, 50 mM, 75 mM, 100 mM and 150 mM and added to growing cultures at an OD_{600} between 0.8 – 1.0. The mutant $\Delta thIA2$ was able to convert >97% at a concentration of 20 mM and 30 mM, further demonstrating its superior performance over the wildtype strain. Conversions of 80% and 76% were achieved at concentrations of 40 mM and 50 mM of acetophenone, although with much larger variance. Less than 5% conversion was observed at concentrations of 75 mM, 100 mM and 150 mM (Fig. 5.5C). These results suggest that the upper limit of acetophenone that the mutant $\Delta thIA2$ expressing LK-ADH can reliably convert to (R)-1-phenylethanol is 30 mM, a 50% increase over the wildtype.

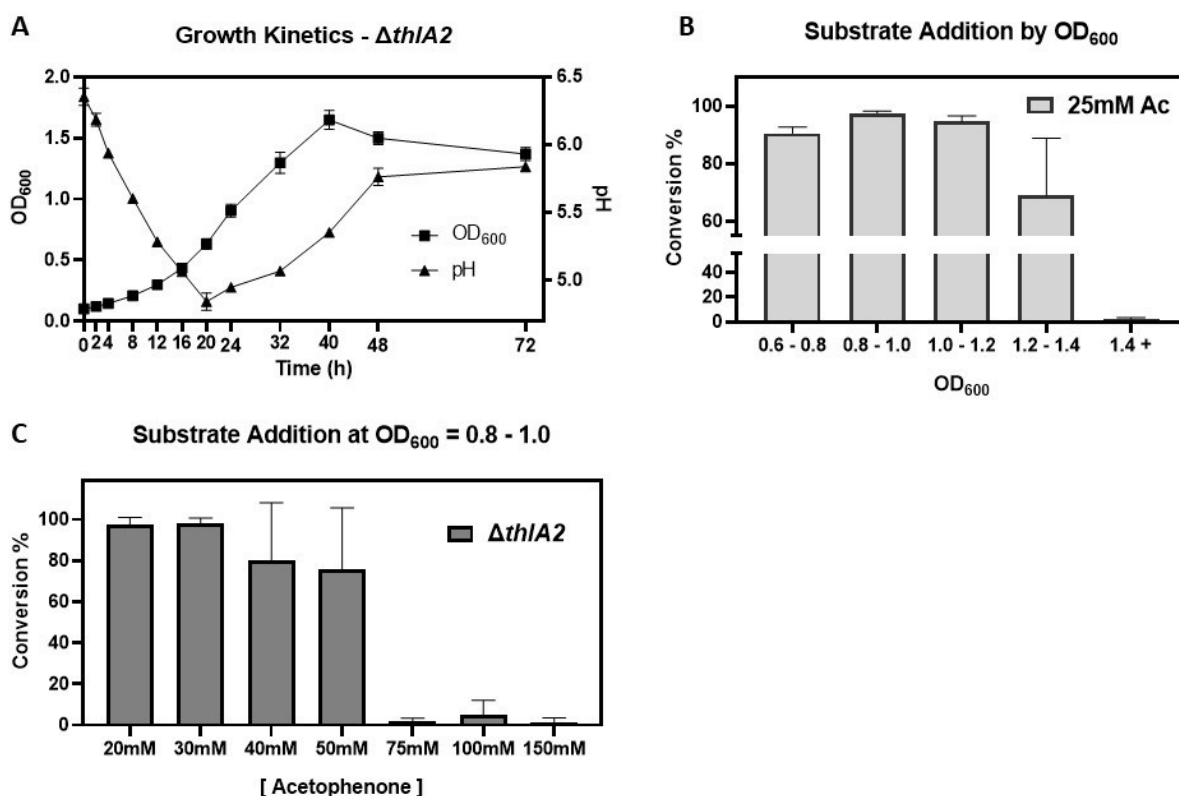


Figure 5.5 | Characterising the performance of the strain $\Delta thIA2$ harbouring LK-ADH for whole cell reduction of acetophenone to (R)-1-phenylethanol. (A) Growth and pH curve of the mutant $\Delta thIA2$ (B) Addition of 25 mM acetophenone mixed 1:1 with ethanol was added according to OD_{600} values rather than time. (C) Addition of increasing concentrations of acetophenone to growing cultures of $\Delta thIA2$. (n = 2). Error bars represent standard error for three technical replicates.

The large variance in conversion achieved at 40 mM and 50 mM, could potentially be reduced through further process optimisation. Regardless, the results demonstrate the superior ability of the mutant $\Delta thlA2$ to reduce acetophenone compared to the wildtype. Although the values of substrate here are 10% of those used in systems using lysed cells or isolated enzymes, these values are comparable (30 mM vs 35 mM) to systems where whole cells are immobilized and represents a first step in the development of *C. pasteurianum* as a whole cell biocatalysts (209,210). To establish *C. pasteurianum* as a platform for whole cell biocatalysis of chiral alcohols, the product spectrum was broadened to include other aromatic ketones.

5.2.5 Expanding the substrate scope to aromatic ketones

Two similar compounds to acetophenone are benzaldehyde and phenylacetone, the former being the simplest aromatic aldehyde consisting of a formyl substituent on a benzene ring and the latter consisting of benzene with an attached acetone substituent, which is the simplest two carbon ketone. Reduction of benzaldehyde and phenylacetone results in the formation of the non-chiral compound phenylalcohol and the chiral compound 1-phenylpropan-2-ol, respectively. Whole cell reduction of benzaldehyde and phenylacetone was explored using wildtype *C. pasteurianum* expressing LK-ADH; concentrations of 20 mM, 30 mM and 40 mM of substrate were added at time of inoculation and after 8 h.

Addition of 20 mM, 30 mM and 40 mM of phenylacetone at time of inoculation resulted in 99%, 97% and 57% conversion, while addition after 8 h resulted in 43%, 16% and 0% conversion to product, respectively. Addition of 20 mM, 30 mM and 40 mM of benzaldehyde at time of inoculation resulted in 97%, 98% and 0% conversion, while addition after 8 h resulted in 91%, 92% and 0% conversion to product, respectively (Fig. 5.6A, 5.6B). For the substrate phenylacetone, conversion was favoured when added at time of inoculation up to a concentration of 30 mM. For the substrate benzaldehyde, conversion to product was favoured when added at time of inoculation up to a concentration of 30 mM. The results suggest that *C. pasteurianum* can tolerate benzaldehyde and phenylacetone to slightly higher concentrations than acetophenone and that the system may be used to reduce other aromatic ketones and thus is not specific to acetophenone. Due to time constraints, these substrates were not tested with the mutant $\Delta thlA2$.

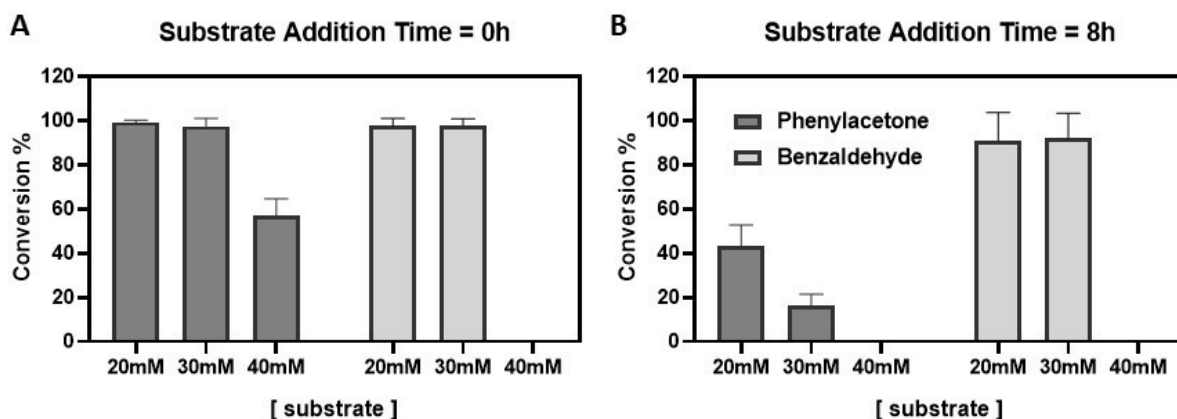


Figure 5.6 | Expanding substrate scope for whole-cell biocatalytic reduction using wildtype *C. pasteurianum* expressing LK-ADH. The substrate scope of the *C. pasteurianum* whole cell system was expanded by testing a range of concentrations of aromatic ketones phenylacetone and benzaldehyde by addition at time of inoculation (A) and after 8 h of growth (B). (n = 2). Error bars represent standard error for three technical replicates.

The results presented in this chapter represent an extension of the initial work in the 1990s which explored the use of clostridia for whole cell biocatalysis. Current synthetic biology tools and techniques were applied for the development of *C. pasteurianum* as a chassis for whole cell biocatalysis. This work also demonstrates that metabolic reducing power can be used to drive biocatalytic redox reactions. The significance and potential future directions of this work is discussed in Chapter 6.

Chapter 6

General Discussion

6.1 Overview of thesis

At the end of the introduction (Chapter 1) in section 1.3, the general research objectives of this thesis were described, which were to further our understanding of *C. pasteurianum* and to develop new tools and strains that are of industrial relevance. These objectives were pursued through research focused on three areas: (1) improving the TE of *C. pasteurianum* through genetic modification, (2) engineering of *C. pasteurianum* to produce a non-native product for the first time, and (3), applying the tools developed and engineered strains of *C. pasteurianum* towards whole cell biocatalysis, the results of which were presented in Chapters 3, 4 and 5, respectively. In this chapter, the limitations, significance, and future directions of the results described in the results chapters are discussed.

6.2 Discussion on improving TE through successive deletion of RM systems in *C. pasteurianum*

The aim of the work completed Chapter 3 was to increase the TE of *C. pasteurianum* by modification of the host genome. The TE achieved in Chapter 3 is the highest reported for *C. pasteurianum* and was enabled by successive deletion of the Type II and Type I RM systems CpaAIP and CpaAII. Although an improved TE of 5.67×10^5 CFUs/ μ g DNA was achieved with the plasmid pMTL_85151, this TE still falls short of that achieved in *C. acetobutylicum* (1.0×10^6 CFUs/ μ g DNA) and 1000-fold lower than that achieved in *E. coli* (1.0×10^9 CFUs/ μ g DNA) (Fig. 3.1A). Interestingly, a higher TE was achieved with the methylated control plasmid compared to the unmethylated version (Fig. 3.5A). This suggests that other RM systems or methylation patterns are likely influencing the TE in *C. pasteurianum*. This conclusion was also shared by Pyne M., who published the transformation protocol utilised in this work and who extensively evaluated various factors influencing TE in *C. pasteurianum* (96,211). The only remaining active RM system in $\Delta pyrE \Delta cpaAIP \Delta cpaAII$ is the uncharacterised Type IIG / N6-adenine RM system and represents a logical target for subsequent deletion. To create the mutant $\Delta pyrE \Delta cpaAIP \Delta cpaAII$, three successive genome modifications were made, with the deletion of the of the Type IIG / N6-adenine requiring a fourth modification. Although less laborious and faster methods of genome modification such as CRISPR have been reported in *Clostridium*, development of multiplex engineering has been hindered due to a lack of recombineering and non-homologous end joining

(NHEJ) tools (94,138,193). These tools would allow for much faster genome modification and without the need for successive procedures that results in tens or hundreds of generations from the original strain.

The mutant $\Delta pyrE \Delta cpaAIP$ generated in this work allows the user to transform *C. pasteurianum* without *in vivo* methylation, providing a more streamlined and simplified workflow for applicative or research purposes. By not repairing the truncated *pyrE* in this strain, future researchers also have a locus in the genome whereby cargo DNA can easily and quickly integrated (184). Such a tool is very useful for the development of a commercially relevant host as the supplementation of any compound (antibiotic, inducer or precursor) for plasmid retention or pathway expression becomes cost prohibitive at large scale (212). Furthermore, the widespread use of antibiotics has been linked to the rise in antimicrobial resistance, and thus alternative methods of selection are being encouraged for use in biotechnology (213–216).

The increase in TE achieved here required the successive deletion of three genes: *pyrE*, *cpaAII* and *cpaAIP*. This work took several months and was laborious. An alternative to increasing TE by deleting RM systems from the genome, is to adapt the DNA being transformed to mimic the host's own, thus evading the RM systems. With this strategy, the transformed DNA is first methylated to mimic the host methylation pattern. Indeed, this is the method used to circumvent the Type II CpaAIP RM system in *C. pasteurianum*; plasmids are propagated in an *E. coli* strain co-harboring a plasmid expressing the M.BepI methyltransferase providing *in vivo* methylation and thus protecting the DNA from the Type II CpaAIP RM system (96,98). This strategy can be enhanced by expressing multiple methylases simultaneously, which led to an improvement in conjugation efficiency up to 1.0×10^4 in the case of *Geobacillus kaustophilus* (217). A similar strategy was also applied to improve the TE by electroporation of *Bifidobacterium adolescentis* up to 5000-fold (218). A simple and logical progression from these ideas would be to integrate all methylases required for the methylation pattern for a strain of interest into an *E. coli* cloning strains such as TOP10 rather than expressing multiple methylases on one or more plasmids. Integration of multiple relevant methylases into a cloning strain of *E. coli* for example would result in a methylation donor strain in which shuttle vectors could be cloned, propagated and protected against active RM systems of the target host. This would streamline the workflow and would have the added benefit that the user would not

obtain a mixture of plasmids following plasmid preps and is a strategy that is currently being explored in the CRG (219). This strategy would provide a more elegant solution negating the need for extensive genome modification to increase the TE in *C. pasteurianum* further.

6.3 Discussion on co-production of 3-HP and 1,3-PDO from glycerol

In Chapter 4, *C. pasteurianum* was engineered to produce a non-native product for the first time by introduction of two pathways, a CoA-dependent and CoA-independent 3-HP pathway. Two mutants, $\Delta dhaDK$ and $\Delta thIA2$ which have never been reported for Clostridia or *C. pasteurianum* were also generated and characterised, confirming the roles of the enzymes, and furthering the understanding of host metabolism.

Through a combination of synthetic biology and metabolic engineering, two final strains (cpDO-421 and cpDO-422) were generated for co-production of 3-HP and 1,3-PDO, resulting in a 9-fold increase of 1,3-PDO relative to the wildtype and an increase in product yield from 11.4 moles to 66 moles of product per 100 moles of glycerol. Still, two general observations from the results could not be explained: (1) although various efforts were made to improve the production of 3-HP, titres remained persistently low with both 3-HP pathways (< 10 mM) and (2) the enzymes introduced, although intended for production of 3-HP, instead resulted in large increases to 1,3-PDO. Although these results were observed for both CoA-dependent and CoA-independent pathways, they will be discussed separately.

6.3.1 Probing potential causes for the low production of 3-HP

The enzymes GabD4 from *C. necator* and the KGSADH from *A. Brasilense* were tested as they had previously been reported to produce upwards of 400 mM 3-HP using glycerol as a substrate (116,117). In both studies enzymes were purified, characterised and shown to have high specific activity (k_{cat} / K_m) with NAD⁺ as a co-factor and using 3-HPA as a substrate, suggesting their effectiveness for catalysing the oxidation of 3-HPA to 3-HP *in vivo*. To ensure strong flux towards the 3-HP and 1,3-PDO pathways and availability of the upstream metabolite 3-HPA, the continued activity of glycerol dehydratase (DhaB or DhaB₁B₂) is essential (Fig. 4.1). This requires the addition of cobalamin (vitamin-B₁₂) and expression of the glycerol reactivating factor (GdrA) if a B₁₂-dependent DhaB is used. Rathnasingh *et al.* demonstrated the

importance of DdrA for increasing 3-HP production when using KGS and DhaB, as its expression was crucial for the continued activity of DhaB (110,116,120). A homolog of GdrA, GdrAB from *K. pneumonia* was included in the work utilising GabD4 (117). Low flux in the conversion of glycerol to 3-HPA was not expected to be the cause of low 3-HP production observed here as *C. pasteurianum* natively produces cobalamin and DdrA (homolog of DdrA). Furthermore, wildtype *C. pasteurianum* has previously shown strong flux towards the reductive glycerol metabolism as evidenced by strong formation of 1,3-PDO (78,80). Lastly, expression of the B₁₂-independent glycerol dehydratase *dhaB₁B₂* from *C. butyricum*, was effective at increasing titres of 1,3-PDO (Fig. 4.5D) (73,80,114). Poor *in vivo* activity of DhaB or DhaB₁B₂ would have resulted in a low *in vivo* pool of the upstream metabolite 3-HPA, another potential cause of poor titres of 3-HP. Again, this was unlikely as 3-HPA, was the same upstream metabolite of 1,3-PDO, and titres of 1,3-PDO reached upwards of 350 mM, suggesting strong flux through this pathway (Fig. 4.20C). It continues to remain unclear why production of 1,3-PDO increased substantially when enzymes specific for conversion of 3-HPA to 3-HP were introduced.

There are two main differences between the above studies using GabD4 and KGSADH and the work reported here. First, the fermentations in the previous studies were completed under aerobic or microaerobic conditions while the work presented here was completed under completely anaerobic conditions. Second, the aforementioned studies utilised glucose and glycerol as co-substrates, which has important *in vivo* implications to the metabolism. These two seemingly unrelated factors may offer an explanation as to why 3-HP titres remained low.

6.3.2 Production of 3-HP is influenced by the availability of NAD⁺

Fermentations completed under aerobic conditions provide a constant source of NADH recycling through oxidative respiration; NADH provides reducing power for generation of a proton gradient in the mitochondria utilised for ATP formation (220). Indeed, the motivation for pursuing co-production of 3-HP and 1,3-PDO in *C. pasteurianum* was the presence of the native 1,3-PDO pathway, which provides a metabolic mechanism for NADH recycling; 1 mole of NADH is generated per mole of 3-HP and 1 mole of NADH is consumed per mole of 1,3-PDO. Theoretically, increases in titres of 1,3-PDO should be equimolar to the production of 3-HP as their co-production is redox balanced. However, this was not the case as expression of KGSADH and GabD4 resulted in production between 0.3 - 1.5 mM 3-HP but increased titres of 1,3-PDO from

43 mM to between 55 mM and 111 mM when expressed from a plasmid (Fig. 4.4B & 4.4C). As previously mentioned, KGSADH and GabD4 were previously characterised and confirmed to have activity using NAD⁺ as a co-factor. However, the experiments where KGSADH and GabD4 were characterised only used NAD⁺ as a co-factor and did not account for the presence of NADH and by extension what is relevant *in vivo*, the intracellular ratio of NAD⁺ / NADH. The intracellular ratio of NAD⁺ / NADH in exponentially growing (aerobic) *E. coli* is $\approx$ 30:1 (221). In contrast, the intracellular ratio of NAD⁺ / NADH in an anaerobic organism, like that of the closely related *C. acetobutylicum* is much lower, $\approx$ 7:1 when fermenting glucose (222). It is well known that enzymes are regulated allosterically or competitively by co-factors. For example, citrate synthase and alpha ketoglutarate are sensitive to the intracellular NAD⁺ / NADH ratio as they are allosterically and competitively inhibited by NADH, respectively (223,224). Furthermore, a plant succinic semialdehyde dehydrogenase (SSADH) from *Arabidopsis* was characterised and reported to be competitively inhibited by NADH (225). GabD4, which is also classified as an SSADH, may also be competitively inhibited by NADH and thus be sensitive to the intracellular NAD⁺ / NADH ratio. Low availability of NAD⁺, which is inherently linked to the intracellular ratio of NAD⁺ / NADH, was suggested by investigators producing 3-HP in *K. pneumonia* as a preventing overproduction of 3-HP (226). In a more recent study, the authors reported that addition of NAD⁺ during the fermentation increased 3-HP production almost 500% and concluded that NAD⁺ was important for 3-HP production (227). Taken together, although KGS and GabD4 were extremely effective for the production of 3-HP in *E. coli*, their sensitivity to low intracellular NAD⁺ / NADH ratios should be taken into consideration for use in anaerobic systems.

6.3.3 Using glycerol as a growth substrate can cause shifts in product formation by influencing the intracellular NAD⁺ / NADH ratio

To build on the conclusions of the previous section, it is important to look at how glycerol can affect the intracellular conditions and metabolism of a microorganism. The motivation for use of glycerol as a feedstock was its low cost due to it being a waste product. However, the reduced nature of glycerol ($\gamma_D=4.67$) compared to glucose ($\gamma_D=4.0$) results in excess reducing equivalents being formed when oxidized; 1 mole of glucose results in 2 moles of ATP and 2 moles of NADH while 2 moles of glycerol results in 2 moles of ATP and 4 moles of NADH (1 mole of glucose has an equivalent amount of carbon as 2 moles of glycerol) (80). This property of

glycerol suggests that using glycerol as a growth substrate would likely lower the intracellular $\text{NAD}^+ / \text{NADH}$ ratio. This is evidenced by the use of glucose-glycerol over glucose only mixtures as a growth substrate in *C. acetobutylicum* and *E. coli* to shift product formation towards more reduced substrates (222,228,229). This is further shown by the decrease in the intracellular ratio of $\text{NAD}^+ / \text{NADH}$ decreasing from 7:1 to 1.33:1 when a glucose-glycerol mix is used compared to solely glucose in *C. acetobutylicum* (222). One does not need to look further than *C. pasteurianum* to observe the effects on growth when fermenting a reduced substrate such as glycerol. Growth on glycerol requires the ability to produce the reduced substrate 1,3-PDO; if the 1,3-PDO pathway is disrupted, another reduced product 1,2-propanediol is generated to maintain redox balance. (80,153). Taken together, a lower intracellular $\text{NAD}^+ / \text{NADH}$ ratio caused by oxidation of glycerol may have had the undesired consequence of reduced activity of the main 3-HP production enzymes KGSADH and GabD4, preventing overproduction of 3-HP.

6.3.4 Microbial production of 3-HP works best under microaerobic conditions

Perhaps it is not by coincidence, that many of the works focusing on the production of 3-HP from glycerol have been completed under aerobic or microaerobic conditions. *E. coli*, *K. pneumonia* and to a lesser extent *B. subtilis* have all been used successfully to produce 3-HP from glycerol or glycerol-glucose substrate mixes with titres ranging from 0.9 – 71.9 g/L (10 – 800 mM) with the (115). In most of these works, glucose was used as the growth substrate, while glycerol was converted into 3-HP by expressing glycerol dehydratase (DhaB), glycerol reactivating factor (GdrA) and a selection of aldehyde dehydrogenases. Thus, by oxidising glucose rather than glycerol, a higher intracellular ratio of $\text{NAD}^+ / \text{NADH}$ is maintained solely due to the inherent nature of each substrate. In one study where anaerobic conditions were used, Huang *et al.* (2012), report the anaerobic co-production of 1.13 g/L of 3-HP in shake flasks using recombinant *K. pneumoniae*; they then claim that by using a fed-batch approach, they were able to increase the production of 3-HP to 24.4 g/L, an increase of more than 20-fold (124). In contrast, in their follow up work they report 16.6 g/L of 3-HP under fed-batch and anaerobic conditions, 30% less 3-HP than previously reported (125). These reports are not consistent with the rest of the literature. For example, Ko *et al.* reported that production of 3-HP and using recombinant *K. pneumoniae* resulted in very low titres under anaerobic conditions and that significant titres could only be achieved using microaerobic or aerobic conditions (136). Another work by Ashok *et al.*

(2013), showed that 3-HP could be produced from glycerol using *K. pneumoniae* in anaerobic conditions, however they used a NAD⁺ regeneration system with nitrate as the electron acceptor (230). This resulted in 40 mM and 250 mM 3-HP in shake flask and fed-batch fermentations, respectively. Another strategy to circumvent this problem of NAD⁺ availability is to engineer the AIDH for enhanced activity; this approach was taken by Park *et al.* who engineered KGSADH using mutational studies to have higher substrate specificity for aldehydes and NAD⁺ while also having less inhibition by NADH resulting in a 40% increase in 3-HP production. (123). In summary, the literature suggests that effective production of 3-HP using the CoA-independent 3-HP pathway requires high availability of the co-factor NAD⁺, and is usually accomplished by completing the fermentation under microaerobic conditions.

6.3.5 The Pdu 3-HP pathway has not been as explored or successful for production of 3-HP

In addition to the CoA-independent 3-HP pathway, the CoA-dependent 3-HP pathway consisting of three Pdu enzymes was also used in this work. This pathway has been explored less than the CoA-independent pathway for production of 3-HP from glycerol (115). Introduction of the Pdu pathway resulted in almost no 3-HP (< 1 mM) but resulted in a large (1.7 – 3.6-fold) increase to 1,3-PDO when initially expressed in *C. pasteurianum* (Fig. 4.3C & 4.3D). The highest amount of 3-HP produced using the Pdu pathway in this work was achieved by integrating the genes for *dhaB₁B₂* and the Pdu enzymes into $\Delta thIA2$ and using static pH conditions during the fermentation resulting in 2.1 mM of 3-HP after 72 h (Fig. 18). In comparison, expression of the Pdu enzymes in *E. coli* resulted in 15.6 mM of 3-HP, however it is not clear if the fermentations were completed under aerobic, microaerobic or anaerobic conditions (128). Similarly, production of 3-HP via the native Pdu pathway in *K. pneumoniae* resulted in 2.0 g/L ($\approx$ 20 mM) of 3-HP after 24 h (132). Using growing cells of *E. coli*, the Pdu pathway was successfully used for production of 1.0 g/L ($\approx$ 10 mM) of 3-HP, however 3-HPA was added into the media rather than being produced from glycerol (201). The Pdu enzymes are native to *L. reuteri* and this has been exploited for the high yield co-production of 3-HP and 1,3-PDO (> 20 g/L) from glycerol as *L. reuteri* lacks the ability to grow on glycerol thus preventing accumulation of oxidative products, however, resting cells were used (199).

One potential factor preventing efficient production of 3-HP using the Pdu pathway is their expression outside of their native host. The Pdu enzymes form part of

the propanediol utilisation pathway and are responsible for catalysing the catabolism of 1,2-propanediol within a bacterial microcompartment (BMC) (231). BMCs can be used to increase reaction rates by concentrating substrates or be used to contain toxic intermediates (232). Thus, by producing the Pdu pathway in an organism where BMCs are not natively expressed, the enzymes may behave differently as their environment is different. Indeed, the PduL from *Moorella thermoacetica*, which is a homolog of the PduL from *L. reuteri*, was shown to exhibit activity towards acetyl-CoA, which may have acted as a competing substrate in the cytoplasm (233). Interestingly, in another work where resting cells of *L. reuteri* were used for production of 3-HP from glycerol using the Pdu pathway, it was reported that acetate, a common fermentation product acted as an inhibitor of the pathway (203). Lastly, the lack of NAD⁺ may have also limited the production of 3-HP using the Pdu pathway as was discussed above for the CoA-independent pathway. This is further supported by recent work where a NAD⁺ regeneration system was used to enhance production of 3-HP using the Pdu pathway (234). In summary, although the Pdu pathway offers an energetic advantage over the CoA-independent pathway, it has not been as explored as a route for production of 3-HP from glycerol due to it being more complex than the simpler CoA-independent route.

6.3.6 An inducible glycerol promoter is an important synthetic biology tool for the fermentation of glycerol

One of the most important tools required for efficient expression of a desired pathway is a suitable promoter. Ideally, a promoter should provide tuneable expression with tight regulation and strong induction. With the rise of synthetic biology there has been an increase in the number of heterologous inducible and synthetic promoter systems reported in the literature in both model and non-model organisms (235–238). We described here a basic characterisation of the promoter driving expression of the genes involved in the reductive pathway of glycerol metabolism. This glycerol inducible promoter had low basal expression during growth on glucose with a 60-fold increase in relative expression under induced (glycerol only) conditions (Fig. 4.5A). It is likely that some regulatory sequences involved in control of the promoter, such as catabolite repression, are located in between bases 122 and 215 directly upstream of the start codon, as there was a significant difference in expression phenotype between promoters P_{dhaBL} and P_{dhaBS} (Fig. 4.5A). The genes *dhaD* and *dhak*, required for oxidation of glycerol are found in a separate operon and their expression

is also likely to be inducible by glycerol but the promoter was not characterised in this work (73). Interestingly, directly upstream of the *dhaD* and *dhaK* operon is a protein annotated as a transcription factor (TF) named PocR. This same PocR TF was found directly upstream of the *pdu* operon and vitamin-B₁₂ biosynthesis gene cluster in *L. reuteri* and found to regulate these gene clusters (239). A similar architecture was found in *Salmonella typhimurium*, where the PocR regulator controlled expression of both vitamin-B₁₂ biosynthesis and *pdu* operons (240). Given that *C. pasteurianum*, like *L. reuteri* is able to synthesize vitamin-B₁₂ *de novo*, it is not unreasonable to hypothesize that glycerol metabolism and vitamin-B₁₂ biosynthesis may be regulated in a similar fashion to *L. reuteri*. The characterisation of the promoter of the *dhaD* and *dhaK* operon and the function of the cis protein PocR are two potential areas for investigation of glycerol metabolism in *C. pasteurianum*. The characterisation of the inducible glycerol promoter P_{dhaBL} provides future researchers with a powerful tool for synthetic biology applications. More in depth characterisation of the inducible glycerol promoter may be necessary prior to its use as a heterologous protein expression system in other microorganisms.

6.3.7 The interesting phenotypes of mutants $\Delta thlA2$ and $\Delta dhaDK$

Two interesting mutants were generated in this work that are not described in the literature. The in-frame deletion of *dhaD* and *dhaK* confirmed the roles of these two enzymes in glycerol metabolism and generated a strain that was unable to grow solely on glycerol. These genes have the same function as their homologs in *K. pneumoniae* as their deletion resulted in the same phenotype (241). The introduction of DhaB₁B₂ and the 3-HP pathways in the mutant $\Delta dhaDK$ was ineffective for conversion of glycerol into 3-HP (Fig. 11, 12, 13). It is possible that catabolite repression prevented full activation of genes related to glycerol metabolism in those experiments where a glucose-glycerol mix was used as a growth substrate. As discussed above, genes related to glycerol metabolism in *L. reuteri*, which is also able to grow on glycerol and produce vitamin-B₁₂, are regulated by PocR and are subject to catabolite repression (239). It may have been more effective to express 3-HP pathways in this mutant using a strong constitutive promoter as previous studies in *E. coli* have done. Another possibility would have been to delete the catabolite repressor proteins ccpA1 and ccpA2 in $\Delta dhaDK$, allowing for growth on glucose without repression of glycerol genes. Although unsuccessful for increasing production of 3-HP in this study, the mutant $\Delta dhaDK$ may be a potentially useful strain to future researchers as it cannot oxidise

glycerol and thus may be effective for production of 1,3-PDO or co-production of 3-HP and 1,3-PDO if the proper mix of pathways and promoters can be found.

The deletion of the thiolase protein in Clostridia has never been reported although it has been actively pursued for many years. Clostridia are able to produce a variety of short and medium chain fatty acids and their respective solvents with carbon lengths ranging from two carbons to eight carbons(70) . These compounds are lengthened by units of two carbons by addition of an acetyl-CoA molecule in what is described as a reverse beta oxidation pathway and this key reaction is catalysed by acetyl-CoA transferase or thiolase enzyme (242,243). The deletion of this key enzyme was expected to produce an interesting phenotype and help understand the redox metabolism of the respective organism. Indeed, deletion of *thlA2* in *C. pasteurianum* yielded an interesting phenotype; fermentation of glucose by $\Delta thlA2$ resulted in a shift in product formation towards ethanol (Fig. 4.17). A similar shift in product formation was observed when glycerol was fermented by $\Delta thlA2$, a large shift in product formation primarily towards 1,3-PDO was observed (Fig. 4.16D). These phenotypes are logical as ethanol and 1,3-PDO are the only products that result in net consumption of reducing equivalents (NADH) when fermenting glucose or glycerol, respectively (Table 4.1). The inability to form butanol, which is a highly reduced product results in the need for reducing equivalents to be recycled elsewhere. These shifts in product formation are more evident in anaerobic systems as NADH recycling must occur by product formation rather than by oxidative phosphorylation. It is easier to quantify these shifts in anaerobic systems as products are more easily measured in solution than quantifying CO₂ formation. The other phenotype observed was the increase in acetate production when fermenting either glucose or glycerol. This is linked to the decreased growth rate and biomass formation observed with $\Delta thlA2$ relative to the wildtype (Fig. 4.14A). As C₄ products cannot be formed in this mutant, specifically butyrate which forms in a 1:1 ratio with ATP, acetate formation becomes the only source for ATP generation (Fig. 4.1).

The main benefit of $\Delta thlA2$ and motivation for creation of this mutant was enhanced product yield. By abolishing C₄ products, the number of products present in the media at the end of the fermentation was reduced from six to four products (Fig. 4.17). Introduction of the 3-HP pathways into $\Delta thlA2$ resulted in increased product yield as carbon was not lost to butanol formation. A similar effect was observed here as in the wildtype, where introduction of 3-HP pathways resulted in increased 1,3-PDO

but not 3-HP. The cause of this phenotype remains unclear in both wildtype and $\Delta thlA2$. Nevertheless, through metabolic engineering, carbon flux was redirected towards product formation pathways resulting in a final yield of 66.6 moles of 3-HP and 1,3-PDO per 100 moles of glycerol compared to 11.4 moles of 1,3-PDO in the wildtype. Strains cpDO-421 and cpDO-421 are extremely efficient at converting glycerol into 1,3-PDO without the need for antibiotics for plasmid selection (pathways were integrated) and without the need for an inducer molecule, as an inducible glycerol promoter drives pathway expression.

6.4. Discussion on the use of *C. pasteurianum* as a platform for whole cell biocatalytic reduction of ketones to chiral alcohols

The work reported in Chapter 5 represents the initial characterisation of *C. pasteurianum* as a potential platform host for whole cell reduction of ketones. Acetophenone was the initial substrate used to optimise the system and test various parameters, including premixing with ethanol, time of addition and the range of concentrations that *C. pasteurianum* could tolerate. The reduction of acetophenone by LK-ADH requires the cofactor NADH, which was supplied by the native metabolism of *C. pasteurianum*. One of the main advantages of using whole cell biocatalysts is the reduced cost of the system which can be as much as 10-fold lower in some cases relative to a purified enzyme system (244). This reduction in cost is due to not requiring purified recombinant enzyme and no costly addition of co-factors. Another advantage of using a whole cell biocatalyst is prolonged preservation of enzymatic activity due to the enzyme being localised internally and thus shielded from potentially harsh conditions of the media (245). This is especially true if using unconventional media such as organic solvents in lipase-catalysed applications or in biphasic systems (246,247). These systems are required when the substrate has poor solubility in an aqueous environment and can limit the effectiveness of a biocatalyst (248). Indeed, it was observed in this work that mixing acetophenone with ethanol aided in conversion to product, presumably due to the increased solubility of acetophenone in the presence of ethanol. If biocatalysts are robust enough to be utilised in non-aqueous environments this allows for the use of substrates that are unstable in aqueous environments (249). In this example, whole cells of *E. coli* were lyophilised and were used directly in neat substrates containing isopropanol and acetophenone reaching

extremely high productivities (500 g/L). The use of growing cells then becomes an impediment to the effectiveness of the biocatalyst. As described in Chapter 5, growing cells of *C. pasteurianum* were affected by increasing concentrations of acetophenone, with growth arresting at concentrations above 100 mM. An alternate strategy would be the traditional use of whole cell biocatalysts, where cells are grown first for biomass formation and then washed and prepared for use as a biocatalyst (245).

The improved performance by the mutant $\Delta thlA2$ for the reduction of acetophenone compared to the wildtype *C. pasteurianum* suggests that metabolic engineering of a strain to increase internal reducing power can translate to stronger performance of the whole cell biocatalytic system. This strategy has been gaining traction in recent years as metabolic engineering is increasingly being applied to biocatalysis. Expression of single and multistep pathways is increasingly becoming common as is the genetic modification of the host to increase co-factor regeneration, to increase the pool of precursors, to block competing pathways or eliminate toxic by-products (250). More evidence of the importance of engineering the host for increased productivity is the influence of intracellular NAD^+ / $NADH$ ratio. By introducing a co-factor regeneration system, the NAD^+ / $NADH$ ratio was decreased resulting in high productivities of acetoin using *B. subtilis* (251). By eliminating native oxidative enzymes, cytosolic levels of $NADH$ were increased to enhance whole cell biocatalytic activity (252). This is in a similar vein to the use of the $\Delta thlA2$ in this work. Other strategies employ replacing NAD^+ specific enzymes in host metabolism with $NADP^+$ specific enzymes. This strategy can be efficiently exploited by targeting key enzymes in the central carbon pathways such as the TCA cycle or the oxidative portion of the pentose phosphate pathway (165). Rather than engineering central pathways in the metabolism, different pathways in metabolism can be linked by introducing heterologous genes to provide substrates to enzymes that regenerate a key cofactor (253).

To further validate *C. pasteurianum* as a platform host for whole cell reduction of ketones, more research needs to be completed to enhance the robustness of the system. First, the substrate scope of LK-ADH needs to be expanded to include cyclic ketones, aliphatic ketones as well as beta-keto esters, all of which were tested during the characterisation of LK-ADH (170). This data would validate that the system is not substrate specific when using LK-ADH. Second, other alcohol dehydrogenases apart from LK-ADH would need to be tested; positive results from this would demonstrate

that the internal cofactor regeneration system is not specific to LK-ADH. Lastly, the system could be tested using resting cells rather than growing cells to decouple biocatalysis from growth, which can be influenced by the concentration and toxicity of the substrate. Together, these experiments would represent a natural progression from the work reported here and would solidify *C. pasteurianum* as a platform host for whole cell reduction of ketones.

Chapter 7

Collaborative Projects & Industrial Placement

7.1 Description of non-core PhD activities

This PhD was part of the 4-year BBSRC Doctoral Training Program (DTP) offered by the University of Nottingham in industrial biotechnology. Although the PhD was technically 48 months, a significant amount of time was spent outside of the core research focus of the PhD. This chapter is meant to document the time spent on those projects and what was accomplished.

The PhD began in October of 2015, although the project carried out at the CRG / SBRC, did not begin until May of 2016. The first 6 – 7 months of this PhD were focused on training workshops and lab rotations before deciding on this PhD project, a defining and extremely attractive feature of the DTP.

Another three months were spent in an industrial placement, which in my case was completed at the Biocircuits laboratory headed by Professor Richard Murray in the Bioengineering Department at the California Institute of Technology between April and June of 2018. The research completed in this industrial placement was focused on cell-free protein expression systems. Using my background in metabolic engineering, the longevity of the system was improved through optimisation of the reaction conditions and metabolism to favour prolonged protein expression.

In summer of 2017, a PhD researcher from India named Shymali Sarma joined the CRG / SBRC for approximately six months. Between September and December 2017, I was tasked with aiding her in her research project as she was also interested in the genetic manipulation of *C. pasteurianum*. I helped her plan out experiments, order DNA primers, carry out fermentations and analysis as well as lent her several key tools that had been developed as part of my research. Specifically, the use of the glycerol inducible promoter P_{dhaBL} and the plasmid Q5_82251, which had improved transformation efficiency. Her efforts resulted in a paper published in 2019 which included me as a co-author. (79).

The last bit of work carried out that took up a significant amount of time was a collaboration our group had with a group at the Swiss Federal Institute of Technology Lausanne, headed by Associate Professor Rizlan Bernier-Latmani. A PhD student in their group by the name of Karen Viacava was researching the use of *C. pasteurianum* for bioremediation purposes, specifically the uptake and metabolism of arsenic. To validate some of her research, two strains of *C. pasteurianum* carrying two separate in-frame deletions and their complements were requested and I was tasked with

generating these strains. Over the course of 3 months, alongside my own work, I created the strains H4 $\Delta arsM$, H4 $\Delta arsB$ and the complemented strains, H4 $\Delta arsM$ *pyrE::arsM* and H4 $\Delta arsB$ *pyrE::arsB*. These strains were shipped to Karen who completed the necessary experiments to validate her hypothesis. Her work is expected to result in a publication at some point in 2020.

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