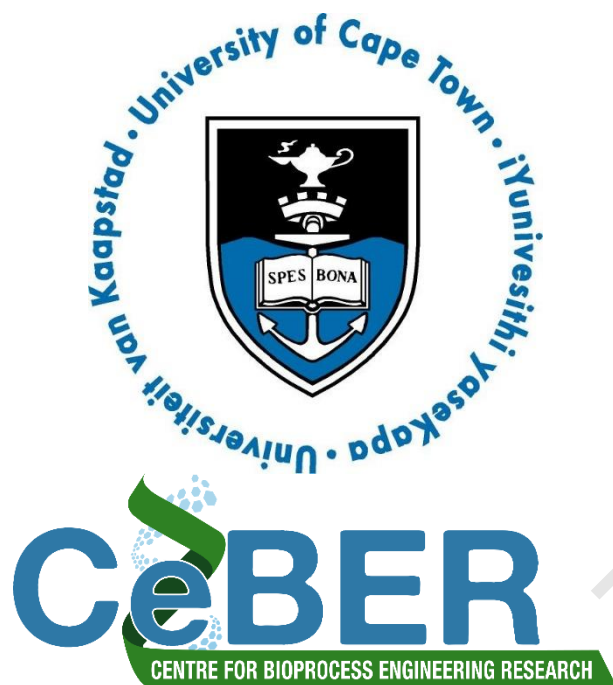




Beyond Bottlenecks:

**Expression of complementary enzymes
and permeabilization of cell membranes
to improve performance of CYP153A6 in
Escherichia coli whole cell biocatalysis**

Bronwyn Elizabeth White

***In fulfilment of the requirements for the degree of
Doctorate of Philosophy in Chemical Engineering***

Supervisor: Prof Susan TL Harrison
Co-supervisor: Prof Martha S Smit
External Co-supervisor: Dr Caryn Fenner

Department of Chemical Engineering
Faculty of Engineering and the Built Environment
University of Cape Town

August 2021

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

Plagiarism Declaration

1. I know that plagiarism is wrong. Plagiarism is to use another's work and to pretend that it is one's own.
2. I have used the Harvard system for citation and referencing. Each significant contribution to, and quotation in, this report from the work, or works, of other people has been attributed, and has been cited and referenced.
3. This report is my own unaided work, except for assistance received from the teaching staff.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signed by candidate

Signature

Abstract

Cytochrome P450s are a diverse and versatile class of enzymes, able to carry out oxyfunctionalisation of hydrocarbons with exceptional regio- and stereospecificity. They show great promise within the medical and fine chemical industries, and could potentially be applied for the activation of linear alkanes into platform chemicals. However, bulk chemicals require higher titres, rates and yields than fine chemicals to be economically viable. Application of these enzymes *in vitro* is hampered by low biocatalyst stability, inhibitory upstream processing costs, and the need for costly cofactor supplementation. *In vivo* operation can overcome these issues, but brings its own set of limitations.

This thesis presents research on the *in vivo* biotransformation of n-octane to 1-octanol by CYP153A6 and its natural redox partners, ferredoxin (Fdx) and ferredoxin reductase (FdR), from *Mycobacterium* sp. HXN-1500. These three proteins were heterologously expressed in *Escherichia coli* BL21DE3. Experiments were carried out at the millilitre scale, using resting cells.

Potential limitations for the whole-cell biocatalyst include insufficient expression of the CYP153A6 or its redox partners, leading to a lack of biocatalytic “active sites”; lack of oxygen for biotransformation and cell metabolism; poor transport of n-octane from the organic phase into the cytoplasm, leading to limitations in substrate; toxicity resulting from the accumulation of substrate or product; and insufficient availability of NADH cofactor.

With regards to heterologous enzyme expression, the decision was made to focus on CYP153A6 expression levels (with FdR and Fdx expression levels forming part of a related project). To investigate the effects of intracellular concentration of active CYP153A6, it was necessary to find a reliable method of varying P450 expression. The haem precursors δ -aminolevulinic acid (δ -ALA) and ferric chloride were applied during the growth and expression stage, and it was found that P450 expression could be optimised by varying the concentration of these chemicals, with co-addition of the two chemicals having a synergistic effect on expression levels. However, higher intracellular levels of active CYP153A6 did not lead to increased biotransformation rates or product titres in biotransformations carried out with whole cells. This demonstrated that the biotransformation of n-octane was not limited by the availability of active CYP153A6, even at concentrations as low as $0.3 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$. Furthermore, these results suggest that *optimising* the expression of CYP153A6 in whole-cell biocatalysts does not equate to *maximising* its expression. Aminolevulinic acid constitutes a substantial portion of media formulation costs in the proposed whole-cell process, and these findings allow for more efficient application of this and other precursor chemicals.

Based on an analysis of the literature, it was hypothesised that oxygen was unlikely to be a limiting substrate, due to the relatively low biotransformation rates being recorded, as well as the reduced oxygen demand of resting cells. Nevertheless, two sets of experiments were carried out to assess the effects of oxygen supply to the system. In the first set, the quantity of oxygen in the headspace was increased by enlarging the vials, while keeping cross-sectional area constant. In the second set, the headspace was replenished at regular intervals to increase the average concentration of oxygen in the headspace over the course of the biotransformation. In both sets of experiments, the differences between lower-oxygen vials and higher-oxygen vials were neither substantial nor consistent. This demonstrated that biotransformations by resting cell cultures at a density of $4 - 5 \text{ g}_{\text{DCW}}.\text{L}^{-1}$ were not limited by the quantity of oxygen available.

The transport of hydrocarbons across the cell envelope affects the availability of n-octane substrate in the cytoplasm, and impacts on the *in situ* extraction of 1-octanol product. Thus, hydrocarbon transport has the potential not only to be a rate-limiting step, but also to control levels of cytotoxicity. To explore the significance of cross-membrane transport, cell membranes were permeabilised via the application of chemicals or via mechanical breakage, and biotransformations were also carried out using cell free extract. To investigate cofactor limitations, the NADH regeneration rate of *E. coli* was enhanced through over-expression of glycerol dehydrogenase (GLD) and supply of glycerol as sacrificial substrate. These interventions were implemented both separately and concurrently, and the results compared to biotransformations by low-GLD, non-permeabilised cells. The interactions between substrate transport and cofactor regeneration were found to be complex.

In cultures of intact or chemically permeabilised cells, strains with enhanced NADH regeneration capacity produced equal, and sometimes lower, titres than control cells carrying only the empty vector. The co-expression of GLD tended to reduce product titres in high-density cultures, by exacerbating instability of the whole-cell biocatalyst, which was already substantially reduced relative to low-density cultures. In low-density cultures, co-expression of GLD was not harmful, but it did not lead to any notable improvements in product formation rates. However, across numerous cultures, enhanced cofactor regeneration capacity correlated with enhanced CYP153A6 stability. Furthermore, in many low-density cultures, the co-expression of GLD provided marginal improvements in overall stability of the whole-cell biocatalyst when biotransformations were extended beyond 48 h. Thus, under the conditions tested, n-octane biotransformation rates were not limited by the availability of NADH. At the same time, the results suggest the possibility of harnessing enhanced cofactor regeneration to extend biotransformation times, presumably by improving the overall metabolic state of the whole cell – but also show that such a strategy has a ‘tipping point’, related to cell density, beyond which the presence of additional dehydrogenase becomes harmful.

The application of Triton X-100 or Polymyxin B substantially increased initial product formation rates relative to untreated cells. In low-density cultures, this led to chemically permeabilised cells achieving significantly higher final titres than untreated cells. However, the use of these chemicals in high-density cultures reduced the stability of the whole-cell biocatalyst; biotransformations levelled off rapidly, limiting the maximum titres that could be obtained. The co-expression of GLD was not beneficial in these cases.

Conversely, the physical breakage of cell envelopes (via passage through a high-pressure homogeniser) improved product titres and space time yields relative to unpermeabilised cells, provided strains were co-expressing GLD. This clearly demonstrated the importance of balancing the supply of alkane and cofactor, with the expression of additional GLD compensating for reductions in metabolic capacity associated with the physically broken membranes. Mechanically permeabilised cells and cell free extract remained stable over extended periods of biotransformation, even though culture densities were high in these cases. Due to their capacity to maintain initial reaction rates at high cell density, mechanically permeabilised cells produced the highest final titres of any system tested in this study. If GLD was not co-expressed (or if CYP153A6 and GLD were expressed in separate cells), the biotransformation rates of mechanically permeabilised cells were substantially reduced.

These results identified cross-membrane transport of hydrocarbons as the key limitation in the biotransformation of n-octane by whole-cell biocatalysts. Supply of substrate was a rate-limiting step in the reaction (hence the increase in product formation rates in low-density cultures of permeabilised cells). The degree of cell permeabilization also affected how the cell responded to substrate and product toxicity (hence the stability of high-density cultures of mechanically permeabilised cells). However, cofactor regeneration rates quickly became a limiting factor if the cofactor pool was disrupted (as was the case when the cell envelope was physically broken).

To improve cross-membrane transport without destroying the cell membrane, *E. coli* strains were engineered to express AlkL, a passive membrane transporter protein that evolved to facilitate the growth of *Pseudomonas putida* on alkanes. The expression strategy included manipulating the supply of haem precursors δ -ALA and FeCl₃, in a drive to achieve comparable expression of CYP153A6 in strains with and without AlkL. Even though the AlkL expression strategy was not fully optimised, cells co-expressing the transport protein demonstrated 50 – 100 % improvements on their yield on biomass after 48 h. These results confirmed the importance of overcoming the cross-membrane transport limitation.

Overall, the findings highlight the importance of balancing reaction components. Cross-membrane transport of hydrocarbons emerged as the key limitation in biocatalysis by CYP153A6 in whole cells. This limitation could be alleviated by permeabilising the cell. Mechanical breakage of the cell envelope was particularly effective, but this in turn impacted negatively on the cell’s ability to maintain its cofactor pool, necessitating the co-expression of glycerol dehydrogenase to boost NADH regeneration rates. In this regard, the membrane channel protein AlkL showed great promise, allowing cells to increase their rates of product formation even while relying on their native cofactor pool. These performance enhancements were achieved despite low intracellular concentrations of active CYP153A6 in cells co-expressing AlkL. Furthermore, in biotransformations with intact cells, intracellular concentration of CYP153A6 could be substantially reduced (via a reduction in the concentration of haem precursor),

without any negative impact on biotransformation being observed. Increasing the cell density of biotransformation cultures allowed for rapid product accumulation, but substantially reduced the stability of the whole-cell biocatalyst. This instability correlated closely with the volumetric rate of product formation, suggesting that the effect was linked to reaction or product toxicity. Isolated improvements in enzyme expression, substrate supply, cofactor regeneration, or biomass loading did not translate into improved biocatalytic performance overall. This demonstrates that the creation of a useful P450-based biocatalyst will depend on the simultaneous optimisation of multiple components.

In the field of biocatalysis, the focus tends to be on the characterisation and modification of the enzymes themselves. While this enzymatic understanding is crucial, the equally important task of understanding host physiology is often not explored in the same exacting detail. Unlocking the full potential of biocatalysis within the bulk chemical space requires an understanding of the cell architectures within which the enzymes of interest must function. This study has served to shed light on the links between cells, enzymes, and reactants – and on how much there still is to understand when it comes to whole cell performance.

Publications and presentations arising from this work

Articles:

White BE, Fenner CJ, Smit MS and Harrison STL, 2017. *Effect of cell permeability and dehydrogenase expression on octane activation by CYP153A6-based whole cell Escherichia coli catalysts*. Microbial Cell Factories 16(156)

White BE, 2016. Cracking chemistry's toughest bonds. Quest 12(3) pp 5-7

Presentations:

White BE, Fenner CJ, Smit MS and Harrison STL, 2017. *Activation of n-octane by CYP153A6 in a whole cell E. coli system*. Presented at c*change Symposium, 17-19 November, Johannesburg, South Africa

White BE, 2017. *Bio-based production of petrochemicals*. Presented at the University of Cape Town Three Minute Thesis competition, 22 February, Cape Town, South Africa

White BE, Shaw M, Swart S and Harrison STL, 2016. *Exploiting CYP153A6 for paraffins activation*. Presented at CATSA 2016, 6-9 November, Drakensberg, South Africa

White BE, Fenner CJ, Smit MS and Harrison STL, 2016. *Octane activation by a whole-cell CYP153A6-based biocatalyst*. Presented at c*change Symposium, 4-6 November, Drakensberg, South Africa

White BE, Kotsiopoulos A, Shaw M, Swart S, van der Bergh N and Harrison STL, 2016. *Alkane Activation*. Presented at CeBER Research Day, 31 August, Cape Town, South Africa

White BE, Fenner CJ, Smit MS and Harrison STL, 2015. *Minimising metabolic burden and maximising enzyme stability while enabling cofactor regeneration and cross-membrane transport in Escherichia coli expressing cyp153A6 for octane activation*. Presented at c*change Symposium, 13-15 November, Kleinmond, South Africa

White BE, Fenner CJ, Kotsiopoulos A, Shaw M, Swart S, van der Bergh N and Harrison STL, 2015. *Activation of linear alkanes*. Presented at CeBER Research Day, 16 September, Cape Town, South Africa

White BE, Smit MS and Harrison STL, 2014. *Biocatalytic conversion of octane by an E. coli system expressing CYP153A6: minimizing mass transfer limitations of the cell*. Presented at CATSA 2014, 9-12 November, Pretoria, South Africa

White BE, Fenner CJ, Smit MS and Harrison STL, 2014. *Minimising metabolic burden and maximising enzyme stability while enabling cofactor regeneration and cross-membrane transport in Escherichia coli expressing cyp153A6 for octane activation*. Presented at c*change Symposium, 7-9 November, Pretoria, South Africa

White BE, Opperman DJ, Savvi S, Smit MS and Harrison STL, 2014. *The Role of Cross-Membrane Transport in the Biocatalytic Conversion of Octane by an E. coli System Expressing CYP153A6*. Presented at the International Conference on Chemical Thermodynamics and South African Institute of Chemical Engineers, 27 July – 1 August, Durban, South Africa

White BE, Fenner CJ, Savvi S, Smit MS and Harrison STL, 2013. *Minimising metabolic burden and maximising enzyme stability while enabling cofactor regeneration and alkane transport in E. coli expressing CYP153A6 for alkane activation*. Presented at c*change Symposium, 15-17 November, Wild Coast, South Africa

Posters:

White BE, Fenner CJ, Smit MS and Harrison STL, 2017. *Biological Paraffin Activation: What's Holding Us Back?* Presented at the University of Cape Town EBE Postgraduate Research Expo, 25 May, Cape Town, South Africa

White BE, Fenner CJ and Harrison STL, 2017. *A microbial cell factory for the activation of n-octane: co-expressing proteins for biocatalysis, cofactor regeneration and transport.* Presented at the Royal Society of Chemistry Catalysis for Fuels Faraday Discussion, 24-26 January, Cape Town, South Africa

White BE, Fenner CJ and Harrison STL, 2015. *Optimal Expression of CYP153A6 by Controlling the Concentration of Heme Precursors.* Presented at CATSA 2015, 15-18 November, Kleinmond, South Africa

White BE, Fenner CJ, Smit MS and Harrison STL, 2015. *Overcoming bottlenecks in P450 alkane activation.* Presented at 12th Biotrans Conference, 26-30 July, Vienna, Austria

White BE, Fenner CJ, Savvi S, Smit MS and Harrison STL, 2013. *Cofactor regeneration as an approach to augment n-octane activation using an E. coli-based whole cell CYP153A6 catalyst.* Presented at CATSA 2013, 17-20 November, Wild Coast, South Africa

Acknowledgements

Regardless of what the university's standard plagiarism declaration would have us attest, there is really no such thing as one's "own unaided work". No achievement, however personal, takes place in a vacuum. This doctoral thesis would never have been written without input from a wide cast of helpful characters.

In the first place, research would be impossible without financial support. This project was conducted using funding from the DST-NRF Centre of Excellence in Catalysis (c*change), as well as from the SARChI Chair in Bioprocess Engineering. I was also able to present part of this work at the 2015 BIOTRANS conference in Vienna, thanks to travel funding from c*change, alongside the University of Cape Town's Conference Travel Grant. I am extremely grateful to all the entities mentioned above for their financial backing over the years.

But no matter how much funding had been thrown at it, this project would not be what it is, and I would not have grown to be the young researcher that I am, without the guidance of Professor Sue Harrison. I owe a great deal to her intellect, experience and wisdom, and I am thankful to her for providing me with opportunities to develop as an independent thinker, as a teacher, and as a person. My co-supervisor Professor Martie Smit, from the University of the Free State, has been invaluable when it comes to providing insights from a fresh perspective, in addition to graciously hosting me in her own laboratory at UFS for several key experiments. Dr Caryn Fenner, although she has since left academia, was an attentive co-supervisor during the early years of this project, and an invaluable source of support as I transitioned from undergraduate to postgraduate studies.

I offer up my heartfelt gratitude to the staff and my fellow students in CeBER, all of whom generously shared space, skills, critiques, solutions and the occasional laugh with me. I would particularly like to acknowledge those who were directly involved in the paraffins project, especially Shanna and Thanos, who have been there pretty much throughout my time as a postgraduate. Special mention goes to Mariette Smart, who helped me navigate through the genetic engineering aspect of this work.

Last but not least, I am most grateful to those people who had nothing and everything to do with this project. I'd like to thank my mother, who finds my work incomprehensible but is proud of me anyway; my father, who throughout my life has given his unswerving support to any and all of my intellectual pursuits; and my sister, who for years has put up with my odd interests, odder moods, and inconvenient demands to be picked up from the lab in the middle of the night. Finally, I'd like to thank my friends: Alex, Ruth, Brendon, Vinay, Jess, Muya, Bruce, Aidan, Maylis, Emily, Jack: if I had never met you, my years at UCT would ultimately have meant very little.

Table of Contents

Abstract	i
Acknowledgements	vii
Table of Contents	ix
List of Figures	xi
List of Tables	xiii
Glossary of Terms	xv
Acronyms and Abbreviations	xvii
1 Introduction	1
1.1 Why hydroxylate alkanes?	1
1.2 What will it take to turn CYP153A6 into an industrial catalyst?	2
1.3 Project scope	3
1.4 Structure of dissertation	4
2 Literature Review	5
2.1 Introduction	5
2.2 Natural Context	5
2.2.1 Oxidoreductases, Monooxygenases, and Alkane Hydroxylases	5
2.2.2 Alkane hydroxylation in nature	6
2.2.3 Cytochromes P450	11
2.3 Industrial Context	18
2.3.1 Industrial applications of monooxygenases	18
2.3.2 Modifying cytochromes P450 for industrial synthetic applications	19
2.3.3 The hard-to-reach standards of industrial success with monooxygenases	20
2.4 Cytochromes P450 for Biocatalysis	21
2.4.1 The choice between <i>in vitro</i> and <i>in vivo</i> operation	21
2.4.2 Considerations relating to heterologous expression	23
2.4.3 Optimal expression of cytochromes P450 and their redox partners	26
2.4.4 Optimal expression levels for <i>in vivo</i> biocatalysis	27
2.4.5 Culture conditions, cell physiology, and biotransformation	29
2.4.6 Process operation in whole-cell systems	31
2.5 Potential Bottlenecks Affecting Whole-Cell Alkane Hydroxylation	32
2.5.1 Balancing enhancement of hydrocarbon transport with host cell viability	33
2.5.2 Overcoming the transport limitations associated with cell membranes	36
2.5.3 Ensuring sufficient supply of NADH cofactor	39
2.5.4 Supplying oxygen for respiration and as a substrate	44
2.6 Summary	44
2.7 Defining the Research Project	45
2.7.1 Problem Statement and objectives	45
2.7.2 Research hypotheses and key questions	46
3 Methodology	49
3.1 Chemicals, bacterial strains, and plasmids	49
3.2 Cell growth and protein expression	54
3.3 Biocatalyst preparation	54
3.3.1 Harvesting	54
3.3.2 Whole cells	54
3.3.3 Mechanically broken cells	54
3.3.4 Chemically permeabilised cells	54
3.4 Biotransformation	55
3.5 Sampling and analysis	56
3.5.1 Quantification of dry cell mass	56
3.5.2 Quantification of CYP153A6	56

3.5.3	Extraction and analysis of organic phase	57
3.5.4	Key performance indicators for comparison of biotransformation experiments.....	57
3.6	Experimental Blocking	57
4	Expression and Efficacy of CYP153A6	59
4.1	Effect of precursor concentrations on P450 expression	59
4.2	Effect of additional heterologous expression on P450 expression	62
4.3	Effect of enzyme loading on biotransformation.....	69
4.4	Oxygen supply.....	76
4.5	Summary of investigations into P450 expression and oxygen limitations.....	81
5	Cofactor regeneration and hydrocarbon transport	83
5.1	Effects of membrane permeabilization in cell suspensions with additional glycerol dehydrogenase.....	86
5.1.1	Combining A6/FdR/Fdx and GLD: co-expression versus co-culturing.....	86
5.1.2	Enhanced cofactor regeneration in preparations of disintegrated cells	89
5.2	Effects of glycerol dehydrogenase co-expression in intact cells.....	91
5.2.1	Effects of glycerol dehydrogenase at high cell density	91
5.2.2	Effects of glycerol dehydrogenase at low cell density.....	94
5.3	Effects of glycerol dehydrogenase co-expression in mechanically broken cells.....	98
5.4	Effects of glycerol dehydrogenase co-expression in chemically permeabilised cells	102
5.4.1	Combining glycerol dehydrogenase and chemical permeabilization at high cell density.....	102
5.4.2	Combining glycerol dehydrogenase and permeabilization at low cell density.....	105
5.5	Membrane integrity, NADH regeneration capacity, and biocatalyst performance.....	108
5.5.1	Stability of CYP153A6.....	108
5.5.2	Stability of the whole-cell biocatalyst.....	111
5.5.3	By-product formation.....	113
5.6	Conclusions from chemically and mechanically permeabilised cells	114
5.7	Expression of the membrane channel protein AlkL	115
5.7.1	Variability in enzyme expression and stability between strains	116
5.7.2	Production of 1-octanol	117
6	Concluding Remarks.....	123
6.1	Summary of key results	123
6.1.1.1	Expression and efficacy of CYP153A6 in intact whole cells.....	123
6.1.1.2	Oxygen supply	123
6.1.1.3	Regeneration of NADH.....	124
6.1.1.4	Cross-membrane transport of hydrocarbons.....	124
6.2	Re-examination of hypotheses	124
6.3	Literature Context.....	126
6.4	Recommendations for future work.....	130
	References.....	133

List of Figures

Figure 2.1: Cytochromes P450 in the broader context of the oxidoreductase supergroup	6
Figure 2.2: Typical preferred substrate ranges of the various types of alkane hydroxylases	9
Figure 2.3: Reaction cycle of cytochrome P450s	13
Figure 2.4: Diversity and classification of cytochromes P450 on the basis of the structure of their electron transfer chains, the types of cofactor and mediator protein(s) they interact with, and their location in the cell	15
Figure 2.5: Simplified reaction scheme for aliphatic alkane activation by CYP153A6	33
Figure 3.1: Gel fragment patterns resulting from restriction digests and subsequent electrophoresis of plasmids	51
Figure 3.2: Schematic representation of the cloning process followed to create the plasmids pCDFDuet(AlkL) and pCDFDuet(GLD+AlkL)	52
Figure 3.3: Apparatus for the replacement of vial headspace (not to scale)	55
Figure 4.1: Effect of haem precursor concentration on expression of active CYP153A6	60
Figure 4.2: Data from both experiments depicted in Figure 4.1, normalised based on P450 concentrations at the common condition of 50 μ M added ferric chloride and no added aminolevulinic acid	62
Figure 4.3: Concentration of CYP153A6 in strains expressing various additional heterologous proteins	63
Figure 4.4: Concentration of active and inactive CYP153A6 in cells expressing A6/FdR/Fdx, at various levels of δ -ALA addition	65
Figure 4.5: Concentration of active and inactive CYP153A6 in cells expressing A6/FdR/Fdx and co-expressing glycerol dehydrogenase, at various levels of δ -ALA addition	66
Figure 4.6: Concentration of active and inactive CYP153A6 in cells expressing A6/FdR/Fdx and co-expressing AlkL, at various levels of δ -ALA addition	67
Figure 4.7: Application of varying levels of δ -ALA addition in order to moderate the expression of active CYP153A6 across cultures expressing various additional heterologous proteins	68
Figure 4.8: Intracellular concentration of active CYP153A6 during biotransformations with cultures grown and expressed with different concentrations of δ -ALA and FeCl_3	70
Figure 4.9: Relationship between the concentration of active CYP153A6 per cell and the apparent average turnover frequency after 4 h of biotransformation	71
Figure 4.10: Relationship between the concentration of CYP153A6 per cell and the apparent average turnover frequency at various points in the biotransformation	72
Figure 4.11: Schematic showing a theoretical relationship between the concentration of biocatalytic enzyme per cell and the apparent turnover frequency (moles substrate converted per mole enzyme per unit time)	73
Figure 4.12: Effect of active CYP153A6 concentration on 1-octanol production in six separate cultures, each having a different intracellular P450 concentration	74
Figure 4.13: Effect of active CYP153A6 concentration on space time yields	75
Figure 4.14: Effect of headspace volume on the production of 1-octanol per gram of dry cell weight	77
Figure 4.15: Effect of headspace volume on space time yield	78
Figure 4.16: Effect of headspace replenishment on the production of 1-octanol per gram of dry cell weight	79
Figure 5.1: Effect of different membrane permeabilization techniques on the biotransformation of n-octane by <i>E. coli</i> expressing A6/FdR/Fdx and co-expressing glycerol dehydrogenase	87
Figure 5.2: The experiment described in Figure 5.1 was repeated with a cell preparation consisting of a 40:20 mix of <i>E. coli</i> expressing A6/FdR/Fdx and <i>E. coli</i> expressing glycerol dehydrogenase	87
Figure 5.3: Effect of glycerol dehydrogenase on the titre and yield on biomass obtained by suspensions of disintegrated <i>E. coli</i> cells	90
Figure 5.4: Effect of glycerol dehydrogenase co-expression on biotransformation of n-octane by high-density suspensions of intact cells	92

Figure 5.5: Key performance indicators for biotransformation systems consisting of high-density suspensions of intact cells	93
Figure 5.6: Effect of glycerol dehydrogenase co-expression on biotransformation of n-octane by low-density suspensions of intact cells	95
Figure 5.7: Effect of culture density on the average rates of product formation over various periods of the biotransformation, for intact cells with and without additional glycerol dehydrogenase	96
Figure 5.8: Effect of culture density on the stability of CYP153A6	97
Figure 5.9: Effects of glycerol dehydrogenase co-expression on cell free extract and mechanically broken cells.....	99
Figure 5.10: Average rates of product formation for ruptured cells with enhanced cofactor regeneration, relative to intact cells	99
Figure 5.11: Effects of glycerol dehydrogenase co-expression on the concentration of active CYP153A6 in cell free extract and mechanically broken cells.....	100
Figure 5.12: Effects of acetone and Triton X-100 on the biotransformation of n-octane at high cell density	103
Figure 5.13: Intracellular concentrations of active CYP153A6 across two days of biotransformation, for intact cells, cells washed with 5% (v/v) acetone, and cells mixed with 0.25% (v/v) Triton X-100	104
Figure 5.14: Key performance indicators for biotransformation systems consisting of whole cells washed with 5% (v/v) acetone prior to biotransformation, or mixed with 0.25% (v/v) Triton X-100 in the biotransformation buffer	105
Figure 5.15: Combining chemical permeabilization and dehydrogenase co-expression in low-density suspensions of resting cells	106
Figure 5.16: Effect of chemical permeabilization and glycerol dehydrogenase co-expression on biocatalyst stability, as indicated by changes in space time yield over the course of the biotransformation	107
Figure 5.17: Intracellular concentrations of active CYP153A6 over the course of various resting-cell biotransformations	110
Figure 5.18: Rate of biocatalytic performance loss as a function of space time yield over the first 10 – 12 h of biotransformation. Data points were drawn from both low- and high-density systems, and include intact, mechanically broken, and chemically permeabilised cells.....	112
Figure 5.19: Volumetric titre following two days of biotransformation with various cell preparations	114
Figure 5.20: Space time yields and yields on biomass over two to five days of biotransformation, for four strains expressing different combinations of A6/FdR/Fdx, glycerol dehydrogenase, and AlkL	118
Figure 5.21: Effects of AlkL and glycerol dehydrogenase co-expression on 1-octanol formed per unit of biomass over two days of biotransformation.....	119
Figure 5.22: Effects of AlkL and glycerol dehydrogenase co-expression on 1-octanol formed per unit of biomass over five days of biotransformation.....	120
Figure 6.1: Graphic representation of maximum activity as a function of intracellular enzyme concentration	130

List of Tables

Table 2.1: Comparison of n-alkane activation by natural and engineered P450s and other hydroxylases	17
Table 2.2: Attempts to facilitate biotransformation via treatment of <i>Escherichia coli</i> *, <i>Schizosaccharomyces pombe</i> **, or <i>Pseudomonas putida</i> *** whole cells with emulsifying chemicals and permeabilising agents.....	36
Table 2.3: Expression of AlkL in <i>Escherichia coli</i> whole cells expressing an alkane hydroxylase for biotransformation.....	39
Table 2.4: Comparison of formate, glucose, and glycerol for use in dehydrogenase-based cofactor regeneration systems	43
Table 3.1: List of plasmids used to create biotransformation strains.....	49
Table 3.2: List of strains used in this study, indicating which aspects of work made use of each strain	53
Table 3.3: Derivation of key performance indicators.....	57
Table 4.1: Intracellular concentrations of CYP153A6 for four different strains, exposed to various δ -ALA starting concentrations in the growth and expression medium	64
Table 4.2: Concentration of active CYP153A6 in <i>E. coli</i> in six different strains exposed to various δ -ALA and FeCl_3 starting concentrations in the growth and expression medium.....	70
Table 4.3: Metabolic oxygen demand estimated from values reported in literature for various aerobic cultures.	80
Table 5.1: List of experiments investigating the interactions between cofactor regeneration and cross- membrane transport, and their impact on biocatalyst performance	85
Table 5.2: Whole-cell biotransformation of n-octane by CYP153A6 and its redox partners, with the availability of glycerol dehydrogenase increased via either co-expression or co-culturing.....	88
Table 5.3: Maximum turnover frequencies for the various mechanically permeabilised systems	101
Table 5.4: Intracellular concentrations of active CYP153A6 at the beginning and end of biotransformations with chemically permeabilised cells at low density	107
Table 5.5: Rate of loss of biocatalytic performance relative to five key performance indicators, for 16 different biotransformation systems.....	111
Table 5.6: Active CYP153A6 concentration at the beginning and end of the biotransformation period, for systems with varying levels of cofactor regeneration capacity and alkane transport capacity	117
Table 6.1: Activation of n-octane by alkane hydroxylases in whole-cell biocatalysts in the literature	128
Table 6.2: Selected data from Table 6.1	130

Glossary of Terms

Activation	In the context of alkanes, this term refers to the formation or insertion of a functional group (double bond, oxygen molecule, -OH group, halogen, etc.) into an alkane molecule
Aliphatic	Organic compound which is not aromatic
Aromatic	Organic compound containing at least one ring of delocalized pi electrons
Cofactor	In the context of this study, the term is used specifically to refer to the cellular cofactors that play a role in energy generation, such as ADP/ATP, FAD/FADH ₂ , and especially NAD(P) ⁺ /NAD(P)H.
Coupling efficiency	The ratio of substrate conversion to NAD(P)H consumption; a low coupling efficiency indicates that enzymes are expending cofactors on futile reaction cycles.
Disintegrated cells	Cells following passage through a high-pressure homogenizer at 30 kpsi, in order to break the cell membrane into pieces.
Electron transport protein	In the context of this study, the term refers to a protein which transfers reducing equivalents from the cellular cofactor to the biocatalytic enzyme
Hydrocarbonoclastic	Capable of metabolizing hydrocarbons
<i>In vitro</i>	Biotransformation carried out using a solution of free enzymes, i.e. enzymes are not confined to the cell
<i>In vivo</i>	Biotransformation carried out in whole cells
Monooxygenase	Enzyme which catalyzes the insertion of a hydroxyl group into a substrate
Oxidoreductase	Enzyme which catalyzes reactions requiring the transfer of electrons from a reductant to an oxidant
Platform chemical	Chemical which can be used to produce other, higher-value chemicals
Precursor	In the context of this study, the term refers to chemical additives which promote the expression of biocatalytic enzyme
Promiscuity	The tendency of an enzyme to accept multiple substrates
Redox partner	See 'electron transport protein'
Ruptured cells	Cells following passage through a high-pressure homogenizer at 15 kpsi, in order to create breaks in the cell membrane.
Selectivity	The ability of a catalyst or enzyme to target a particular atom or functional group within the substrate molecule
Specificity	The ability of a catalyst or enzyme to target a particular substrate within a mixture of compounds (opposite of promiscuity)
Two-liquid-phase system	Enzymatic systems containing both a bulk aqueous and a bulk organic phase
Uncoupling	Futile reaction cycles in which a cytochrome P450 activates oxygen rather than the hydrocarbon substrate, forming water or reactive oxygen species.

Acronyms and Abbreviations

°C	Degrees centigrade
CO	Carbon monoxide
BEHP	Bis(2-ethylhexyl) phthalate
CFE	Cell free extract
δ-ALA	δ-aminolevulinic acid
DCW	Dry cell weight
<i>E. coli</i>	<i>Escherichia coli</i>
FdR	Ferredoxin reductase (one of two redox partners of CYP153A6)
Fdx	Ferredoxin (one of two redox partners of CYP153A6)
FeCl ₃	Ferric chloride
g	Gram(s)
GLD	Glycerol dehydrogenase
h	Hour(s)
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kpsi	1 kpsi = 1000 pounds per square inch = 6.895 x 10 ⁶ Pascals
L	Litre(s)
M	Moles per litre
mM	Millimoles per litre
min	Minute(s)
mmol	Millimole(s)
mol	Mole(s)
NAD(P)/NAD(P)H	Cellular cofactor, nicotinamide adenine dinucleotide (phosphate); 'H' indicates the reduced form of the compound
O ₂	Diatomic oxygen
<i>P. putida</i>	<i>Pseudomonas putida</i>
P450	Cytochrome P450; typically used to refer to the active form of the enzyme, as determined by spectrophotometric assay
P420	Inactive form of cytochrome P450
STY	Space time yield
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
TOF	Turnover frequency
WCW	Wet cell weight

1 Introduction

This project investigates the *in vivo* conversion of n-octane to 1-octanol by CYP153A6 and its native redox partners, which were heterologously expressed in *Escherichia coli* BL21DE3. CYP153A6 is a monooxygenase native to *Mycobacterium* sp. HXN-1500, a hydrocarbonoclastic species first isolated from a hexane scrubber (van Beilen et al., 2002). It is classified as a Class I bacterial P450 (Hannemann et al., 2007), relying on two electron transport proteins, ferredoxin reductase and ferredoxin, and utilising the cellular cofactor NADH as an electron source. CYP153A6 is able to insert molecular oxygen into C₅ – C₁₁ linear alkanes, with extreme selectivity for the terminal carbon atom, thus producing primary alcohols (Funhoff et al., 2006).

1.1 Why hydroxylate alkanes?

Alkanes represent a challenge to conventional chemical catalysts because they consist entirely of C-C and C-H bonds. Such bonds share all electrons covalently, with low-acidity hydrogen atoms and no lone pairs or low-lying empty orbitals for oxidants to target. Consequently, they are the most thermodynamically stable bonds in nature (Arnold et al., 2015). However, many naturally-occurring fossil fuel deposits consist of a mixture of predominantly linear alkanes, so despite the unreactive nature of these molecules, their functionalisation is the first step in the production of most petrochemicals. Directly or indirectly, petrochemicals play an indispensable role in all aspects of modern life, thanks to their diverse and versatile properties. They are used in industries such as pharmaceuticals, cosmetics, food and feed, flavour and fragrance, and materials – all of which are demonstrating sustained growth in demand (Becker and Wittmann, 2015; Bergman, 2007; J. M. Choi et al., 2015; S. Choi et al., 2015; Kaluzna et al., 2016; Soussan et al., 2016). For example, 1-octanol has direct applications as an agrochemical, as antifoam, and as a solvent in various types of surface coatings and perfumes. It is also a precursor for chemicals such as ethoxylates and alkyl and ether sulphates (Vallon et al., 2013). The efficient activation of alkanes into oxygenated products is therefore of great relevance to the fine and bulk chemical sectors.

Processes such as cracking and thermal hydrogenation represent the established routes for alkane functionalisation. Due to the thermodynamic stability of the C-C and C-H bonds, these processes have high energy demands. Meanwhile, activation of alkanes produces compounds that are more reactive than the substrate, leading to low process selectivity (Arnold et al., 2015; Bergman, 2007; Soussan et al., 2016). Chemists therefore continue to search for methods of alkane activation that are less intensive and easier to control. Substantial progress has been made in elucidating mechanisms of reaction for C-H bonds, and catalysts have been developed based on ligand complexes containing d-block or f-block metal ions. Depending on the complex, a reaction can proceed via σ -bond metathesis, 1,2 addition, oxidative addition, or H-atom abstraction. However, only a handful of these developments have proved applicable to saturated hydrocarbons such as alkanes. Necessary conditions in these cases frequently include temperatures above 50 °C and pressures of up to 150 atmospheres, and the reactions proceed in toxic solvents such as toluene, cyclohexane, tetrahydrofuran, or strong acids (Arnold et al., 2015; Bergman, 2007; Shul'pin, 2016; Shul'Pin et al., 2018). Furthermore, these catalysts continue to suffer from product over-oxidation or poor regioselectivity due to the generation of hydroxyl and peroxide radicals (Shteinman, 2015; Shul'pin, 2016; Shul'Pin et al., 2018; Soussan et al., 2016). This is problematic, given that the goal is to develop “gentler” processes with higher selectivity than existing methods.

There has thus been much interest in the enzymatic hydrocarbon activation which occurs in nature. Enzymes typically display excellent stereo- and regioselectivity. Having evolved within living organisms, they tend to function at temperatures and pressures close to ambient, in water-based environments, making use of readily-available oxidants such as molecular oxygen. There is ongoing research into catalysts that mimic enzymatic hydrocarbon oxyfunctionalisation by directing transformation through iron-oxygen intermediates, rather than typical oxidative addition mediated by platinum-group metals. However, enzymes are highly complex – the role of the larger protein structure surrounding the active site is known to be important, but often is either not fully understood, or cannot be easily replicated by

a synthetic catalyst (Bergman, 2007). Meanwhile, microorganisms capable of metabolising alkanes are well-known in the field of bioremediation. Alkanes (along with other aliphatic and some aromatic hydrocarbons) are readily available in nature. They are produced in low quantities by many insects, plants, and microorganisms, as well as emanating from human activities. Consequently, numerous microorganisms have evolved to take advantage of this niche carbon source. Multi-step enzymatic processes convert alkanes, via alcohol and aldehyde intermediates, into carboxylic acids suitable for β -oxidation (Abbasian et al., 2015). One such family of enzymes is the cytochromes P450, which also occur widely in fungi, plants, and animals. Since the 1960s, there has been substantial research into P450-mediated hydroxylation. Initially, most of this research focused on the metabolism of drugs, steroids, and carcinogens in the livers of humans and common laboratory test animals. However, there was also considerable research into bacterial cytochromes P450 capable of metabolising bioactive compounds such as camphor and barbiturates (Guengerich, 2019). In recent decades, there has been growing interest in the use of cytochromes P450 for biocatalysis, including alkane hydroxylation.

Although many cytochromes P450 exhibit some degree of substrate promiscuity, their specificity for alkane substrates over the oxidised reaction products tends to be high, as does their regio- and stereoselectivity. In this sense they offer significant advantages relative to existing chemical catalysts (Abbasian et al., 2015; Kaluzna et al., 2016; Sellés Vidal et al., 2018; Soussan et al., 2016). Compared to many catalysts under development, they also display comparable or higher turnover frequencies, under far milder conditions (Holtmann and Hollmann, 2016). Since the required process conditions are less harsh than those of traditional chemical processes, cytochrome P450-based processes could potentially reduce both energy costs and safety risks associated with alkane activation (Nielsen and Keasling, 2016). There is growing demand for chemicals produced in ways that minimise exploitation and harm to people, the environment, and scarce resources. This creates a need to develop processes for the petrochemical industry that offer long-term stability and sustainability (J. M. Choi et al., 2015; Ladkau et al., 2016). There is some debate regarding whether bioprocesses are universally more environmentally friendly than traditional chemical routes. They typically require less energy input and fewer process steps, and produce fewer emissions and toxic chemical waste streams (J. M. Choi et al., 2015). However, they can produce large quantities of other waste, particularly aqueous waste, and often have a large water footprint (S. Choi et al., 2015), thus their relative environmental burden, while promising, is case-specific. Regardless, biocatalysis remains environmentally appealing in the sense that the catalysts can be produced from renewable resources (Nielsen and Keasling, 2016). Based on their selectivity, potential for energy efficiency, and sustainability, many researchers have argued that oxidoreductases represent the future of industrial oxidation processes (Girvan and Munro, 2016; Holtmann et al., 2014; Holtmann and Hollmann, 2016; Lundemo and Woodley, 2015).

In the case of alkane oxidation specifically, CYP153A6 is a promising candidate. It is able to metabolise linear alkanes from pentane (Koch et al., 2009) through to undecane (Funhoff et al., 2006), thus covering a reasonable fraction of crude oil. It requires no genetic modification to accept these alkanes, which are already its preferred substrate – as suggested by its original discovery within a hexane scrubber (van Beilen et al., 2002) and confirmed by binding studies which demonstrated substantially reduced K_d values in the presence of sterically-hindering methyl groups (Funhoff et al., 2006). Crucially, CYP153A6 has demonstrated selectivities of over 95% for terminal hydroxylation, with activities of up to $60.8 \pm 10.6 \text{ mol}_{\text{substrate}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$ *in vitro* (Funhoff et al., 2006). It is therefore a very promising enzyme to develop for alkane biocatalysis.

1.2 What will it take to turn CYP153A6 into an industrial catalyst?

Although alkane hydroxylases show great promise for the conversion of alkanes to platform chemicals, they are still a long way from being suitable industrial catalysts for production of commodity products. The space time yields of alkane hydroxylase-based processes usually fail to meet minimum requirements for economic feasibility (Bernhardt, 2006; Lundemo and Woodley, 2015). Purified enzymes display low stabilities when used *in vitro*, which is problematic given the numerous complex process steps required for their production (de Carvalho, 2017; Schrewe et al., 2013a; Wachtmeister and Rother, 2016), especially since alkane hydroxylation targets the comparatively higher-volume, lower-cost bulk chemicals industry. Furthermore, most known cytochromes P450 require electrons

obtained from a cellular cofactor (usually NAD(P)H), often via one or two partner enzymes. All of this means that cytochromes P450 lend themselves towards *in vivo* processes. However, *in vivo* operations have their own set of challenges. Logically, the performance of a whole-cell biocatalyst might be expected to depend not only on the kinetics of the cytochrome P450 enzyme, but also on its expression levels, as well as the expression levels and efficient functioning of the redox partners that constitute the electron supply chain. The reaction relies on cellular metabolism to regenerate the NAD(P)H that provides it with reducing power; hence catalytic performance must be impacted to some degree by cell physiology. Alternatively, the reaction may be limited by the supply of oxygen (due to poor mixing of the gas and liquid phases) or alkane (due either to its low solubility in the aqueous phase, or its poor transport across the cell membrane). The cytochrome P450 is capable of converting the oxidant into harmful reactive oxygen species such as hydrogen peroxide (Bordeaux et al., 2012; Bühler and Schmid, 2004; Holtmann and Hollmann, 2016; Kadisch et al., 2017). Furthermore, living organisms are sensitive to hydrocarbon toxicity, so substrate and product inhibition thresholds are typically low (Holtmann and Hollmann, 2016; Kaluzna et al., 2016; Olaofo et al., 2013; Soussan et al., 2016). Finally, the performance of the process as a whole is a function of both the performance at a cellular level, and the culture density that can be achieved and maintained. The interactions between all these factors are poorly understood, since whole-cell processes are often developed by treating the microorganism as a “black box” whose internal machinery takes care of itself. Especially in the case of complex multi-component P450 systems, it is not clear which factors are most limiting to performance, or how those limitations could be successfully overcome.

There are thus many potential avenues of attack when it comes to improving the performance of a whole-cell biocatalyst, ranging from genetics (for example, directed evolution to modify specificity or kinetics), right through to bioreactor design (for example, varying air supply or agitation rate to improve oxygen transfer). This study is focused at the level of the host cell; in particular, it investigated expression levels of the cytochrome P450, cofactor regeneration capacity, and cross-membrane transport of alkane substrate and biotransformation products.

1.3 Project scope

After a thorough review of the literature, the following factors were identified as potentially significant limitations in the conversion of n-octane to 1-octanol by CYP153A6 in whole cells: intracellular concentration of the P450 enzyme, availability of oxygen for biotransformation and cell metabolism, transport of alkane substrate and alcohol product across the cell membrane, and regeneration of NADH cofactor.

The overarching objective of this study was to determine whether, and to what degree, the factors described above are limiting the biotransformation process in the whole-cell biocatalyst system. The characterisation of bottlenecks can direct future research towards the interventions likely to have the highest impact. Similarly, the comparison of strains and biocatalyst preparation techniques can differentiate between approaches which are worth pursuing further, and those which are of limited potential.

Creating sustainable, economical routes for the activation of linear alkanes would unlock significant opportunities in the petrochemicals industry. Such routes remain elusive due to the inherent lack of reactivity of the alkane backbone. Cytochromes P450 could overcome this challenge, forming the basis of renewable, mild, selective processes – but only if the limitations of these enzyme-based processes can be understood and overcome.

The scope of the study was limited to a single substrate, and n-octane was selected as the model; the assumption was that information regarding performance improvements at the cellular level would apply, at least to some degree, to other aliphatic hydrocarbons. The enzyme CYP153A6 was selected (along with its native redox partners, ferredoxin and ferredoxin reductase) due to its ability to target linear alkanes for terminal hydroxylation with high selectivity. The operon containing all three proteins was cloned into the pET28b+ plasmid and transformed into *E. coli* BL21DE3; alcohol dehydrogenase activity in *E. coli* is tightly regulated and appears only under anaerobic conditions (Holland-staley et al., 2000), meaning that this host should have negligible capacity to degrade the desired product. Some cells were

further modified by the addition of plasmids containing genes for the expression of glycerol dehydrogenase or the membrane transporter protein AlkL, or by permeabilising the membranes via physical or chemical methods. This allowed for the creation of whole-cell biocatalysts with varied capacities for cofactor regeneration or hydrocarbon transport. The study could thus test various combinations of enhanced P450 expression, enhanced cofactor regeneration, and enhanced n-octane transport. The influence of process conditions such as oxygen supply and cell density were also examined to a limited extent.

This study aimed to demonstrate that the performance of the CYP153A6 biocatalyst depends not only on the *availability* of enzyme, substrate, or cofactor, but on balancing the *interactions* between these components.

1.4 Structure of dissertation

This thesis begins with a review of the relevant literature around enzymatic alkane hydroxylation (Chapter 2), including the nature of cytochromes P450 and the CYP153 family, issues relating to *in vivo* enzyme catalysis, transport of hydrocarbons across cell membranes, and supply or regeneration of cellular cofactor. It was necessary to determine the state of knowledge regarding P450 processes and alkane hydroxylation processes generally, as well as the CYP153A6 system specifically. Why were the apparent activities reported in whole cells orders of magnitude lower than the maximum activities demonstrated by free enzymes? Why were so many systems levelling off before reaching the 1-octanol toxicity limits predicted by doping studies? Why was the application of dehydrogenases extremely beneficial in some systems, but unhelpful in others? Why was the application of dehydrogenases or the addition of cofactor proving to be beneficial at all, when theoretical models suggested that cellular metabolism was more than capable of supporting biotransformation targets? Numerous studies pointed to the potential benefits of enhanced cofactor regeneration or substrate transport, but there was limited indication of how these factors were interacting with each other or with cellular metabolism, and it was this gap that this project set out to address.

The experimental methods used in the study are then detailed in Chapter 3, covering the host system and the combinations of genes and plasmids that were utilised to create the various recombinant strains; procedures for the growth and preparation of the whole-cell biocatalyst; details of the biotransformation process; and analytical methods.

The results are presented and discussed concurrently in Chapters 4 and 5. These begin with aspects relating to P450 concentration: the effects of precursor concentration on CYP153A6 expression (Section 4.1), the relationship between heterologous expression load and CYP153A6 expression levels (Section 4.2), and the effect of the intracellular CYP153A6 concentration on the performance of the whole-cell biocatalyst (Section 4.3). This is followed by an account of experiments relating to oxygen availability, which was explored through variations in headspace volumes and headspace replacement (Section 4.4). Cofactor regeneration capacity and hydrocarbon transport are discussed together, as it was found that these factors interacted with one another in significant ways. Methods of permeabilising the cell membrane were compared (Section 5.1). The effects of dehydrogenase co-expression were explored in intact cells (Section 5.2), mechanically broken cells (Section 5.3), and chemically permeabilised cells (Section 5.4). Section 5.5 elaborates on the effects the various treatments had on biocatalyst stability and by-product formation. Overarching findings relating to dehydrogenase co-expression and cell permeabilization are summarised in Section 5.6. Finally, Section 5.7 covers experiments with strains co-expressing the membrane channel protein AlkL for enhanced hydrocarbon transport.

The thesis concludes with a summary of the results (Chapter 6), which are discussed in context of prior knowledge from the literature. The significance of the results is interrogated, leading to recommendations for future work on the CYP153A6 process, as well as broader insights that may be applicable to the P450 space more generally.

2 Literature Review

2.1 Introduction

This project centres on heterologous expression of the oxidoreductase enzyme CYP153A6 and its electron partner proteins, ferredoxin and ferredoxin reductase, in resting *Escherichia coli* BL21(DE3) whole cells, in order to carry out the biotransformation of linear alkanes, specifically n-octane to 1-octanol.

The literature review contextualises the scientific and industrial interest in cytochromes P450 more broadly, by providing an overview of the prevalence and diversity of alkane hydroxylation in nature. This is followed by a detailed discussion of the forms and functions of the cytochrome P450 family of monooxygenases, leading into a summary of the literature concerning CYP153A6 specifically. CYP153A6 is then compared to other alkane hydroxylases that have received attention in the literature, highlighting the unique properties that make this enzyme worth developing. Further context is provided by summarising the adaptation and application of monooxygenases for commercial use, including a discussion of the various standards for predicting industrial success.

The relative strengths and weaknesses of *in vivo* and *in vitro* operation are compared, considering the particular nature of CYP153A6 and its redox partners. Current approaches to whole-cell biotransformation and their key challenges are discussed, including issues relating to choice of microbial host, expression of heterologous proteins, culture conditions and their effects on cell physiology, and process operation in whole-cell systems.

Finally, within the context of *in vivo* operations, key aspects of the hydrocarbon oxyfunctionalisation reaction are discussed. These include issues relating to availability and toxicity of hydrocarbon substrates and products, the manipulation of cell membranes to enhance substrate accessibility and product export, the regeneration of nicotinamide cofactors, and the supply of oxygen for maintenance metabolism and for biotransformation.

2.2 Natural Context

This section provides an overview of alkane metabolism in nature, including its prevalence, purpose, and mechanisms. It introduces the various types of enzyme responsible for hydroxylating alkanes. An overview of the cytochrome P450 superfamily is then provided, including the common features of P450 enzymes, the reaction cycle for terminal hydroxylation by P450s, and how the vast numbers of P450s are differentiated and classified. The intersection of these two spaces contains the cytochromes P450 capable of hydroxylating alkanes, which are briefly discussed and compared. Finally, information specific to CYP153A6 is presented.

2.2.1 Oxidoreductases, Monooxygenases, and Alkane Hydroxylases

The capacity to hydroxylate alkanes is found in a variety of organisms and is carried out by a range of different enzymes. All these enzymes are oxidoreductases and, with the exception of those found in anaerobic microorganisms and a few species of fungi, most are monooxygenases. Monooxygenases rely on a metallic centre or a flavin moiety to activate molecular oxygen, incorporating one oxygen atom into the substrate molecule while reducing the remaining oxygen atom to water.

Key monooxygenase subgroups include the haem-based cytochromes P450 and the flavin-dependent monooxygenases (FMOs). Most P450s and FMOs are not alkane hydroxylases. P450s catalyse more than twenty different kinds of reaction besides hydroxylation, including desaturation, denitrification, demethylation, decarboxylation, epoxide formation, coupling of dimers, formation and contraction of aromatic rings, oxygenation or removal of heteroatoms, and intramolecular rearrangement (Bernhardt, 2006; Girvan and Munro, 2016; Kelly and Kelly, 2013; Sellés Vidal et al., 2018). They play a key role in sterol synthesis and in the metabolism of xenobiotics. Examples of their natural functions include the degradation of antibiotics and phytotoxins in bacteria; the synthesis of complex secondary metabolites

in plants and fungi, including flavonoids, alkaloids, and hormones; and the metabolism of drugs and production of steroids in humans (Ayala and Torres, 2004; Grogan, 2011; Guengerich and Munro, 2013; Kelly and Kelly, 2013). FMOs carry out comparable functions, such as the degradation of (poly)aromatic compounds and the production of secondary metabolites. For example, they are involved in the metabolism of nicotine and caffeine, have been associated with Huntington's disease and Alzheimer's disease in humans, are responsible for the gall-causing activity of certain plant pathogens, and play a role in the regulation of cell shape and motility in various bacteria. Types of reaction catalysed by FMOs include hydroxylation, epoxidation, sulfoxidation, halogenation, and Baeyer-Villiger oxidation (Huijbers et al., 2014). Given the wide distribution of monooxygenases across all kingdoms of life, and their diverse roles in the synthesis and degradation of complex or challenging metabolites in nature, it is not surprising that this type of enzyme would be heavily involved in the metabolism of alkanes and other hydrocarbons.

The intersection of monooxygenases, cytochromes P450, and alkane activation is represented schematically in Figure 2.1. Various enzymes, which are discussed in more detail in Sections 2.2.2 and 2.2.3, are also put into context.

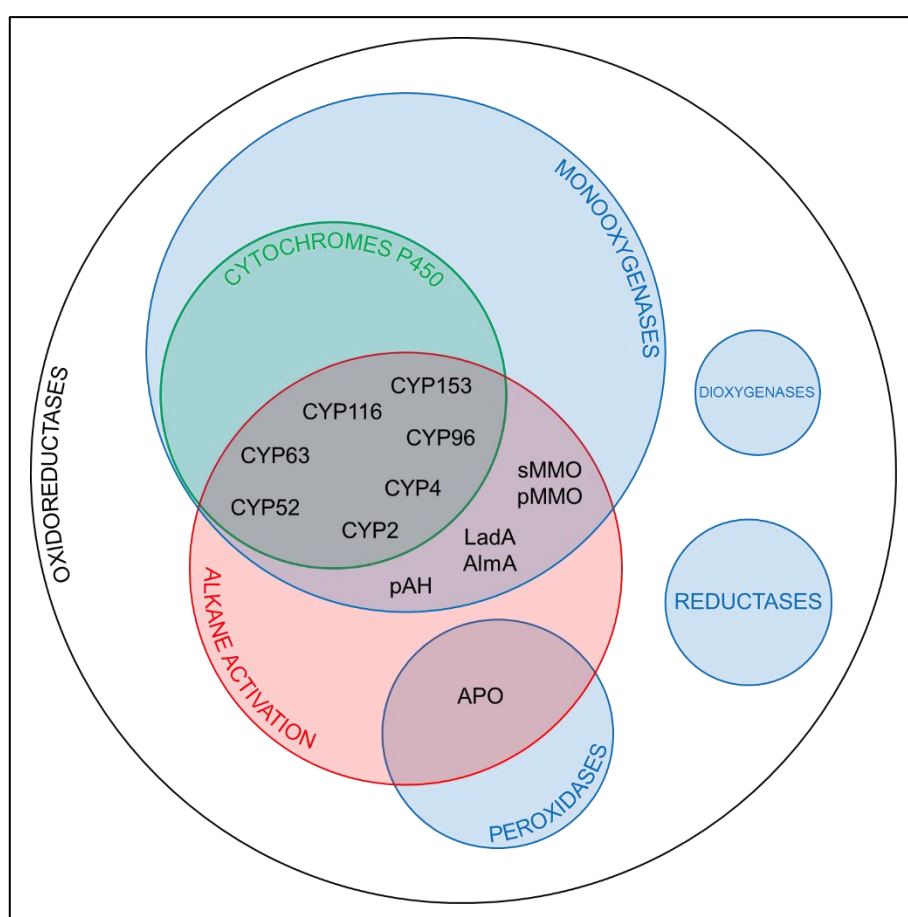


Figure 2.1: Cytochromes P450 in the broader context of the oxidoreductase supergroup

2.2.2 Alkane hydroxylation in nature

Prevalence of alkanes in the environment

Microbial alkane degradation has been noted since early in the 20th Century, when it was discovered that various species of mycobacteria and pseudomonads were responsible for the disappearance of oil slicks. However, although alkane degraders thrive in oil-polluted environments, they are also prevalent in uncontaminated sediments. Studies of samples from soils contaminated by petrol, diesel, or coal tar, as well as samples from uncontaminated soils, led to the isolation of sixty-one species of bacteria capable of degrading hexadecane, spanning six different genera. This suggests that the capacity to

degrade alkanes first evolved to take advantage of naturally-occurring hydrocarbons (Bouchez-Naitali et al., 1999; Rojo, 2009; Van Beilen and Funhoff, 2007).

Alkanes are naturally present in low concentrations in most soil and water environments. Certain strains of cyanobacteria produce C₁₅ – C₁₉ alkanes and alkenes to modulate their membrane fluidity, thus enhancing growth at low temperatures. Some microorganisms utilise alkanes as a metabolic overflow product. Plants produce alkanes as a component in their cuticular waxes, which are vital to moisture retention, protection against UV damage, and defence against bacteria, fungi, and insects. Alkanes also play a role in various insect pheromones (Berla et al., 2015; Schirmer et al., 2010; Zhang et al., 2020). However, the widespread exploitation of petroleum by humans since the Industrial Revolution means that microbial communities are exposed to hydrocarbons more frequently and at higher concentrations (Abbasian et al., 2015), creating new environmental niches for alkane-degrading organisms.

Role of alkane degradation in microbial metabolism

Alkane degradation capacity appears across a range of gram-positive and gram-negative bacteria. The genera *Mycobacterium* and *Pseudomonas* are particularly well-represented, although roughly twice as many *Actinobacteria* as *Proteobacteria* have been found containing alkane hydroxylases. Alkane degraders have also been found in the phyla *Bacteroidetes*, *Chlorobi*, *Deinococcus-Thermus*, and *Firmicutes*. Alkane degradation capacity is common amongst yeasts and other fungi, and has been noted in some algal species. The majority of known alkane degraders are mesophiles, but numerous extremophiles, spanning multiple genera, have been identified via bioprospecting in volcanic environments and oil reservoirs. Known obligate alkanotrophs are rare, and most have been isolated from marine environments. Examples include *Alcanivorax borkumensis* and *Thalassolituus oleivorans*, which appear to have evolved as a direct response to anthropogenic petroleum-based pollution. The majority of organisms shown to be capable of degrading hydrocarbons are also able to metabolise more typical carbon sources, such as sugars, which they tend to prefer over alkanes (Nie et al., 2014; Rojo, 2009; van Beilen et al., 2002; Van Beilen and Funhoff, 2007; Wentzel et al., 2007).

Most microorganisms hydroxylate linear alkanes at the terminal carbon atom to form primary alcohols. These undergo further oxidation, first into aldehydes and then into carboxylic acids, which can be utilised for the synthesis of acyl-coenzyme A to feed into the cell's β -oxidation pathway (Wentzel et al., 2007). Alternatively, some species produce ω -hydroxylic or α - ω -dioic acids rather than aldehydes (Abbasian et al., 2015). While terminal oxidation of alkanes is the most common enzymatic approach, subterminal oxidation has also been reported, in some cases coexisting with terminal oxidation. Cells convert secondary alcohols into ketones, which are then converted into esters by Bayer-Villiger monooxygenases, thus functioning as a storage mechanism for excess carbon (Rojo, 2009).

When it comes to oxyfunctionalisation of C-H bonds, the enzymes involved in anabolism tend to have a very narrow substrate scope, while those involved in catabolism typically accept a much broader range of substrates (Hollmann et al., 2011). Hence the capacity to hydroxylate linear alkanes is frequently accompanied by activity on cycloalkanes, alkylbenzenes, and fatty acids in species of *Acinetobacter*, *Alcanivorax*, *Burkholderia*, *Candida*, *Caulobacter*, *Mycobacterium*, *Rhodococcus*, *Sphingomonas*, and *Yarrowia*. Many strains capable of methane oxidation can also hydroxylate short-chain halogenated alkanes, cyclic alkanes, and alkenes (Soussan et al., 2016; Van Beilen and Funhoff, 2007). The species *Rhodococcus fascians* 154-S, which is able to grow on C₆ – C₁₆ linear alkanes as sole carbon source, can also be cultured on the branched alkane squalane (van Beilen et al., 2002). Numerous *Alcanivorax* species can degrade branched-chain alkanes (Rojo, 2009); the degradation of branched alkanes and alkenes proceeds either via epoxidation or via terminal oxidation (Abbasian et al., 2015). Some bacteria are also able to metabolise cycloaliphatic and aromatic hydrocarbons. *Rhodococcus erythropolis* can metabolise cyclohexane and naphthalene as sole growth source, but grows best on diesel, which contains a mixture of linear alkanes, cycloalkanes, and aromatics (Sazykin et al., 2019). *Acinetobacter* species can convert cyclohexane into adipic acid; monooxygenases are responsible for two of the six steps in the process. *Rhodococcus ruber* SC1 follows a similar route to convert cyclododecane into dodecanedioic acid (Abbasian et al., 2015).

Although the majority of alkane degraders operate under aerobic conditions, the ability to metabolise alkanes has been observed in both facultative and obligate anaerobes (Abbasian et al., 2015; Nie et

al., 2014). These organisms are slow-growing, with doubling times in some cases exceeding three days (Abbasian et al., 2015; Wentzel et al., 2007). Postulated mechanisms of anaerobic alkane activation include fumarate addition, subterminal hydroxylation followed by carboxylation to fatty acids, and dismutation of oxygenated nitrogen species (Ji et al., 2019; Rojo, 2009; Wentzel et al., 2007). Substrate ranges are usually narrow: C₃ – C₄ for the sulphate-reducing *Desulfococcus* BuS5, C₆ – C₈ for the denitrifying *Azoarcus* sp. HXN1, and C₁₂ – C₂₀ for the sulphate-reducing *Desulfobacterium* Hdx3 (Ji et al., 2019). Phototrophs such as *Blastochloris sulfovirdis* and *Pelobacter acetylenicus* have also shown the ability to anaerobically degrade hydrocarbons (Abbasian et al., 2015). Certain microbial communities have demonstrated anaerobic methanogenic degradation of C₁₆ – C₂₀ n-alkanes; *Smithella* and *Desulfatibacillum* species appear to be the ones carrying out the alkane degradation, and are assumed to produce carbon dioxide and formate which is then used by species such as *Methanocalculus* (Ji et al., 2019). Methane oxidation has also been observed under anaerobic conditions (Rojo, 2009); evidence suggests that this proceeds via reversal of the methanogenesis pathway. Methyl-coenzyme M reductase carries out the initial oxidation step via coupling of methane with coenzyme M, and the resulting metabolic intermediate can be converted to methanol by a variety of enzymes (Mueller et al., 2014).

Types of alkane hydroxylases

The enzymes responsible for the initial step in alkane degradation pathways are typically hydroxylases. Alkane hydroxylases can be differentiated into a number of types based on substrate range and active site composition. Figure 2.2 presents the different types of alkane hydroxylase, arranged in order of substrate preference from short to long chain: particulate methane monooxygenases (pMMO), soluble methane monooxygenases (sMMO) and their close relatives the propane monooxygenases (PMO) and butane monooxygenases (BMO), aromatic peroxygenases (APO), particulate alkane hydroxylases (pAH), cytochromes P450 (P450), and long-chain alkane hydroxylases such as LadA and Alma. Alkane hydroxylases are discussed with respect to their relevance to increasing chain length.

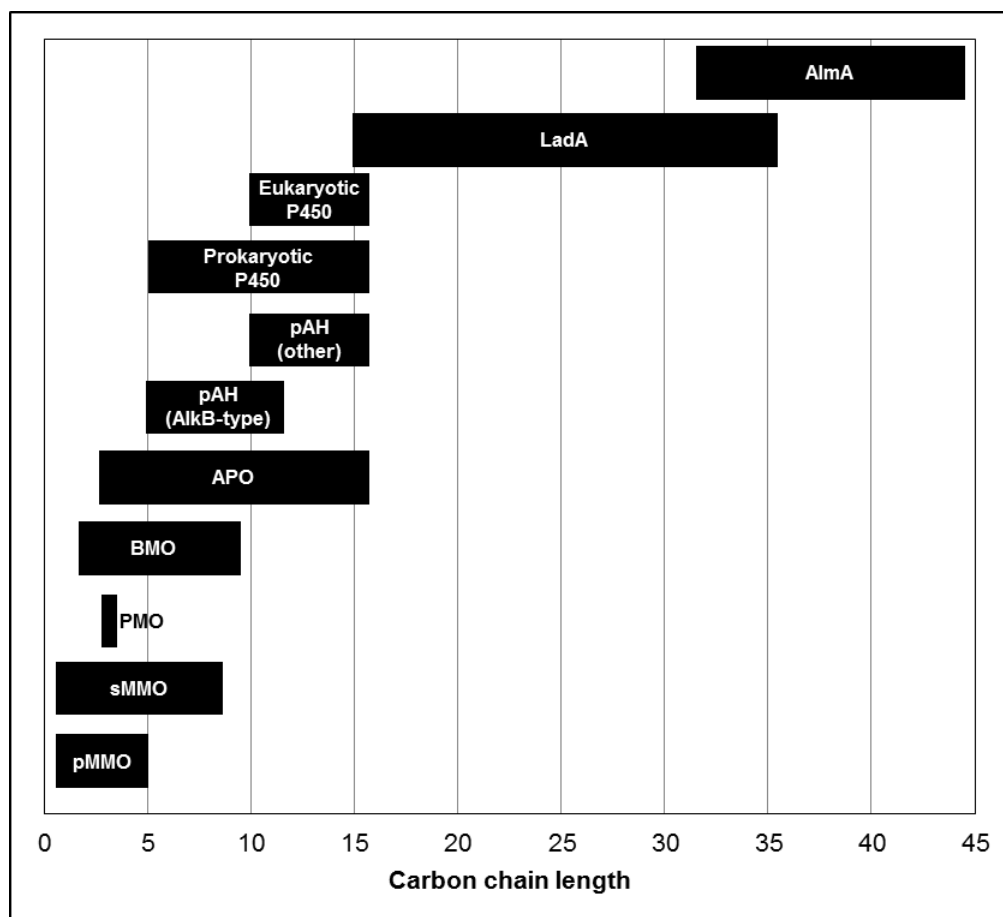


Figure 2.2: Typical preferred substrate ranges of the various types of alkane hydroxylases. *pMMO*: particulate methane monooxygenase; *sMMO*: soluble methane monooxygenase (Abbasian et al., 2015; Ayala and Torres, 2004; Bordeaux et al., 2012; Rojo, 2009; Van Beilen and Funhoff, 2007); *PMO*: propane monooxygenase; *BMO*: butane monooxygenase (Rojo, 2009); *APO*: aromatic peroxygenase (Bordeaux et al., 2012; Peter et al., 2011); *pAH*: particulate alkane hydroxylase (Bordeaux et al., 2012; Rojo, 2009; van Beilen et al., 2002; Van Beilen and Funhoff, 2007); *P450*: cytochrome P450 (Abbasian et al., 2015; Bordeaux et al., 2012; Girvan and Munro, 2016; Rojo, 2009); *LadA* and *AlmA* are soluble flavin-based monooxygenases (Bordeaux et al., 2012; Nie et al., 2014; Rojo, 2009; Wentzel et al., 2007).

The shortest alkane chain lengths ($C_1 - C_4$) are targeted by the methane, propane, and butane monooxygenases. Methane monooxygenases (MMOs) are capable of oxidising methane to form methanol, which is converted into formaldehyde by alcohol dehydrogenases. Formaldehyde can feed into anabolic pathways, or can be used as an energy source via conversion into formic acid and then carbon dioxide (Rojo, 2009). MMOs can also handle other gaseous alkanes, and many can degrade linear alkanes with chain lengths up to n-octane; some are promiscuous and can oxidise a variety of hydrophobic molecules, such as alkenes, aromatics, and acyclic or heterocyclic compounds (Abbasian et al., 2015; Ayala and Torres, 2004; Bordeaux et al., 2012; Van Beilen and Funhoff, 2007). Particulate MMOs contain two copper atoms in their active sites and can be found in almost all aerobic methanotrophs. However, some of these organisms also have genes coding for a soluble MMO based on non-haem diiron; these sMMOs are expressed under conditions of low copper availability (Bordeaux et al., 2012; Rojo, 2009). In an evolutionary sense, pMMOs and sMMOs are not closely related (Ayala and Torres, 2004). However, studies have identified multiple groups of soluble diiron monooxygenases similar to sMMOs, comprising dozens of novel sequences (Van Beilen and Funhoff, 2007). Characterised examples include butane monooxygenases capable of degrading linear alkanes from ethane to nonane, found in *Mycobacterium vaccae* sp. JOB5, *Nocardioides* sp. CF8, and *Pseudomonas butanovora*; and propane monooxygenases in *Gordonia* sp. TY-5, *Mycobacterium* sp. TY-6, and *Pseudonocardia* sp. TY-7, which appear limited to propane oxidation only (Rojo, 2009).

Most of the enzymes that oxidise alkanes of intermediate chain length ($C_5 - C_{15}$) fall into one of two categories: the haem-based cytochromes P450, or the non-haem diiron-based particulate alkane hydroxylases. Particulate alkane hydroxylases (pAHs) are membrane-associated and require additional

proteins to facilitate their interaction with the cellular cofactors which supply reducing equivalents for alkane activation (Bordeaux et al., 2012; Van Beilen and Funhoff, 2007). Some pAHs, of which AlkB from *Pseudomonas oleovorans* is the most well-studied example, carry out terminal oxidation of C₅ – C₁₂ linear alkanes. Dozens of AlkB homologues have been identified, with considerable sequence diversity (Rojo, 2009). Even a small community of bacteria, isolated from the same environment, will produce a range of AlkB homologues with sequence identities as low as 51%. On the other hand, different strains, isolated from distinct environments, can contain AlkB homologues with sequence identity of up to 99.8% (van Beilen et al., 2002). Other pAHs, distinct from AlkB, have been found to hydroxylate C₁₀ – C₁₆ linear alkanes; functional expression and knockout studies suggest that these pAHs are even more prevalent than the AlkB-type pAHs (Van Beilen and Funhoff, 2007).

The cytochromes P450 are a superfamily of oxidoreductases whose substrates include hydrocarbons, xenobiotics, steroids, and prostaglandins. The active site of all P450s contains a haem moiety, which receives reducing equivalents from cellular cofactors, via flavin-based mediator proteins. P450s can be classified into over 100 families, of which several show an affinity for linear alkanes, most notably the yeast-based CYP52 family and the bacterial CYP153 family. Eukaryotic P450s tend to be membrane-bound and display low catalytic efficiency; their preferred substrate range is C₁₀ – C₁₆. Prokaryotic P450s are soluble and typically accept C₅ – C₁₆ linear alkanes (Abbasian et al., 2015; Bordeaux et al., 2012; Girvan and Munro, 2016; Rojo, 2009).

Besides the pAHs and P450s, soluble, H₂O₂-dependent aromatic peroxygenases have also been isolated from the agaric mushrooms *Agrocybe aegerita* and *Coprinellus radians*. These enzymes, which are notable for their stability in organic solvents, can hydroxylate C₃ – C₁₆ alkanes, usually at the secondary or tertiary carbon atom (Bordeaux et al., 2012).

Enzyme systems for the metabolism of C₁₆₊ linear alkanes are not as well-studied as those for shorter alkanes (Rojo, 2009). LadA, a soluble flavin-based monooxygenase from *Geobacillus thermodenitrificans* NG80-2, is known to carry out terminal oxidation of C₁₅ – C₃₆ alkanes (Bordeaux et al., 2012; Nie et al., 2014; Rojo, 2009; Wentzel et al., 2007). A similar enzyme, AlmA, allows *Acinetobacter* sp. DSM 17874 to grow on linear alkanes from C₃₂ – C₄₀. A number of species possess genes homologous to those coding for AlmA, including *Acinetobacter* sp. RAG-1, *Marinobacter aquaeolei* sp. VT8, *Oceanobacter* sp. RED65, *Nocardia farcinica* IFM10152, and several members of the genera *Mycobacterium*, *Photobacterium*, *Psychrobacter*, and *Ralstonia* (Wentzel et al., 2007). There are also several putative dioxygenases which can handle C₁₀ – C₃₀ linear alkanes (Abbasian et al., 2015).

Most alkane-metabolising bacteria contain multiple alkane hydroxylases. Each enzyme accepts a limited but complementary range of linear alkanes (and sometimes other hydrophobic substrates), endowing the cell with a broad substrate range overall – some cultures have demonstrated the capacity to metabolise alkanes from gaseous butane through to solid paraffin waxes (van Beilen et al., 2002; Van Beilen and Funhoff, 2007; Wentzel et al., 2007). 19% of microorganisms known to contain an AlkB homologue, and 36% of microorganisms known to contain a member of the CYP153 family, also carry multiple other alkane hydroxylases, with sequence similarities that can be as low as 30% (Nie et al., 2014). Genome sequencing suggests that *Mycobacterium tuberculosis* and *Streptomyces coelicolor* may contain up to twenty distinct cytochrome P450s; ten different CYP52 genes have been successfully cloned from *Candida tropicalis* ATCC 20336, and numerous species of bacteria have been found to contain multiple distinct AlkB homologues (Van Beilen and Funhoff, 2005). Many organisms contain both pAHs and P450s, although there are plenty of bacteria that appear to contain only pAHs, and only a few that contain only P450s, such as *Rhodococcus rhodochrous* NCIMB 12566 (7E1C) and several strains of *Mycobacterium* (Nie et al., 2014; van Beilen et al., 2002). The ratio of AlkB homologues to P450s varies substantially between marine, freshwater, and terrestrial environments (Nie et al., 2014). However, an organism's preference for pAHs or P450s has little bearing on its ability to degrade C₁₆₊ linear alkanes (van Beilen et al., 2002), suggesting that enzymes such as LadA and AlmA coexist alongside both pAHs and P450s. Alkane hydroxylases appear to spread easily via horizontal gene transfer, presumably due to their tendency to generate reactive oxygen species under conditions of oxidative stress (Nie et al., 2014; Sazykin et al., 2019).

2.2.3 Cytochromes P450

Diversity and conserved features of the P450 enzyme class

The organisms, enzymes, and metabolic processes responsible for alkane hydroxylation first attracted interest through research into bioremediation of human-associated pollutants such as petroleum (Bouchez-Naitali et al., 1999). Research into cytochromes P450 began for completely unrelated reasons, in the field of drug metabolism. The first P450 to be reported in the literature was isolated from rat livers, where it was responsible for hydroxylating 17-hydroxyprogesterone (Omura and Sato, 1964; cited in Guengerich, 2019). Early research focused exclusively on mammalian P450s (Guengerich, 2019; Meunier et al., 2004). As the ubiquity and diversity of cytochromes P450 became more apparent, there was a growing interest in harnessing these enzymes for diagnostic applications and industrial synthesis. The research space for P450 biocatalysis expanded considerably, and remains very active.

Cytochromes P450 are found across all domains of life, sometimes in abundance: humans, for example, have 57 distinct P450s; thale cress has 286. Over 18 000 sequences have been found bearing the genetic “signature” of the superfamily; most of the associated proteins have yet to be characterised, and their physiological functions remain unknown. The cytochromes P450 that have been studied are known to carry out a wide range of oxidation reactions, including decarboxylation, dehalogenation, dehydration, demethylation, desaturation, dimer couplings, epoxidation, heteroatom dealkylation, heteroatom oxygenation, hydroxylation, isomerisation, nitration, oxidative deamination, ring formation, ring coupling, and ring contraction. P450 reduction reactions appear to be much rarer, but P450s have been observed to reduce nitric oxide in fungal and animal cells. A handful of P450s are even suspected of catalysing acid-base chemistry, such as the mammalian P450s which carry out hydrolysis in a manner similar to the enzyme phospholipase D, and the *Saccharomyces coelicolor* P450s which carry out the non-redox rearrangement of bicyclic pentanone (Bernhardt, 2006; Girvan and Munro, 2016; Guengerich and Munro, 2013; Hannemann et al., 2007; Kelly and Kelly, 2013; Sellés Vidal et al., 2018). The genetic diversity of cytochromes P450 reflects this remarkable range of functions. Individual enzymes can exhibit sequence similarities of less than 16%. Grouping P450s into families of genes sharing at least 40% sequence identity produces close to a thousand different families: 19 among vertebrates, 67 among insects, 127 among plants, well over 300 among bacteria, and about 400 among fungi (Durairaj et al., 2016; Hannemann et al., 2007; Meunier et al., 2004).

Despite their low sequence similarity overall, the amino acid content of the P450 active site is highly conserved across a broad range of P450s (Gricman et al., 2015; Hannemann et al., 2007; Munro et al., 2013; Van Beilen et al., 2005). The Phe-XX-Gly-Xb-XX-Cys-X-Gly motif is found throughout the superfamily; of the variable residues, glutamine and threonine occur frequently (Harris et al., 2018; McIntosh et al., 2014; Meunier et al., 2004). Mutation of the cysteine or glycine residues has been shown to cause complete protein deactivation (Munro et al., 2013; Zhang et al., 2020). P450 activity is based on the haem moiety – an iron atom coordinated by four equatorial pyrrole ligands and an axial thiolate ligand – bound to a cysteine residue. Cysteine thiolate is a good electron donor, lowering the reduction potential in the active site, and encouraging the protonation of iron-oxygen intermediates necessary for O-O bond scission. Cysteine thiolate is also the cause of the eponymous absorbance spectrum that is characteristic of all cytochromes P450, which peaks at 450 nm when the protein is exposed to carbon monoxide (Bernhardt, 2006; Gricman et al., 2015; Guengerich et al., 2009; Guengerich and Munro, 2013; Hannemann et al., 2007; McIntosh et al., 2014; Meunier et al., 2004; Munro et al., 2013; Sellés Vidal et al., 2018).

The active site is contained within a triangular prism-shaped fold, which is formed by a small domain consisting of four or five β -sheets and a larger domain usually constructed from 12 α -helices. Like the composition of the active site itself, this fold topology is highly conserved between P450s, even when overall sequence similarity is low (Fiorentini et al., 2018; Fürst et al., 2019; Gricman et al., 2015; Meunier et al., 2004; Munro et al., 2013). By contrast, surface residues and regions in and around the substrate access channel vary widely, even between closely related P450s. These parts of the protein are known to play key roles in substrate recognition and binding, although exact structure-function relationships are still opaque in many cases (Arnold et al., 2012; Fiorentini et al., 2018; Fürst et al., 2019; Hannemann et al., 2007; Jung et al., 2016; Meunier et al., 2004). Variations in surface residues also affect

thermostability and solvent tolerance; monooxygenases tend to be extremely flexible, undergoing significant conformational changes and long-range movement of residues (Fürst et al., 2019), but thermostable P450s are substantially more compact, less flexible, and richer in salt-bridged networks, due to mutations in peripheral residues dispersed throughout the protein (Harris et al., 2018; McIntosh et al., 2014). Irrespective of other variations in topology, the nucleotide binding domain responsible for interactions with electron donors tends to be fairly well conserved, as it is among all NAD(P)H-dependent oxidoreductases (Bernhardt, 2006; Sellés Vidal et al., 2018).

Reaction cycle and electron transport chain

This project focuses on terminal hydroxylation of n-octane. Numerous cytochromes P450 are capable of catalysing terminal hydroxylation, using molecular oxygen as oxidant and the cellular cofactor NAD(P)H as a source of reducing equivalents. Figure 2.3 depicts the relevant P450 reaction cycle. When a cytochrome P450 enzyme is in its resting state (1), the iron atom of the haem group is in a ferric, low-spin form. A molecule of water functions as a sixth ligand, taking up a position opposite the cysteine thiolate ligand and loosely coordinating with the haem. The substrate displaces this water molecule, shifting the iron atom to a high-spin form (2), with an associated shift in redox potential that promotes electron acceptance; the reaction cycle is thus initiated by substrate binding. The first electron to be transferred to the haem reduces the iron atom from ferric to ferrous form (3). In its Fe^{2+} state, the haem is capable of binding molecular oxygen (4). Transfer of a second electron generates a negatively charged ferric-peroxo intermediate (5), which is rapidly protonated to generate a ferric-hydroperoxo intermediate (6). A second protonation step causes heterolytic cleavage of the dioxygen bond, resulting in the release of a water molecule and the formation of a high-valent iron-oxo intermediate (7). This short-lived electrophilic species is responsible for activation of the substrate, resulting in formation of the hydroxylated product and return of the iron atom to a ferric state (8); the most likely reaction mechanism is hydrogen extraction and subsequent oxygen rebound. Interactions between the hydroxylated product and key active site residues promote a polar environment, drawing water molecules into the active site, thus displacing the product and returning the enzyme to its resting state (1) (Bordeaux et al., 2012; Guengerich and Munro, 2013; Meunier et al., 2004).

In addition to the main reaction cycle, there are several side reactions which can occur. After the initial binding of oxygen, the resulting ferric-oxo intermediate (4) can dissociate to release a superoxide anion. However, this occurs at an extremely low rate, since the ferric-oxo species is fairly stable at room temperature (Meunier et al., 2004). Similarly, although the oxygen atom from the reactive iron-oxo species (7) can be hijacked to form a water molecule instead of being inserted into the substrate, this requires two electrons and two hydrogen atoms to be delivered in rapid succession, which is difficult for the electron transport chain to achieve under most circumstances. A more common side reaction is the misprotonation of the ferric-hydroperoxo intermediate (6), leading to release of hydrogen peroxide. This process can also be reversed, making it possible to generate the reactive species without relying on electron donors, via application of H_2O_2 instead of oxygen. In many cases, the associated haem degradation decreases the overall efficiency of this reaction pathway, but several thermophilic P450s have evolved to take advantage of this so-called “peroxide shunt” (Harris et al., 2018; Holtmann et al., 2014; Meunier et al., 2004; Munro et al., 2013).

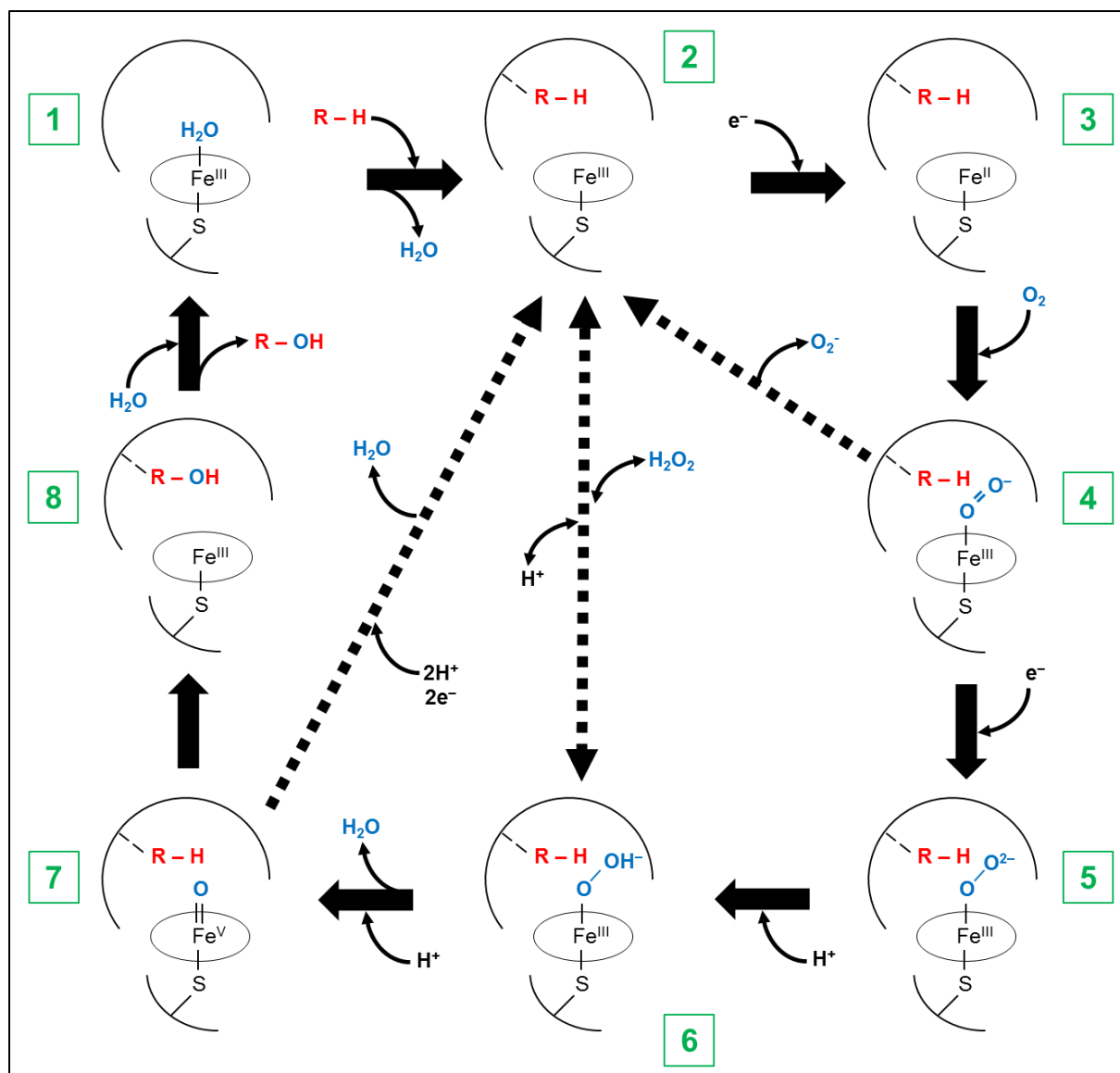


Figure 2.3: Reaction cycle of cytochrome P450s (schematic based on reviews by Meunier et al. (2004) and Bordeaux et al. (2012)).

The transfer of electrons and protons can be several orders of magnitude slower than the binding of substrate or oxygen; the transfer of the second electron is a particularly slow step (Meunier et al., 2004; Munro et al., 2013). Mechanisms of protonation are not well-defined, although the threonine residue present in many P450 active sites is thought to play a key role (Meunier et al., 2004). The ultimate source of electrons is usually the cellular cofactor NAD(P)H, but P450s require a single electron to be delivered before and after the oxygen binding step, and NAD(P)H provides reducing equivalents in the form of hydrides. Therefore, the cofactors cannot interact directly with the P450, and the enzyme relies on flavin-based mediator proteins for electron transfer (Holtmann et al., 2014; Sellés Vidal et al., 2018). P450-mediator interactions require strong organisation in the form of salt bridges and hydrogen bonds; multiple amino acids will be involved in the movement of electrons from the mediator binding domain to the haem domain (Meunier et al., 2004).

A variety of mediator protein systems have evolved to service the different cytochromes P450. Common systems consist of a ferredoxin or flavodoxin mediator combined with a ferredoxin reductase, or a cytochrome b5 mediator in combination with a cytochrome P450 reductase (CPR) or cytochrome b5 reductase. Some P450s interact directly with either CPR or a phthalate-family oxygenase reductase, and do not require a second mediator protein. In certain cases, the reductase or mediator domain will be naturally fused with the P450 domain into a single polypeptide chain, but multi-protein systems are more common (Hannemann et al., 2007): product over-oxidation is more prevalent in single-complex

systems as opposed to systems with an electron transport chain, and multiple proteins also provide cells with multiple points of metabolic control (Bühler and Schmid, 2004; Holtmann et al., 2014). Often, a particular electron transport system can service multiple types of P450, sometimes even heterologous ones; but many P450s have a preferred transport system, and their catalytic efficiency, selectivity, or even substrate specificity may be affected if they are forced to utilise a different system (Bernhardt, 2006; Durairaj et al., 2016; Fiorentini et al., 2018; Hannemann et al., 2007).

Classification of cytochromes P450

These differences in electron transfer systems directly inform how cytochromes P450 are classified. The substrate promiscuity that many cytochromes P450 exhibit, and the diversity of reaction types that they carry out (both as a superfamily and, in some cases, individually) makes a reaction-based classification system impractical. Rather than by reaction type, a convenient way of differentiating P450s is by considering their location in the cell, the architecture of their electron transport chains, and the types of cofactor, reductase, and mediator proteins that they interact with, as outlined in Figure 2.4.

The simplest distinction is between prokaryotic systems, which tend to be soluble and cytosolic, and eukaryotic systems, in which some or all of the components are membrane-bound. Eukaryotic P450s are typically localised to the endoplasmic reticulum, but some are found in the mitochondria (Hannemann et al., 2007; Kelly and Kelly, 2013; Munro et al., 2013).

Adjacent to this distinction, five major types of electron transport architecture can be distinguished: (A) systems consisting of a separate P450, reductase, and mediator protein; (B) systems consisting of only a P450 and a separate reductase protein; (C) systems where the P450 and mediator domains are fused into a single polypeptide, while the reductase domain is a separate protein; (D) systems where the P450 and reductase domains are fused, and no additional mediator protein is required; and (E) P450s that require neither mediator proteins nor reductases (Soussan et al., 2016).

The P450 superfamily can be more finely divided into ten classes based on the exact type of reductase and mediator that the P450 interacts with (Hannemann et al., 2007).

Class I P450s interact with ferredoxin mediator proteins, which transfer electrons from reductases based on the flavin adenine dinucleotide (FAD) moiety, which in turn can derive reducing equivalents from NADH, NADPH, or both. Most bacterial P450s fall into Class I. They utilise a variety of ferredoxins and their associated ferredoxin reductases, usually of the [2Fe-2S] cluster type, but in some cases [3Fe-4S] and [4Fe-4S] types. There are also some eukaryotic Class I P450s, which are bound to the inner membrane of the mitochondria. In contrast to the diverse electron transfer systems serving prokaryotic Class I P450s, eukaryotic P450s typically rely on a membrane-associated adrenodoxin reductase, with a soluble adrenodoxin shuttling electrons between the reductase and the P450. Bacterial and mitochondrial ferredoxins are usually not interchangeable in heterologous systems. Typical examples of Class I are CYP101, also known as P450cam, which allows *Pseudomonas putida* to metabolise camphor and which has been extensively studied; and members of the CYP11 family involved in the synthesis of steroid hormones in humans (Bernhardt, 2006; Girvan and Munro, 2016; Hannemann et al., 2007).

Class II P450s utilise either cytochrome P450 reductase (CPR) or cytochrome b5 reductase, in some cases with cytochrome b5 as a mediator protein. Most eukaryotic P450s fall into Class II. In mammals, these P450s are deployed mainly for the degradation of xenobiotics; the CYP2 family, for example, is involved in metabolising common drugs such as nicotine and the beta blocker propranolol. In plants and fungi, Class II P450s are involved in primary metabolism as well as the synthesis of secondary metabolites; an industrially relevant example is the CYP51 family, which plays a key role in fungal steroid production and is subsequently the target of many anti-fungal treatments. Generally, proteins in Class II systems are anchored to the endoplasmic reticulum. The only known exception is CYP105A3 from *Streptomyces carbophilus* which, like other bacterial P450s, is soluble and cytosolic. It is capable of hydroxylating mevastatin to form pravastatin, making it an industrially useful enzyme (Bernhardt and Urlacher, 2014; Durairaj et al., 2016; Girvan and Munro, 2016; Hannemann et al., 2007; Munro et al., 2013).

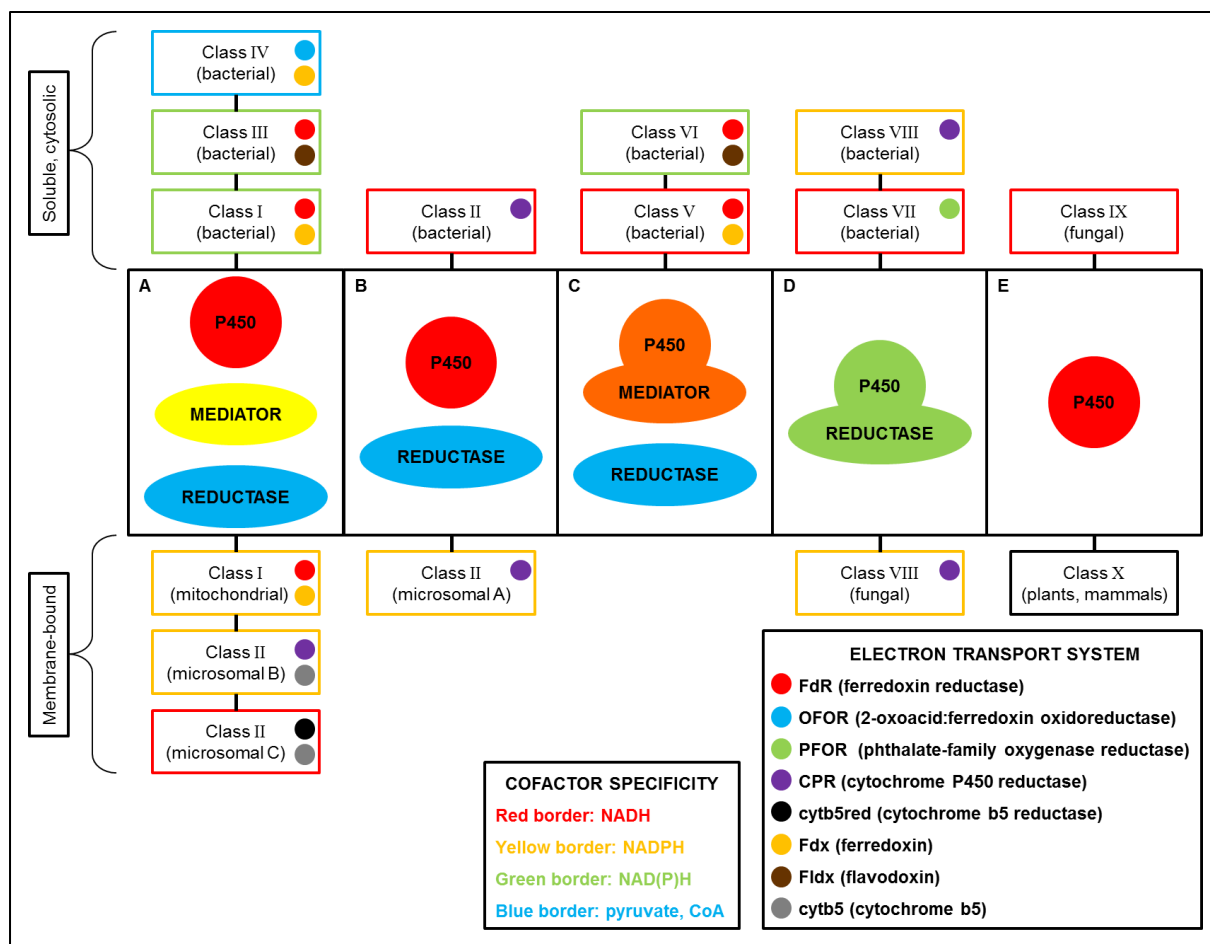


Figure 2.4: Diversity and classification of cytochromes P450 on the basis of the structure of their electron transfer chains, the types of cofactor and mediator protein(s) they interact with, and their location in the cell. Bacterial and fungal P450 systems are soluble and cytosolic; microsomal P450 systems are anchored to the endoplasmic reticulum; P450 systems located in the mitochondria consist of a membrane-bound P450, membrane-associated reductase, and soluble mediator; and plants and mammals have membrane-bound P450s localised to the endoplasmic reticulum. Border colours indicate cofactor specificity. Coloured circles indicate type of reductase and mediator protein. Based on information presented by Bernhardt (2006) and Hannemann et al. (2007).

The majority of P450 proteins can be classified as either Class I or Class II; a handful of exceptions to this rule make up the remaining classes. Classes III and IV are similar to Class I, but rely on significantly different mediator proteins, reductases, or cofactors. In Class III, the mediator protein is an FMN-based flavodoxin instead of an iron-and-sulphur-based ferredoxin; the archetypal Class III P450 is CYP176A1 from *Citrobacter braakii*, which oxidises the monoterpene cineole that is produced by many plants. Class IV derives reducing equivalents from pyruvate or Coenzyme A rather than NAD(P)H, and thus requires a modified 2-oxoacid:ferredoxin reductase, which interacts with a ferredoxin mediator; this class includes numerous thermophilic P450s, such as CYP119 from *Sulfolobus solfataricus*, CYP175A1 from *Thermus thermophilus* HB27, and several P450s from *Sulfolobus tokodaii* (Girvan and Munro, 2016; Hannemann et al., 2007).

In Classes V and VI, the ferredoxin or flavodoxin mediator domain is fused to the P450 domain, but the reductase domain exists as a separate protein. The only known example of Class V is CYP51FX from *Methylococcus capsulatus*, which is involved in the demethylation of sterols. Class VI P450s have been found in *Rhodococcus rhodochrous* strains 11Y, DN22, and YH1; they are largely uncharacterised, but are known to be capable of degrading the military explosive RDX, making them useful for bioremediation purposes (Hannemann et al., 2007).

Class VII is the only class to make use of a phthalate dioxygenase reductase for electron transfer. This PFOR domain, which contains both FMN and [2Fe-2S] subdomains, is fused to the P450 domain. It is highly unusual for FMN to be the initial receiver of electrons, rather than FAD. Class VII includes

CYP116B2 from *Rhodococcus* sp. NCIMB 9784, and other members of the CYP116 family from *Rhodococcus ruber* DSM44319, *Ralstonia metallidurans*, *Ralstonia eutropha* JMP134, *Gibberella zeae* PH-1, and three pathogenic *Burkholderia* species. The CYP116B subfamily can be used for remediation of thiocarbamate pesticides (Ciaramella et al., 2017; Hannemann et al., 2007).

Class VIII are also monomeric fusions, but here P450 is fused to a CPR-like domain which contains both a FAD equivalent and an FMN equivalent. Class VIII has been found widely distributed amongst prokaryotes and lower eukaryotes. Class VIII includes the soluble CYP102A subfamily from *Bacillus megaterium* and *Bacillus subtilis*, along with uncharacterised CYP102 homologues from the pathogens *Bacillus anthracis* and *Bacillus cereus*; these P450s perform the initial terminal hydroxylation step in the metabolism of C₁₂ – C₂₂ fatty acids. Class VIII also includes the membrane-bound CYP505A subfamily, which hydroxylates C₉ – C₁₈ saturated fatty acids in fungi such as *Fusarium oxysporum* and *Phanerochaete chrysosporium* (Ciaramella et al., 2017; Durairaj et al., 2016; Hannemann et al., 2007).

The sole member of Class IX is the nitric oxide reductase CYP55 from *Fusarium oxysporum*, which plays a key role in denitrification under oxygen-limiting conditions. Besides its unusual abilities to use an oxidant other than oxygen and to accept electrons directly from NADH, CYP55 is also the only known soluble eukaryotic P450. Genome analyses suggest that CYP55 is present in numerous fungal species (Hannemann et al., 2007).

Class X P450s are found in plants and mammals. They are all membrane-bound; their key feature is that they utilise oxygen and reducing equivalents from the substrate itself, and thus do not require cellular cofactors or electron transfer proteins. Class X includes enzymes from the CYP74 family, which play key roles in the lipoxygenase synthesis pathway in plants, as well as prostacyclin synthase and thromboxane synthase, which are part of the mammalian arachidonic acid cascade; both pathways are important for cell signalling and damage response (Ciaramella et al., 2017; Hannemann et al., 2007).

Cytochromes P450 for hydroxylation of linear alkanes

As discussed in Sections 2.2.2 and 2.2.3, not all enzymes capable of hydroxylating linear alkanes are cytochromes P450. Similarly, only some cytochromes P450 are capable of hydroxylating linear alkanes: Class I enzymes from the bacterial CYP153 family, Class II enzymes from the yeast-based CYP52 family as well as a handful from the fungal CYP63 family and the mammalian CYP4 and CYP2 families, some members of the bacterial CYP116 family from Class VII, and at least one member of the plant-based CYP96B subfamily. Bacterial P450s such as the Class I CYP101 and the Class VIII CYP102A have also been genetically engineered for activity on linear alkanes, as will be discussed further in Section 2.3.2. Table 2.1 compares the relative strengths and weaknesses of various alkane hydroxylases.

The Class II P450s are difficult to utilise *in vitro* or in heterologous hosts due to their multi-component and microsomal nature, which complicates heterologous expression and protein purification. Furthermore, these enzymes tend to be promiscuous, and linear alkanes are not always their primary substrates, resulting in sub-optimal activity or regioselectivity. Soluble monomeric hydroxylases such as CYP116B5, mutants of CYP102A, and the fungal aromatic peroxygenases all suffer from low regioselectivity, and sometimes from low activity or stability as well. The exceptional regioselectivity of AlkB and the Class I P450s make them favoured candidates for alkane hydroxylation studies; the soluble nature of the CYP153s is an additional advantage (Bernhardt, 2006; Bordeaux et al., 2012; Funhoff and Van Beilen, 2007). The enzymes in the CYP153 family display a particularly high affinity for aliphatic alkanes, suggesting that these might be their preferred substrate. They also show a distinct preference for terminal oxidation, which is extremely difficult to achieve with either genetically engineered enzymes or traditional synthetic catalysts (Fiorentini et al., 2018; Funhoff and Van Beilen, 2007).

Table 2.1: Comparison of n-alkane activation by natural and engineered P450s and other hydroxylases

	Enzyme	Species	Advantages	Disadvantages	Reference
Natural P450s	CYP153A6	<i>Mycobacterium</i> HXN-1500	High regioselectivity Soluble	Multi-component Only moderate activity	Funhoff et al., 2006
	CYP116B5	<i>Acinetobacter radioresistens</i>	Monomeric Soluble	Low regioselectivity	Minerdi et al., 2015
	CYP96B5	Rice		Not yet fully characterised	Zhang et al., 2020
	CYP63A2	<i>Phanerochaete chrysosporium</i>		Low activity Low substrate specificity Multi-component Microsomal	Syed et al., 2013
	CYP52A3	<i>Candida maltosa</i>		Only moderate activity Low regioselectivity Multi-component Microsomal	Scheller et al., 1998
	CYP4B1	Rabbit	High regioselectivity	Multi-component Microsomal Only moderate activity	Fisher et al., 1998
	CYP2E1	Various mammals		Low activity Multi-component Microsomal	Funhoff and Van Beilen, 2007
Engineered P450s	CYP102 var. PMO-R1	<i>Bacillus megaterium</i>	High activity Monomeric Soluble	Low regioselectivity Low stability	Fasan et al., 2007
	CYP102 var. 9-10A-A328V	<i>Bacillus megaterium</i>	High activity Monomeric Soluble	Low regioselectivity Low coupling efficiency	Peters et al., 2003
	CYP101 var. F87W/Y96F/T101L/V247L	<i>Pseudomonas putida</i>	High activity High regioselectivity Soluble	Multi-component Targets subterminal carbon	Bell et al., 2002
Other alkane hydroxylases	AlkB	<i>Pseudomonas putida</i>	High regioselectivity	Also active on alcohol products Membrane-bound	Grant et al., 2012, 2011
	APO1 AaP	<i>Agrocybe aegerita</i>	Monomeric Soluble	Low regioselectivity Only moderate activity	Peter et al., 2011
	LadA	<i>Geobacillus thermodenitrificans</i> NG80-2	High regioselectivity	Very low activity Multi-component	Bordeaux et al., 2012
	pMMO	<i>Methylococcus capsulatus</i>	High regioselectivity	Very low activity Low stability Multi-component Membrane-bound	
	sMMO	<i>Methylosinus trichosporium</i> OB3b	High activity High regioselectivity	Low stability Multi-component	

CYP153A6 and its redox partners

CYP153A6 is part of the native alkane-catabolising pathway of *Mycobacterium* HXN-1500. This species was isolated from amongst organisms colonising a trickling-bed hexane scrubber (van Beilen et al., 2002). The preferred substrates of *Mycobacterium* HXN-1500 appear to be C₅ – C₁₂ linear alkanes, but the species is also capable of growth on longer alkanes (Van Beilen et al., 2005), and CYP153A6 has shown *in vitro* hydroxylation activity on ethane and propane (Arnold et al., 2012), certain branched

alkanes, terpenes, and N-substituted pyrrolidines (Funhoff et al., 2006; Van Beilen et al., 2005) and on C₆ – C₈ cyclic alkanes to a limited extent (Pennec et al., 2015). However, it is most active on n-octane and n-nonane, and *in silico* docking models show optimal docking in the active site for C₆ – C₉ linear alkanes (Funhoff et al., 2006).

CYP153A6 is a Class I P450, relying on ferredoxin and ferredoxin reductase to transfer electrons from the cellular cofactor NADH. *In vitro* studies found that the enzyme has a high affinity for n-octane, with low substrate binding coefficients of $0.17 \pm 0.03 \mu\text{M}$. It has been shown to convert n-octane to 1-octanol with over 95% specificity and an *in vitro* activity of $60.8 \pm 10.6 \text{ mol}_{\text{substrate}} \cdot \text{mol}_{\text{enzyme}}^{-1} \cdot \text{min}^{-1}$ (Funhoff et al., 2006). Funhoff et al. (2006) estimated the theoretical maximum activity to be approximately $90 \text{ mol}_{\text{substrate}} \cdot \text{mol}_{\text{enzyme}}^{-1} \cdot \text{min}^{-1}$, which would fit well with studies of purified CYP/FdR/Fdx which showed NADH consumption levelling off at $90 \text{ mol}_{\text{cofactor}} \cdot \text{mol}_{\text{enzyme}}^{-1} \cdot \text{min}^{-1}$ (Arnold et al., 2012). This would make CYP153A6 capable of achieving double or even triple the typical activities of the CYP52 family, and although CYP101 and CYP102A enzymes can reach much higher activities, their selectivity for terminal oxidation does not exceed 10% (Funhoff et al., 2006).

Unfortunately, *in vivo* activities of under $22 \text{ mol}_{\text{product}} \cdot \text{mol}_{\text{enzyme}}^{-1} \cdot \text{min}^{-1}$ have typically been reported when CYP153A6 and its redox partners have been expressed in *P. putida* and *E. coli* for the production of 1-octanol or perillyl alcohol (Alonso-Gutierrez et al., 2013; Cornelissen et al., 2013, 2011; Gudimichin et al., 2012, 2011; Kochius et al., 2018; Olaofe et al., 2013; Pennec et al., 2015; Van Beilen et al., 2005; Willrodt et al., 2015). This suggests that additional system limitations are prevalent in whole-cell biotransformations.

2.3 Industrial Context

The direct activation of alkanes would be industrially beneficial, but the use of alkane hydroxylases has not yet been established in the platform chemicals industry. Furthermore, while the application of enzymes for catalysis has become widespread, the monooxygenases remain an underutilised enzyme class. This section discusses the industrial usage of monooxygenases, highlighting cases of commercial application of monooxygenases in general, outlining the engineering of cytochromes P450 for non-natural purposes, and elaborating on the challenges of exploiting cytochromes P450 for industrial biocatalysis.

2.3.1 Industrial applications of monooxygenases

The commercial potential of the oxidoreductase enzyme class has been recognised since the 1960s, and has been exploited to synthesise a range of products across the bulk chemicals, fine chemicals, and pharmaceuticals industries (Sellés Vidal et al., 2018; Wentzel et al., 2007). Many industrial processes based on multi-enzyme pathways, both natural and engineered, involve at least one monooxygenase. In many cases, a cytochrome P450 is critical to the process. Still, the number of commercialised processes pales in comparison to the number of industrially interesting reactions that cytochromes P450 have been shown to perform at laboratory scale (for examples, see reviews by Ayala and Torres, 2004; Bernhardt, 2006; Durairaj et al., 2016; Girvan and Munro, 2016; Grogan, 2011; Hollmann et al., 2011; Holtmann et al., 2014; Julleson et al., 2015; Kelly and Kelly, 2013; Lundemo and Woodley, 2015; and Munro et al., 2013). The potential of monooxygenases in general, and cytochromes P450 in particular, remains vastly under-exploited because the activities and stabilities of these systems typically fail to meet the standards for industrialisation (Bernhardt, 2006; Gröger, 2014; McIntosh et al., 2014).

It is worth noting that out of hundreds of oxidoreductase processes, both commercial and potential, the majority are concerned with the synthesis of pharmaceuticals or fine chemicals (Woodley, 2013). There are far fewer examples of biotransformation of hydrocarbons into platform chemicals, and even fewer that utilise alkanes as a feedstock. Commercially, *Candida tropicalis* has been engineered to produce dicarboxylic acids, especially C₁₂₊ acids, from n-alkanes or plant-derived fatty acids via fermentation (Kroha, 2004). The biotransformation of dodecanoate into dodecanol and dodecanal, by *E. coli* expressing AlkB and its facilitator proteins, moved into pilot-scale studies in 2013 (Gröger, 2014), but direct conversion of linear alkanes into alcohols has yet to be commercialised.

2.3.2 Modifying cytochromes P450 for industrial synthetic applications

The natural diversity of cytochromes P450 makes them promising candidates for the synthesis of a wide variety of commercially valuable products. However, many of these P450s are challenging to apply *in vitro* or in heterologous hosts, and their natural hosts may not be suited to industrial application. Often, the P450 that carries out the reaction of interest suffers from low stability or turnover frequency, while the most active and stable P450s do not act on the necessary substrate. Therefore, there has been substantial work to genetically engineer P450s to be more suitable for industrial applications. Attempts have been made to alter their substrate preference, increase their activity and selectivity (especially on non-natural substrates), improve the efficiency of their electron transport chains, enhance their stability at high temperatures or in the presence of organic solvents, improve their expression in heterologous hosts, and exploit their naturally occurring (but usually disfavoured) peroxide shunt pathway. Both directed evolution and rational design techniques have been used, and more recently researchers have begun combining directed evolution, *in silico* analysis, and rational design (Bernhardt, 2006; Ceccoli et al., 2014; Cheng et al., 2015; J. M. Choi et al., 2015; Currin et al., 2015; Woodley, 2013). Besides protein engineering, process interventions such as immobilisation and chemical modification have been shown to alter substrate specificity, product selectivity, or catalytic activity in certain cases (Ayala and Torres, 2004).

CYP102A1 and CYP101, having demonstrated the highest activities of any enzymes in the cytochrome P450 family, have been extensively engineered to carry out non-natural reactions. In the field of alkane activation, there has been particular interest in modifying these and other enzymes – including CYP153A6 and the non-haem alkane hydroxylase AlkB – to act on gaseous alkanes. Substantial progress has been made in engineering mutants that can hydroxylate butane, propane, and even ethane. However, increased activity on short-chain linear alkanes is associated with decreased activity on medium chain lengths, and mutants show a strong preference for subterminal activation. There has also been very limited success in modifying cytochromes P450 to activate methane (Bernhardt, 2006; Bordeaux et al., 2012; Currin et al., 2015; Kelly and Kelly, 2013; Koch et al., 2009; Soussan et al., 2016). CYP102A1 has, however, been induced to hydroxylate methane and other gaseous alkanes via the application of inert decoy molecules such as perfluorinated fatty acids, although this strategy also leads to altered regioselectivity. Unfortunately, coupling efficiencies are low, and the additional downstream processing required to remove the decoy molecules may limit the industrial applicability of this approach (Bordeaux et al., 2012; Holtmann et al., 2014; Kawakami et al., 2015; Koch et al., 2009).

As an alternative to changing the substrate scope of high-performance enzymes like CYP102 and CYP101, cytochromes P450 which exhibit desirable substrate or product spectra can be engineered to be more effective catalysts. For example, the alkane hydroxylation activity of CYP153A13a was enhanced by artificially fusing its P450 and reductase domains into a single protein, and the coupling efficiency of CYP153A G307A was improved by fusion with the reductase domain of CYP102A1. Similar fusion engineering has been undertaken with other P450 systems; solubilising a membrane-bound P450 by fusing it with a bacterial reductase domain is thought to be a particularly promising approach within the sphere of pharmaceuticals and fine chemicals. However, the creation of active fusion proteins remains something of a hit-and-miss exercise. Fusion proteins are also particularly prone to cleavage, resulting in low catalyst stabilities (Bordeaux et al., 2014, 2012; Ciaramella et al., 2017; Fiorentini et al., 2018; Hannemann et al., 2007; Lundemo et al., 2016).

Stability is a major focus of protein engineering efforts, both for enzyme catalysis in general and cytochromes P450 in particular. Thermostability is frequently used as a screening criterion in protein engineering trials, alongside specific activity; substantial work has been done to make enzymes such as CYP102A1 and its mutants more thermostable (Arnold, 1998; Arnold et al., 2012; Harris et al., 2018). Thermophilic enzymes are frequently more stable in the presence of organic solvents than their mesophilic counterparts (Hollmann et al., 2011), which is another important consideration for hydrocarbon-based processes.

The peroxide shunt has also attracted interest as a means of avoiding cofactor dependency. The use of molecular oxygen in the cytochrome P450 catalytic cycle requires two electrons to be delivered directly to the haem group, but P450s are capable of binding hydrogen peroxide instead of oxygen, thus creating the necessary reaction intermediate without the need for NAD(P)H and facilitator proteins

(Meunier et al., 2004). However, it is difficult to maintain enzyme efficiency and stability in the presence of hydrogen peroxide (Holtmann and Hollmann, 2016; Julsing et al., 2008; Soussan et al., 2016). Several P450s have therefore been genetically modified with a view to achieving and maintaining higher activities with hydrogen peroxide as oxidant, with varying degrees of success (Bernhardt, 2006; Currin et al., 2015; Holtmann and Hollmann, 2016; Joo et al., 1999; Julsing et al., 2008; Kelly and Kelly, 2013).

Mutants frequently demonstrate trade-offs between activity, stability, and selectivity (Soussan et al., 2016). Nevertheless, a more detailed molecular-level understanding of enzyme design is emerging as new technologies and strategies are developed (Cheng et al., 2015), and ongoing advances in protein engineering will serve to steadily increase the industrial potential of cytochromes P450.

2.3.3 The hard-to-reach standards of industrial success with monooxygenases

In general, the main advantages of enzyme catalysis are mild operating conditions and high selectivity, as this implies an opportunity to substantially reduce a process's energy consumption, waste formation, and safety risk. Enzymes may also present opportunities to synthesise products with regiochemistries or enantiopurities that cannot be achieved via traditional chemistry. Furthermore, enzymes can be produced from renewable sources, and many of them demonstrate activity on traditionally recalcitrant substrates such as hydrocarbons (Ayala and Torres, 2004; Bordeaux et al., 2012; J. M. Choi et al., 2015; Hollmann et al., 2011; Holtmann et al., 2014). However, the characterisation of a commercially interesting enzyme does not guarantee that it will be developed on a commercial scale. Industrial success requires economic feasibility.

Even within the low-volume pharmaceutical industry, most whole-cell processes achieve space time yields exceeding $2 \text{ g.L}^{-1}.\text{h}^{-1}$ and product titres exceeding 20 g.L^{-1} for growing cells (Lundemo and Woodley, 2015). However, pharmaceuticals can theoretically be economical from as little as $0.001 \text{ g.L}^{-1}.\text{h}^{-1}$ and 0.1 g.L^{-1} . Fine chemical production imposes stricter criteria, demanding minimum space time yields of $0.1 \text{ g.L}^{-1}.\text{h}^{-1}$ and minimum titres of 1 g.L^{-1} (Julsing et al., 2008). For resting cells, suggested benchmarks are $10 - 35 \text{ g}_{\text{product}}.\text{g}_{\text{DCW}}^{-1}$ for pharmaceuticals, $70 - 230 \text{ g}_{\text{product}}.\text{g}_{\text{DCW}}^{-1}$ for fine chemicals, $140 - 400 \text{ g}_{\text{product}}.\text{g}_{\text{DCW}}^{-1}$ for specialty chemicals, and $700 - 2000 \text{ g}_{\text{product}}.\text{g}_{\text{DCW}}^{-1}$ for bulk chemicals (Tufvesson et al., 2011). A resting cell process which ran for 48 h at a biomass concentration of $10 \text{ g}_{\text{DCW}}.\text{L}^{-1}$, and which met the recommendations for bulk chemical production, would be achieving a space time yield of $250 \text{ g.L}^{-1}.\text{h}^{-1}$ or more – substantially higher than the space time yields required of growing cells. In essence, productivity requirements increase by orders of magnitude when moving from pharmaceuticals to bulk chemicals, placing significant demands on biocatalyst activity and stability.

Every biotransformation has its own window of space time yields that are both acceptable and achievable, defined by upper and lower bounds on both product concentration and residence time. Downstream processing becomes impractical and uneconomical unless a minimum product concentration is achieved; maximum allowable concentration is defined by the tolerance of the enzyme or cell to product toxicity and inhibition. Reaction rate and catalytic deactivation set the lower and upper bounds for residence time: taking the biocatalyst out of the reactor before the reaction is complete is wasteful, and leaving the biocatalyst in the reactor after it ceases to be active is inefficient (Schrewe et al., 2013a). Space time yield is therefore a function of expression, activity and stability: how much enzyme is available, how quickly can that enzyme carry out the reaction of interest, and for how long can the enzyme remain active?

In this context, the challenges facing the application of cytochromes P450 can seem daunting. Catalytic turnover rates of most monooxygenases are extremely low by industrial standards, and cytochromes P450 typically struggle to reach levels of activity and stability that would make them commercially viable (Ayala and Torres, 2004; Bernhardt, 2006; Bühler and Schmid, 2004; Gröger, 2014; Julsing et al., 2008; Lundemo and Woodley, 2015). This is due in part to their complex, multi-component nature. Cytochromes P450 require nicotinamide cofactors as a source of electrons, and up to two mediator proteins to assist in electron transfer. Thus, the expression and interaction of multiple proteins must be coordinated. This makes cytochromes P450 particularly challenging to apply *in vitro*, since limitations in cofactor supply or electron transport can result in low activities (Ayala and Torres, 2004; Hollmann et al., 2011; Julsing et al., 2008). The enzymes themselves are often highly sensitive to temperature, shear, and organic solvents. Whole cell processes can mitigate these issues, but will typically impose

their own limits in terms of enzyme expression levels and toxicity or inhibition thresholds for product, substrate and solvent (Ayala and Torres, 2004; Hollmann et al., 2011). The low space time yields exhibited by cytochrome P450-based reactions means that industrial interest in P450 biocatalysis has largely been confined to low-volume, high-value products, such as pharmaceuticals and fine chemicals (Holtmann et al., 2014). Although enzyme catalysis is by now well-established in industry, the field of bulk chemicals is still dominated by traditional chemical processes (Gröger, 2014), and successful bulk bioprocesses are mostly limited to fermentations for the production of growth-associated chemicals (Becker and Wittmann, 2015; Lundemo and Woodley, 2015). Nevertheless, cytochromes P450 are widely considered to be worth the effort it will take to overcome these challenges, thanks to the unique reactions that they are capable of carrying out – the direct terminal activation of linear alkanes by CYP153A6 being a prime example. Aside from significant work being done in the field of protein engineering (as discussed in the previous section), there is still considerable scope to optimise biocatalyst production and to improve the conditions of the biotransformation via process engineering approaches. These process interventions will be the focus of the second half of the Literature Review.

2.4 Cytochromes P450 for Biocatalysis

Before experimental work on this project could begin, several early-stage key questions had to be answered:

- Would the project focus on free enzyme systems or whole-cell systems?
- Which microorganism(s) would be chosen for P450 expression and/or biotransformation?
- What manner of expression system would be used?
- If a whole-cell process was selected, would it use growing or resting cells?
- How was the P450 likely to interact with the host cell's metabolism, and how could negative interactions be dealt with?
- What range of operational conditions would be examined?

Based on a synthesis of the literature, Section 2.4 argues the case for the use of whole-cell systems as opposed to free enzyme, and advocates for *Escherichia coli* as a suitable host system. The challenges that arise when working with microbial cells are discussed, including how these issues might be avoided or mitigated. The section presents an overview of the factors relating to expression of heterologous proteins in general and cytochromes P450 in particular, the response of cell physiology to P450 expression levels and process conditions, and the relationship between cell physiology and biocatalytic performance. Operational concerns for P450 biotransformations are also briefly discussed, with a focus on two-liquid-phase systems.

2.4.1 The choice between *in vitro* and *in vivo* operation

In vitro operation is the form of biocatalysis most analogous to conventional chemical catalysis. The enzyme is expressed, purified, and applied in either soluble or immobilised form; the enzyme itself is the catalyst – the basic unit of biotransformation. In the case of *in vivo* operation, although it is still the enzyme itself which makes the reaction of interest possible, the unit of biotransformation is now the microbial cell. The cell functions as a “black box” which takes up substrates and excretes products, while carrying out any number of reactions around and alongside the reaction of interest. Whole-cell systems are thus significantly more complex than purified enzymes: cells carry the risk of unwanted side reactions, rely on intricate interconnected cycles which the biotransformation both influences and is influenced by, and impose additional mass transfer limitations on the reaction (Holtmann et al., 2014; Schrewe et al., 2013a; Soussan et al., 2016). However, cells also come with built-in control networks which shield enzymes from organic solvents, reactive oxygen species, and variations in temperature, pH, and osmolarity (de Carvalho, 2017; Kadisch et al., 2017; Nielsen and Keasling, 2016; Wachtmeister and Rother, 2016). Enzymes that are directly exposed to hydrophobic substrates or solvents may undergo conformational changes, leading to altered selectivity, reduced activity, or even complete deactivation. Isolated proteins are also vulnerable to denaturation at the gas/liquid interfaces of aerated systems (de Carvalho, 2017; Schrewe et al., 2013a). Moreover, the most common method of deactivation for monooxygenase enzymes in particular is via destruction of the active site by reactive oxygen species, which can be generated by uncoupling in the P450 reaction cycle, as described in

Section 2.2.3. Living cells will naturally counter this oxidative stress, but isolated enzyme systems require the addition of catalase or fresh cell extract to scavenge hydrogen peroxide and other reactive oxygen species (Bordeaux et al., 2014; Kochius et al., 2018; Schrewe et al., 2013a; Van Beilen and Funhoff, 2007). Overall, whole-cell operation reduces sources of enzyme instability and aids the system in withstanding stressful industrial conditions, thus extending reaction time, which is key to the development of commercially viable processes (Bordeaux et al., 2012; Holtmann et al., 2014; Lundemo and Woodley, 2015; Schrewe et al., 2013a; Soussan et al., 2016).

Enzyme immobilisation aims to exploit the benefits of cell-free operation, while minimising its drawbacks. Immobilisation techniques can increase the stability and recoverability of purified enzymes, which may allow for high catalyst loading and catalyst recycling, and thus justify the intensive upstream processing necessary for catalyst production (J. M. Choi et al., 2015; Soussan et al., 2016; Wachtmeister and Rother, 2016). For example, immobilisation of chloroperoxidase together with glucose oxidase in polyurethane foam has been shown to substantially increase turnover numbers for the oxidation of indole, thioanisole, and heptene (Schrewe et al., 2013a); the application of proliferating cell nuclear antigen to immobilise CYP101, putidaredoxin, and putidaredoxin reductase in trimeric ring structures increased catalytic activity by two orders of magnitude relative to free enzymes (Lundemo and Woodley, 2015); and immobilised transaminases are used industrially for the production of anti-diabetic compounds (J. M. Choi et al., 2015). However, the successful immobilisation of multicomponent enzyme systems remains highly challenging (Bernhardt, 2006; Lundemo and Woodley, 2015).

Free or immobilised, the use of purified enzyme entails extensive upstream processing, which can increase catalyst production costs by an order of magnitude. Conversely, whole-cell processes require increased downstream processing, as well as sterile operation (Bordeaux et al., 2012; de Carvalho, 2017; Holtmann et al., 2014; Tufvesson et al., 2011; Wachtmeister and Rother, 2016). Consequently, cell-free systems may be advantageous when it comes to single-step biotransformations, particularly for the synthesis of high-value, low-volume products such as pharmaceuticals; but whole-cell operation is more suitable for the production of bulk or even specialty chemicals where volumes are high and product purity standards are somewhat lower, especially if the process requires the interaction of multiple enzymes (de Carvalho, 2017; Schrewe et al., 2013a).

Many monooxygenases, including cytochromes P450 and particulate alkane hydroxylases, rely on electrons that originate from cellular cofactors such as NAD(P)H and are delivered by one or more redox mediator proteins. To utilise such enzymes in cell-free systems, it is necessary to purify multiple different proteins; the addition or regeneration of cofactor is also needed. This becomes prohibitive from an economic perspective. The efficiency of interactions between the components may also be reduced when they are removed from the cellular environment. Artificial systems to supply reducing equivalents are under development, but so far cannot compete with the metabolism-linked cofactor regeneration which is automatically provided by whole cells (Ayala and Torres, 2004; Bernhardt, 2006; Bordeaux et al., 2012; Bühler et al., 2008; de Carvalho, 2017; Fasan et al., 2007; Holtmann et al., 2011; Holtmann et al., 2014; Lundemo and Woodley, 2015; Schrewe et al., 2013a; Soussan et al., 2016; Tieves et al., 2016; Van Beilen and Funhoff, 2007; Wachtmeister and Rother, 2016).

Numerous P450 systems have demonstrated better performance *in vivo* than *in vitro*. CYP154A8 demonstrated higher enantioselectivity and seven-fold higher product titres for n-octane hydroxylation in whole-cell *Pseudomonas putida* than as a purified enzyme system (Tieves et al., 2016), CYP153A enzymes from *Polaromonas* sp. JS666 produced roughly five times more 1-octanol *in vivo* than *in vitro* (Lonsdale et al., 2015), and CYP153A6 displayed higher limonene hydroxylation activities in living cells than in cell free extracts (Cornelissen et al., 2011). There is thus a strong case for pursuing the development of whole-cell processes for alkane hydroxylation.

2.4.2 Considerations relating to heterologous expression

Native hosts versus heterologous hosts

There are two ways of applying biocatalytic enzymes *in vivo*: by culturing the organisms that naturally produce these enzymes, or by using genetic engineering tools to express the enzymes of interest in a heterologous host.

The chief advantage of using native hosts for hydrocarbon biocatalysis is that these organisms are specifically adapted to carry out the reaction of interest. They possess efficient mechanisms for solvent tolerance and substrate import, allowing cells to withstand higher levels of exposure to toxic substrates, products, and solubilising agents (de Carvalho, 2017; Favre-Bulle et al., 1991; Lonsdale et al., 2015; Vallon et al., 2013). However, the wild-type strains carry out the biotransformation of interest as part of their metabolic processes. When it comes to the oxidation of alkanes, the desired product is usually an intermediate in these natural pathways and is rapidly degraded by the cells. To avoid this, the complicated task of manipulating natural metabolism must be undertaken. The genomes of many organisms encode multiple different proteins with overlapping functionality, and this is especially true of species which metabolise alkanes (as discussed in Section 2.2.2). For example, the particulate alkane hydroxylase AlkB is part of an operon containing genes for a complete alkane metabolic pathway in *Pseudomonas oleovorans* (Kok et al., 1989). When Grant et al. (2011) investigated the hydroxylation of n-octane and n-dodecane by AlkB, they eliminated the alcohol dehydrogenase protein AlkJ from the operon, but this did not prevent the formation of octanoic or dodecanoic acid. Thus, a few targeted knock-out mutations are not guaranteed to prevent product degradation; extensive genetic engineering may be necessary. The more extensive the alterations, the greater the risk of disrupting metabolic pathways and compromising the viability of the organism (Bühler and Schmid, 2004; de Carvalho, 2017; Favre-Bulle et al., 1991; Lonsdale et al., 2015; Rojo, 2009; Soussan et al., 2016; Vallon et al., 2013). Furthermore, the expression of proteins that act on hydrocarbons is tightly regulated in native hosts, in some cases via multi-tier regulation that controls not only the genes themselves but also their transcriptional regulators. Expression is usually substrate-induced, promoters are often primed by starvation or other stressful conditions, and many systems are highly sensitive to catabolite repression, all of which precludes the supply of sacrificial substrate for energy generation. Products of hydrocarbon activation tend to be cytotoxic, so regulatory control via product inhibition is common, limiting the maximum achievable titres (Moreno and Rojo, 2019; Rojo, 2009; Wentzel et al., 2007). Overall, compared to common industrial strains, native hosts are more likely to suffer from slow growth rates, high or unusual nutritional requirements, high energy demand and associated low product yields, low ceilings for product concentration, and pathogenicity (Becker and Wittmann, 2015; Schrewe et al., 2013a).

In contrast, there are a number of heterologous hosts that have been extensively studied and adapted for both research and industry. These strains come with a multitude of tools for genome editing and gene expression, a large body of existing knowledge regarding cell physiology, and fewer hurdles when it comes to obtaining regulatory approval (Baneyx, 1999; Becker and Wittmann, 2015; Nielsen and Keasling, 2016). Crucially, these strains do not necessarily metabolise the product of interest. Thus, heterologous expression of biotransformation enzymes can reduce or even eliminate metabolic degradation of the desired product (Ayala and Torres, 2004; Bosetti et al., 1992; Grant et al., 2011; Soussan et al., 2016). Heterologous hosts allow for protein expression and product accumulation to levels well exceeding those of native hosts, and can even support the creation of novel pathways not found in nature (Becker and Wittmann, 2015; Dai and Nielsen, 2015; Julleson et al., 2015; Wentzel et al., 2007).

Heterologous hosts are not without their drawbacks. They are often more vulnerable than native hosts to the toxicity of alkane substrate and hydroxylated product, as will be discussed further in Section 2.5.1. Biocatalytic enzymes may interact with host pathways in unexpected ways, resulting in unwanted side reactions, reduced catalytic activities, enzyme instability, or excessive nutrient requirements (Bailey et al., 1996; Bühler and Schmid, 2004; Glick, 1995; Soussan et al., 2016). Nevertheless, while the bulk chemicals industry is often focused on fermentation processes, which favour the selection and modification of promising strains, the biotransformation processes found in the pharmaceutical and fine

chemical industries frequently employ recombinant strains, and most research into biocatalysis makes use of recombinant strains due to their overall robustness and simplicity of application (de Carvalho, 2017).

Choice of heterologous host and expression system

Escherichia coli and *Saccharomyces cerevisiae* are the microbial species most widely used for heterologous expression in research and industry (Dai and Nielsen, 2015). *S. cerevisiae* and other yeasts offer a unique advantage in terms of cytochrome P450 expression, since they contain a range of native P450s and have therefore evolved to carry out efficient and reliable expression of haem-based proteins. This expression machinery can be harnessed to produce heterologous P450s in a properly folded and stable form. By contrast, wild-type *E. coli* do not contain haem-based proteins, so the addition of haem precursors such as δ -aminolevulinic acid is often necessary to ensure good expression of heterologous P450s (Lundemo and Woodley, 2015). Even then, *E. coli* may struggle to carry out the correct post-translational modifications necessary for the expression of an active protein (de Carvalho, 2017). However, when expression can be carried out successfully, the specific activities reached by heterologous P450s in *E. coli* can exceed those reached in *S. cerevisiae* – sometimes by orders of magnitude, as in the case of CYP3A4, which showed a 115-fold increase in testosterone hydroxylation activity when the host was changed from *S. cerevisiae* to *E. coli* (Julsing et al., 2008). *Pseudomonas* species, many of which are known to contain particulate alkane hydroxylases, may enable heterologous P450s to achieve even higher specific activities: the activity of CYP153A6 on limonene was roughly 30 times higher when the enzyme was expressed in *P. putida* rather than *E. coli* (Van Beilen et al., 2005). This is presumably because *Pseudomonas* species are naturally equipped to metabolise hydrocarbons, with cellular mechanisms that enhance substrate transport into the cytoplasm and increase the cell's solvent tolerance (Bühler and Schmid, 2004; Lundemo and Woodley, 2015; Soussan et al., 2016). These mechanisms may also enhance the stability of the whole-cell biocatalyst: *E. coli* and *P. putida* expressing CYP154A8 for alkane hydroxylation produced similar titres after six hours of biotransformation, but the *P. putida* culture then continued to produce alcohols for up to 24 hours, while titres in the *E. coli* system levelled off (Tieves et al., 2016). *Bacillus subtilis* also demonstrates significantly higher tolerance than *E. coli* towards the substrates and solvents used in hydrocarbon-based processes (Lundemo and Woodley, 2015). Unfortunately, *B. subtilis* suffers from plasmid instability, high rates of improper protein folding, and high protease activity (de Carvalho, 2017), and neither *Bacillus* nor *Pseudomonas* hosts offer the convenience of *E. coli* strains, which have been extensively developed for use in laboratories and factories. The genetics and molecular biology of *E. coli* are particularly well-understood (Becker and Wittmann, 2015; Glick, 1995); these bacteria are easy to culture, grow rapidly and to high densities on relatively cheap and widely available substrates, and are compliant with most SHE regulations globally (Baneyx, 1999; Soussan et al., 2016); strains have been designed specifically for heterologous expression, with tight regulatory control, minimal degradation of foreign proteins, and compatibility with a wide range of expression vectors (Rosano and Ceccarelli, 2014), although many have the drawback of losing plasmids rapidly in the absence of selective pressure (Kadisich et al., 2017). However, the most relevant feature of *E. coli* in the case of alkane hydroxylation is that this species strictly regulates the metabolism of alcohols. The transcription of alcohol dehydrogenases in *E. coli* is induced by an excess of NADH, and expression is also highly regulated at the translational level. Even post-expression, the alcohol dehydrogenase is inactivated by the presence of oxygen. In other words, degradation of the alcohol product by the *E. coli* host should only happen under anaerobic conditions, which alkane hydroxylation processes will obviously be seeking to avoid (Holland-staley et al., 2000). Similarly, the β -oxidation system in *E. coli* is not induced by the presence of alcohols but rather by long-chain fatty acids. Hence, *E. coli* shows minimal activity towards any intermediates of the alkane activation pathway (Cornelissen et al., 2013; Favre-Bulle et al., 1991; Grant et al., 2011). In this regard *Bacillus*, *Pseudomonas*, or *Saccharomyces* strains suffer from a similar disadvantage to native hosts, in that they are more likely to possess metabolic pathways for degrading hydrocarbons, and thus may require extensive genome editing to be useful as host cells. For example, in the hydroxylation of limonene to perillyl alcohol, 26% by-product formation was observed when CYP153A6 was expressed in *P. putida*, compared to only 8% when the host was *E. coli* (Cornelissen et al., 2013). Similarly, *B. megaterium* was found to be an unsuitable host for terpenoid hydroxylation processes, because its natural P450s interfered with the products (Janocha and

Bernhardt, 2013). *E. coli* is thus widely used as a heterologous host for P450-based biotransformation processes.

Heterologous expression can be achieved by integrating foreign genetic material directly into the host genome – an approach which generally guarantees stability for the heterologous genes and allows reasonably tight control over enzyme expression levels. Alternatively, heterologous expression can be achieved using plasmids, which are less stable and controllable, but much simpler to apply. Although plasmids are prone to mutations, can be lost during cell replication, result in highly variable expression levels, and impose a greater burden on the host cell than genome editing, they are nevertheless useful in the development phase of a project because they are easy to modify and to move between hosts (Becker and Wittmann, 2015; Glick, 1995; Kadisch et al., 2017; Nielsen and Keasling, 2016). A variety of plasmids have been engineered for use with *E. coli*, each with its own combination of replicons, promoters, selection markers, and affinity tags. These differences mean that biocatalyst performance is affected by choice of plasmid as well as choice of host strain (Jiang and Morgan, 2004; Rosano and Ceccarelli, 2014). Different types of plasmid carry different amounts of genetic material and prompt the host cell to maintain a different number of plasmid copies. The larger the plasmid or the higher the copy number, the more burden is placed on the metabolism of the host cell. This metabolic load results in reduced growth rates or lower achievable culture densities, and promotes the growth of cells that have lost the plasmid, necessitating the application of selective pressure on the culture. It is therefore advantageous to keep plasmid size and copy number as low as possible while still achieving the desired result in terms of heterologous protein concentration (Alonso-Gutierrez et al., 2013; Baneyx, 1999; Cserjan-Puschmann et al., 1999; Glick, 1995; Kadisch et al., 2017; Rozkov et al., 2004; Willrodt et al., 2015). Selective pressure is typically based on the use of antibiotic resistance markers, coupled with the appropriate antibiotics, although this selective pressure diminishes as antibiotics degrade or are inactivated by the resistance marker, making plasmid-based expression expensive to apply anywhere except at bench scale (Baneyx, 1999; Glick, 1995; Rosano and Ceccarelli, 2014). The choice of antibiotic resistance marker can have unexpected effects on a biotransformation. Chloramphenicol resistance, for instance, is conferred via expression of the protein chloramphenicol acetyl transferase (CAT), which has been found to acetylate perillyl alcohol (Alonso-Gutierrez et al., 2013).

Expression and stability of heterologous proteins

In species that naturally metabolise alkanes, there is high variability in the spatial organisation and regulation of the genes for hydrocarbon metabolism. However, very few of these microorganisms are obligate alkanivores, growth on hydrocarbons is less efficient than growth on sugars or amino acids, and the products of hydrocarbon activation tend to be cytotoxic. Therefore, cells exert tight control over their alkane hydroxylase genes, with regulation often extending to control of transcriptional activators. Wild-type expression of alkane hydroxylases is often substrate-induced, and promoter systems may be primed by stress conditions (Ayala and Torres, 2004; Moreno and Rojo, 2019; Rojo, 2009). *Rhodococcus erythropolis*, *Acinetobacter baylyi*, and *Achromobacter xylosoxidans* are all known to modulate their expression of CYP153s based on the type of substrate they are exposed to (Sazykin et al., 2019). Alkane hydroxylase expression may also be subject to strict catabolite repression, as in the case of *Pseudomonas putida*; or it may be sensitive to product inhibition, as with *Pseudomonas butanovora* and *Acinetobacter baylyi* (Moreno and Rojo, 2019; Rojo, 2009; Wentzel et al., 2007).

However, as outlined at the beginning of Section 2.4.2, there are compelling reasons to avoid native hosts when expressing cytochromes P450 for biotransformation. When heterologous genes are introduced into a host cell using plasmids, expression can be controlled through the addition of easily available promoters, such as lactose or isopropyl β -D-1-thiogalactopyranoside (IPTG). However, heterologous expression can be challenging, and specialised strategies may have to be developed for recalcitrant proteins (Ceccoli et al., 2014; Kelly and Kelly, 2013). Post-translational processes vary between organisms, and heterologous proteins may be vulnerable to host proteases, especially when the goal is to accumulate these proteins to high concentrations. Mutations that reduce expression or inhibit activity of host proteases are usually not an option because they have significant negative implications for cell physiology, but common heterologous hosts such as *Escherichia coli* BL21(DE3) have been engineered to tolerate large quantities of foreign protein (Baneyx, 1999; Kadisch et al., 2017; Rosano and Ceccarelli, 2014). *E. coli* also has two sequential systems of molecular chaperones,

plus additional miscellaneous helper proteins, which work to decrease the rate of protein misfolding and associated formation of non-active inclusion bodies (Alonso-Gutierrez et al., 2013; Baneyx, 1999). Nevertheless, co-expression of additional chaperone genes is sometimes necessary, for example when expressing P450s from plants or animals in microbes (Julsing et al., 2008). Heterologous expression can also be improved via codon optimisation: silent point mutations that improve translational efficiency by substituting “rare” amino acids, which the host cell produces at low levels, with more common residues (Becker and Wittmann, 2015; Glick, 1995). For example, the yield of artemisinic acid by an engineered strain of *Saccharomyces cerevisiae* more than doubled following codon optimisation of a plant-based P450 responsible for the final tailoring step (Julsing et al., 2008). However, “rare” codons are usually there for a reason, so codon optimisation needs to be balanced against the risk of reducing or altering the enzyme’s performance (Rosano and Ceccarelli, 2014).

A simpler, yet highly effective method of improving heterologous expression is by optimising process conditions in the growth and expression stage, including temperature, type of carbon source supplied for growth, length of expression period, inducer concentration, and time of induction (Baneyx, 1999; Bordeaux et al., 2014; Glick, 1995; Janocha and Bernhardt, 2013; Jiang and Morgan, 2004). The cell must divert considerable metabolic resources to support heterologous protein expression, which can reduce cell growth rates or final culture densities. In cases where high expression is required, the separation of growth and expression stages can help cells cope with this metabolic burden. This application of “point induction” can allow the culture to reach much higher cell densities. However, cells exposed to continuous induction may achieve higher specific enzyme concentrations (Bordeaux et al., 2014; da Silva et al., 2013; Glick, 1995).

Expression of active CYP153A6 in *E. coli* BL21(DE3) must be performed at a temperature of 20°C to prevent formation of inactive forms of the protein (Olaofe et al., 2013). Furthermore, significant differences in expression have been observed depending on method of induction. Under point induction by IPTG, which was optimal if inducer was added when the culture had an optical density close to 1, CYP153A6 could be accumulated to a maximum concentration of 0.5 – 1 $\mu\text{mol.g}_{\text{DCW}}^{-1}$ over 24 h, after which the enzyme began to be degraded by the cells. Under continuous induction by lactose, enzyme concentration per cell reached similar levels, but showed no signs of degradation after 48 h, even though more than 60% of the heterologous protein was expressed during the first 24 h (Gudimanchi et al., 2012, 2011).

2.4.3 Optimal expression of cytochromes P450 and their redox partners

Cytochromes P450 present additional challenges when it comes to heterologous expression. P450s are haem-based proteins, and thus the synthesis and correct incorporation of haem is necessary for the formation of active enzyme. Furthermore, many P450 systems are multi-component, and will function poorly *in vivo* if the host cell does not express the appropriate electron transfer proteins.

Natural rates of haem biosynthesis in *Escherichia coli* are typically much lower than achievable rates of heterologous protein synthesis. This can lead to wasteful formation of enzymes which lack the haem group that is central to their functioning (Varnado and Goodwin, 2004). In hosts which do produce sufficient haem, P450 over-expression may affect the overall performance of the whole-cell biocatalyst: in *S. cerevisiae*, systems analysis of multiple CYP102A1 mutants found that haem depletion was the most significant stressor arising from heterologous P450 expression, causing host cells to undergo wide-ranging physiological changes (Michener et al., 2012). To combat this, the host cell can be genetically engineered to over-express the *hemA* gene in order to boost haem biosynthesis (Julsing et al., 2008). Additional haem can also be added directly to the culture medium, but this requires heterologous expression of a specialised haem receptor protein (Varnado and Goodwin, 2004). In contrast, heterologous transport systems are not needed for the uptake of δ -aminolevulinic acid (δ -ALA) and iron, which are the key components of the haem moiety. Production of δ -ALA is the rate-limiting step in the haem biosynthesis pathway of *E. coli*, while insufficient iron availability increases the probability that proteins will incorporate protoporphyrin IX before it has been chelated with iron to form haem, resulting in inactive enzymes. Addition of these precursors can therefore lead to higher P450 concentrations or improved activity of whole-cell biocatalysts (Jiang and Morgan, 2004; Lu et al., 2010, 2013; Sudhamsu et al., 2010; Varnado and Goodwin, 2004). For example, CYP153A6 expression in

E. coli was directly proportional to increasing δ -ALA addition up to a concentration of 0.25 mM, and could be more than doubled by the addition of 50 μ M FeCl₃, although cells grown and expressed in Lysogeny Broth rather than chemically defined medium did not benefit from iron addition (Olafe et al., 2013). Consequently, it is standard to supplement with one or both of these compounds in the production of P450-based biocatalysts. Iron is typically supplied as ferric or ferrous citrate, ferric chloride or ferrous sulphate (Bühler and Schmid, 2004; Jung et al., 2016; Ladkau et al., 2016; Lonsdale et al., 2015; Lundemo et al., 2016; Pennec et al., 2015; Ruff et al., 2016; Schewe et al., 2008; Tieves et al., 2016; Vallon et al., 2013; van Nuland et al., 2016; Willrodt et al., 2015). Unfortunately, the necessity of δ -ALA addition can increase the production costs of haem-based biocatalysts substantially (Julsing et al., 2008; Tufvesson et al., 2011).

As discussed in Section 2.2.3, most cytochromes P450 must interact with flavin-based mediator proteins and reductases, which are able to accept reducing equivalents from NAD(P)H in the form of hydrides, and then transfer these reducing equivalents to the haem in the form of individual electrons (Holtmann and Hollmann, 2016). This convoluted transfer of electrons from cellular cofactor to P450 has been identified as a potential bottleneck in the application of P450s for biotransformation processes (Bernhardt, 2006; Lundemo and Woodley, 2015). Most heterologous hosts contain their own suite of flavin-based electron transfer proteins, which are usually capable of interacting with foreign P450s, since reductase or mediator binding sites tend to be very similar across a wide range of monooxygenase proteins (Bernhardt, 2006; Hannemann et al., 2007). However, these electron transfer systems may not offer the same levels of efficiency as the enzyme's natural partners: when CYP105A1 was forced to rely on the native flavodoxin and flavodoxin reductase of *E. coli* JM109, the production of 15-hydroxyabietic acid was reduced almost seven-fold (Janocha and Bernhardt, 2013), and very low rates of n-octane hydroxylation were observed in *E. coli* BL21(DE3) when CYP153A6 was expressed without its natural ferredoxin and ferredoxin reductase co-enzymes (Gudimich et al., 2012). In whole-cell *Pseudomonas putida* systems, choice of redox partners led to a 20% variation in product titres in the hydroxylation of n-alkanes by CYP154A8 (Tieves et al., 2016), and affected rates of testosterone hydroxylation by CYP106A2 by as much as sixteen-fold (Julsing et al., 2008).

Biocatalytic performance is influenced not only by the type of electron transfer proteins that are expressed, but also by their levels of expression within the cell. Bacterial reductases often display much lower heterologous expression levels than their associated P450s (Hannemann et al., 2007), although this can be countered by expression strategies such as placing the genes for the reductase and mediator proteins on a separate plasmid with a higher copy number (Cornelissen et al., 2011; Janocha and Bernhardt, 2013). In the case of *in vitro* production of ω -hydroxy palmitic acid by CYP153A35, product yield could be increased nine-fold by changing the ratio of P450:CamA:CamB from 1:1:10 to 1:5:20. When the heterologous expression system was modified in an attempt to achieve this ratio *in vivo*, product titres increased by 66% (Jung et al., 2016). CYP105D5 demonstrated maximum activities for *in vivo* fatty acid hydroxylation at a molar ratio of 1:3:1 for P450:Fdx:FdR (Chun et al., 2007). For n-octane hydroxylation by CYP153A6, total turnover numbers in purified enzyme systems could be improved by applying a P450:Fdx:FdR ratio of 1:10:1 (Arnold et al., 2012); changing the P450:Fdx:FdR ratio from 1:1:1 to 1:3:3 led to four-fold increases in total turnover number in cell free extracts (Pennec et al., 2015), and molar enzyme ratios of 1:30:0.5 were found to be optimal when working in cell free extract systems (Kochius et al., 2018).

2.4.4 Optimal expression levels for *in vivo* biocatalysis

Heterologous expression can impair normal cellular functioning, by requiring the cell to divert resources such as ATP, GTP, NAD(P)H, aminoacyl-tRNAs, and amino acids to maintain and express foreign DNA and proteins. This burden is exacerbated if the rate of heterologous expression is high, or if the heterologous protein requires amino acids that the cell produces in limiting quantities. This can result in translation errors, or trigger cellular stress responses such as increased protease activity, impacting expression and stability of both heterologous and homologous proteins (Alonso-Gutierrez et al., 2013; Baneyx, 1999; Becker and Wittmann, 2015; Glick, 1995; Kadisch et al., 2017; Nielsen and Keasling, 2016). Heterologous NAD(P)H-dependent oxidoreductases have also been known to interfere with the natural metabolic flux of host cells. This interference may be direct, via degradation of key metabolites or native proteins; or indirect, via competition for energy and common precursors. Even non-growing

cells require energy for overflow metabolism, which is necessary for rapid response to changing conditions, and proteins associated with central metabolism often have a higher affinity for NAD(P)H than heterologous biocatalytic enzymes. The increased demand for cofactors to drive biotransformation may induce cells to increase uptake of carbon sources, and to channel more of that carbon towards acetate formation (Alonso-Gutierrez et al., 2013; Bühler et al., 2008; Dai and Nielsen, 2015; Glick, 1995; Kadisch et al., 2017; Nielsen and Keasling, 2016; Schrewe et al., 2013a; Sellés Vidal et al., 2018; Wu et al., 2016). Excessive metabolic burden may also prompt changes in cellular morphology. The permeability of the cell membrane can increase substantially under conditions of high metabolic load. Host cells may also elongate or decrease in size in order to achieve more favourable ratios of surface area to volume; some have been observed to form extended chains, or to secrete slime that causes them to clump together (Glick, 1995).

Genetic engineering and systems biology strategies can be used to avoid or compensate for metabolic burden. The traditional approach involves strain adaptation by natural selection under conditions relevant to the desired industrial process, but in recent decades, rational design approaches have become possible. This includes optimisation of transcriptional, translational, and post-translational processes, enhancement of cellular respiration, and redirection of metabolic flux by editing genes that play a role in central metabolic networks. However, redirecting metabolic flux is usually an iterative and labour-intensive process, due to the complexity of host pathways which have evolved for tight control and rapid rebalancing of fluxes. Most metabolic engineering focuses on fermentation for the production of metabolites from sugars; limited work has been done on engineering cellular metabolism for optimal biotransformation (Becker and Wittmann, 2015; S. Choi et al., 2015; Dai and Nielsen, 2015; Glick, 1995; Julleson et al., 2015; Nielsen and Keasling, 2016; Wu et al., 2016). However, metabolic engineering of *Escherichia coli* W3110, including removal of two fermentative pathways and partial inactivation of the respiratory chain, along with genetic engineering of a CYP102A1 variant to enable it to carry out the hydroxylation of n-propane using either NADH or NADPH as cofactor, resulted in a resting whole-cell biocatalyst that was three times more efficient when utilising glucose as energy source for biotransformation (Fasan et al., 2011). Alternatively, co-culturing is a straightforward strategy for reducing metabolic burden when expressing multiple different heterologous proteins. With each culture producing one kind of protein, the metabolic load on the cells is less severe (Wachtmeister and Rother, 2016; Wu et al., 2016). However, co-cultures will be more vulnerable to transport limitations.

Given the complexities of balancing metabolic load, it is sensible to avoid unnecessary expression of heterologous proteins. While high expression levels may increase specific activity by ensuring more copies of the enzyme are present in the host cell (Bühler and Schmid, 2004), the associated metabolic burden can have negative effects on overall biocatalyst performance. Hosts such as *E. coli* generally demonstrate good performance under significant heterologous expression loads; for example, *E. coli* BL21(DE3) has been used to successfully express limonene synthase, geranyl diphosphate synthase, and a complete mevalonate synthesis pathway simultaneously (Willrodt et al., 2016). However, the overexpression of monooxygenases has been shown to inhibit *E. coli*-based processes (Bühler et al., 2008), possibly because of the associated high iron demand (Michener et al., 2012) and tendency to produce reactive oxygen species (Holtmann and Hollmann, 2016). In the hydroxylation of dodecanoic acid methyl ester by growing *E. coli* JM101 expressing AlkB and AlkL, biocatalyst stability and productivity per cell could be improved by *reducing* the levels at which the heterologous proteins were expressed, leading to higher titres and space time yields (Kadisch et al., 2016). In the conversion of n-octane to 1-octanol by CYP153A6 in non-growing *E. coli* BL21(DE3), cultures with an initial P450 concentration of $0.76 \mu\text{mol.g}_{\text{DCW}}^{-1}$ lost activity faster and achieved lower maximum titres than cultures expressing only $0.53 \mu\text{mol.g}_{\text{DCW}}^{-1}$, and the best performances in this system have been achieved with resting *E. coli* BL21(DE3) cells with CYP153A6 concentrations of only $0.2 \mu\text{mol.g}_{\text{DCW}}^{-1}$ – a level of expression which also does not lead to any decrease in growth rates or final culture densities relative to non-expressing cells (Gudimich et al., 2012; Olaofe et al., 2013). The production of 2-octanol from n-octane by *P. putida* was also found not to correlate with CYP154A8 expression levels (Jung et al., 2016). However, Jung et al. compared strains with different combinations of redox partners; Tieves et al. (2016), who also found that choice of redox partners was more important than CYP153A35 expression levels in the hydroxylation of palmitic acid, noted that P450 expression *did* correlate with biocatalyst productivity when comparing within a single strain.

The optimal level of heterologous expression when producing a whole-cell biocatalyst thus appears to be situation-specific. It may be considerably lower than the maximum achievable level, although low concentrations of P450 can limit the biotransformation in some cases. The relationship between P450 expression and biocatalyst performance is one that merits further investigation.

2.4.5 Culture conditions, cell physiology, and biotransformation

Although *in vivo* operation has numerous benefits (as outlined in Section 2.4.1), the performance of a whole-cell biotransformation system cannot be optimised unless cell physiology is taken into account. The metabolic state of the cell affects the synthesis and stability of heterologous proteins, the passage of substrate and product across the cell membrane, the rate of cofactor regeneration, and the cell's ability to cope with stresses such as toxicity, resource limitation, and reactive oxygen species. Consequently, cell physiology has a significant influence on biocatalytic properties such as specific activity and stability (Kadisich et al., 2016; Schrewe et al., 2013a; Willrodt et al., 2016). Since cell physiology is sensitive to environmental conditions, the optimisation of pH, temperature, medium composition, and supply of substrates, co-substrates, and dissolved oxygen can substantially improve biocatalyst performance (Julsing et al., 2008).

Cell metabolic state – growing versus resting

The culture used for biotransformation can be in one of several metabolic states: unrestricted growth, exogenously limited growth, non-growth (resting cells), or metabolically inactive (dead cells). The metabolic state is a key determinant in the balance between anabolic and catabolic processes, and hence affects cofactor availability, cell integrity, and enzyme synthesis and degradation (Kadisich et al., 2017). Cofactor-neutral biotransformation cascades do not strictly require metabolic activity, but may still benefit from the protective environment a whole cell provides. However, the application of metabolically active cells is especially advantageous when cofactor regeneration is required (Schrewe et al., 2013a), and such cells also offer increased resistance to external stressors (Chubukov and Sauer, 2014).

Growing cells tend to divert cofactor away from biocatalysis to support growth, while resting cells exhibit fewer side reactions and channel more cofactors towards biocatalysis, resulting in higher specific reaction rates (Bühler et al., 2008; Janocha and Bernhardt, 2013; Kadisich et al., 2017; Lundemo and Woodley, 2015; Schrewe et al., 2013a; Willrodt et al., 2016). Even in cases where growing cells display higher specific activities, resting cell systems may be able to operate at higher biomass concentrations, resulting in better space time yields (Lundemo and Woodley, 2015). The avoidance of biotransformation by-products and undesired growth metabolites can also simplify downstream processing (de Carvalho, 2017). Unfortunately, resting cells are significantly more vulnerable to toxicity, oxidative stress, and metabolic burden, and may ramp up enzyme degradation processes and decrease rates of enzyme synthesis, leading to reduced biocatalyst stability (Bühler et al., 2008; Favre-Bulle et al., 1991; Julsing et al., 2012; Kadisich et al., 2017; Lundemo et al., 2016; Lundemo and Woodley, 2015; Schrewe et al., 2013a; Soussan et al., 2016; Willrodt et al., 2016).

However, the negative effects of resting cell systems can be ameliorated by the correct choice of limiting nutrient, which has wide-reaching effects on overall cellular metabolism. Most microbial species generate energy through catabolism of carbon-based substrates, and will thus reduce their metabolic activity significantly if they are not supplied with a suitable carbon source. In theory, the omission of key nutrients other than carbon can curtail biomass formation while still allowing for energy generation. In practice, cells may respond to multiple forms of nutrient limitation by reducing carbon uptake and metabolic activity. *E. coli* has been observed to reduce its uptake of glucose by 85 – 95% following depletion of sulphur, phosphate, or nitrogen in carbon-rich medium. However, cells did not reduce the expression and maintenance of their primary glucose uptake proteins, but instead began to accumulate metabolites such as α -ketoglutarate, which is known to inhibit such proteins. Interestingly, inducing a resting state via magnesium depletion reduced glucose uptake by only 50% (Chubukov and Sauer, 2014), and this positive discrepancy has been exploited to improve the production of limonene by genetically engineered *E. coli* by a factor of five (Willrodt et al., 2016).

Induction conditions

Besides affecting the expression and stability of heterologous proteins, induction strategy also influences the metabolome of the cell as it enters the biotransformation phase, with potentially wide-reaching consequences for biocatalyst performance. In some host species, environmental conditions may trigger differential expression of native monooxygenase proteins, influencing the stereoselectivity of the target reaction, as in the case of butene and propene epoxidation by growing *Mycobacterium* cultures (Hollmann et al., 2011).

In the case of oxidative desymmetrisation of 3-azabicyclo[3,3,0]octane by whole-cell *Escherichia coli* BL21(DE3) expressing a heterologous monoamine oxidase, initial activities increased up to fifty-fold and final conversions doubled when cells were washed prior to resuspension in biotransformation buffer. The washing step was found to be beneficial in cases where growth and expression of cells had been carried out in Terrific Broth (TB), but not where Lysogeny Broth (LB) had been used, suggesting that certain medium components or growth by-products were hindering biotransformation in the case of TB. Alternatively, it was hypothesised that growth on the TB medium had reduced the permeability of the cell membranes, and that resuspension had led to partial lysis which improved substrate mass transfer (Ramesh et al., 2016).

In the case of n-octane hydroxylation by *E. coli* BL21(DE3) expressing CYP153A6, growth and expression using either LB or chemically defined medium resulted in similar concentrations of active P450 per cell, yet cells grown on LB demonstrated substantially improved biocatalytic performance in subsequent resting-cell biotransformations, including titres that were up to 8% higher, two-fold higher reaction rates, and increases in space time yield of over 60%. This was presumably because the cells cultured on LB had increased expression of glucose uptake proteins, which was beneficial for cofactor regeneration during biotransformation (Olaofe et al., 2013).

Furthermore, point induction of CYP153A6 expression resulted in higher initial activities on n-octane than continuous induction, possibly due to the fact that the specific concentrations of NADH were an order of magnitude higher in the case of point induction (Gudiminch et al., 2012, 2011).

Induction temperature was shown to be crucial for the formation of active CYP153A6. When induction was carried out at temperatures of 25 °C or higher, only inactive forms of the enzyme were detected, and cells were consequently incapable of carrying out the biotransformation of n-octane (Olaofe, 2013).

Availability of carbon for energy generation

High initial activities will not result in high final titres or space time yields if cells cannot generate sufficient energy to sustain the biotransformation (Cornelissen et al., 2011), and this requires the availability of a suitable carbon source. In the case of propane hydroxylation by a CYP102A1 mutant expressed in *Escherichia coli* DH5 α , Fasan et al. (2007) found that 40 – 60% of maximum biocatalytic activity could be restored after product formation had plateaued, simply by transferring resting cells into fresh medium.

The type of carbon source can also have a substantial effect on biotransformation. In the hydroxylation of limonene by *Pseudomonas* cells heterologously expressing AlkB, acetate, citrate, glucose, glycerol, octane, and succinate were tested as co-substrates for supply of energy and cofactor regeneration. Specific activities varied substantially with different co-substrates: from 1.7 – 23.6 $\mu\text{mol.g}_{\text{DCW}}^{-1}.\text{min}^{-1}$ when the host was *P. putida* GPO12, and from 0.8 – 22.6 $\mu\text{mol.g}_{\text{DCW}}^{-1}.\text{min}^{-1}$ when the host was *P. putida* KT2440. Glycerol, octane, and citrate produced the best results in both cases (Cornelissen et al., 2011). However, in the hydroxylation of n-octane by resting *E. coli* BL21(DE3) expressing CYP153A6, cells grown on Lysogeny Broth and induced using IPTG displayed ten-fold lower initial hydroxylation rates when supplied with glycerol rather than glucose. Furthermore, maximum titres of 1-octanol over 48 h increased by roughly 75% when the glucose concentration was increased from 5 g.L^{-1} to 10 g.L^{-1} (Gudiminch et al., 2012). Unfortunately, glucose addition can lead to acetic acid formation. Toxicity studies indicate that as little as 0.25 $\text{g.g}_{\text{DCW}}^{-1}$ of acetic acid can inhibit 1-octanol production by roughly 20%; concentrations exceeding 1 $\text{g.g}_{\text{DCW}}^{-1}$ lead to 75% productivity loss (Olaofe et al., 2013).

Culture density

Assuming that similar levels of production can be maintained in the individual cells, increasing the total concentration of biomass will improve volumetric titres and space time yields. However, at higher culture densities, cells will face greater mass transfer limitations and competition for nutrients and oxygen. Thus, culture density presents a trade-off between increased conversion and decreased biocatalyst stability. For example, when CYP105A1 and other heterologous proteins were expressed in *Escherichia coli* JM109 resting cells, the product formation per gram of cells after the first hour of biotransformation was similar for cultures at 20 g_{DCW}.L⁻¹ and cultures at 25-30 g_{DCW}.L⁻¹, but volumetric titres after 24 h were highest in the cultures with lower biomass concentration (Janocha and Bernhardt, 2013). When CYP153A6 was expressed in *E. coli* BL21(DE3) resting cells, biomass concentrations of 7, 14, and 28 g_{DCW}.L⁻¹ resulted in similar initial production rates per cell, but production levelled off after 5 h in the high-density culture, while production in the lower-density cultures could be maintained for 15 h. Furthermore, enzyme selectivity was 94% at a biomass concentration of 7 g_{DCW}.L⁻¹, but only 91% at 28 g_{DCW}.L⁻¹, despite lower volumetric titres at the higher biomass loading (Gudiminch et al., 2012). This may have been due to poor mass transfer of substrate and product promoting product over-oxidation, as will be discussed in Section 2.5.1. Cultures of *E. coli* BL21(DE3) with specific CYP153A6 concentrations of 0.53 and 0.76 μmol.g_{DCW}⁻¹ were both unstable at cell densities of 28 g_{DCW}.L⁻¹, but cultures of *E. coli* BL21(DE3)pLysE, which expressed only 0.15 μmol.g_{DCW}⁻¹ CYP153A6, were stable for 24 h at a cell density of 20 g_{DCW}.L⁻¹ (Gudiminch et al., 2012). It is possible that the metabolic burden associated with higher heterologous expression load makes cells more vulnerable to limitations in nutrient or oxygen supply, or that the over-expression of the monooxygenase increases the formation of reactive oxygen species.

Temperature and pH

Mesophilic organisms naturally operate within relatively narrow ranges of temperature and pH, but heterologously expressed proteins may impose even stricter requirements on operating conditions. In the hydroxylation of n-octane by *Escherichia coli* BL21(DE3) expressing CYP153A6, increasing the reaction temperature from 20°C to 37°C reduced final 1-octanol titres and productivities per cell by roughly a factor of three, despite a four-fold increase in initial activity per unit of enzyme at the higher temperature (Olaofe et al., 2013).

Similarly, the pH of the biotransformation medium can influence biocatalyst activity and stability. Optimisation of pH strategy led to a 22% increase in the conversion of n-octanes to dioic acids by a mutant strain of *Candida tropicalis* (Liu et al., 2004). *In vitro*, CYP153A6 can tolerate a pH range of 5.0 – 9.5 without the protein precipitating. However, hydroxylation of aliphatic alkanes was negatively affected at pH values outside the range of 6.1 – 7.5, and the purified enzyme displayed optimal activity at pH 6.8 – 7.0 (Funhoff et al., 2006). This is compatible with *E. coli*'s preferred pH range of 6.5 – 7.5.

2.4.6 Process operation in whole-cell systems

The combination of microbes and hydrocarbons presents unique process engineering challenges. The substrates and products of biotransformation can interfere with cell membranes, impairing metabolic functions and sometimes even resulting in cell death (as will be discussed in Section 2.5.1), so regulated substrate supply and rapid product removal are necessary to avoid toxicity effects (Bühler and Schmid, 2004; de Carvalho, 2017). Furthermore, whole cells require excellent mass transfer, but the volatility of hydrocarbon substrates and products makes them vulnerable to stripping when combined with oxygen sparging, as well as presenting an explosion hazard. Novel approaches to reactor design are therefore required (Bühler and Schmid, 2004; Schmid et al., 1998; Schrewe et al., 2013a; Soussan et al., 2016; Wachtmeister and Rother, 2016). Biofilms, membrane reactors, gas loop reactors, solid phase substrate supply or product extraction, and two-liquid-phase systems have all been investigated for the biotransformation of hydrocarbon substrates (Hollmann et al., 2011; Lundemo and Woodley, 2015; Schrewe et al., 2013a; Soussan et al., 2016).

Biofilm set-ups can improve resistance to hydrocarbon toxicity, allowing for extended periods of biocatalytic activity in some cases (Hollmann et al., 2011; Soussan et al., 2016). The stability of whole cells can also be improved via immobilisation: *Candida bombicola* has been immobilised on films

composed of octadecylamine to enhance cells' recoverability and reusability (Bernhardt, 2006), *Escherichia coli* cells have been encapsulated in polyvinylidene difluoride "bubbles" to allow operation in neat hydrocarbon substrate (Wachtmeister and Rother, 2016), and the encapsulation of cells within polyvinyl alcohol gel particles known as Lentikats® has been shown to double the number of times recombinant *E. coli* can be reused, relative to free cells, for the production of lactones (Rebroš et al., 2014). Although they are sensitive to fouling by whole cells, membrane reactors can also minimise toxicity while allowing for substrate removal and biocatalyst retention (Bühler and Schmid, 2004; Soussan et al., 2016). However, all these strategies impose additional, sometimes debilitating, barriers to mass transfer (Bühler and Schmid, 2004; Kadisch et al., 2017). The application of adsorbent or hydrophobic resins such as Optipore L-493 and Amberlite XAD-4 for *in situ* extraction can be highly effective, but is limited by the pricing and chemical properties of available resins, making it an unsuitable approach outside of the fine chemical industry (Bühler and Schmid, 2004).

In two-liquid-phase systems, the cells are suspended in a bulk aqueous medium, which is in contact with a bulk hydrocarbon phase. The substrate is therefore available in stoichiometric excess, but its feed rate is limited by its low solubility in the aqueous phase, mitigating toxicity to the cell. The bulk organic phase also provides *in situ* product extraction, thus enhancing reaction thermodynamics and improving biocatalyst selectivity and stability (Bühler and Schmid, 2004; Schrewe et al., 2013a; Woodley, 2013). Biphasic processes have substantially larger water footprints and greater mass transfer limitations than neat substrate systems; they also require more complex downstream processing due to high levels of organic emulsification. However, since the use of neat substrate systems is confined to biocatalysts with extreme solvent tolerance, and hampered by low operational stability, two-liquid-phase operation is the most widely applied approach for the biotransformation of hydrocarbons (Bühler and Schmid, 2004; Schmid et al., 1998; Schrewe et al., 2013a; Soussan et al., 2016; Wachtmeister and Rother, 2016).

In most cases, the substrate itself acts as the bulk organic phase. In some cases, however, the addition of a solvent chemical can improve biocatalyst performance, typically by solubilising or emulsifying the hydrocarbons and hence improving interphase transport (Soussan et al., 2016). However, certain solvents can be cytotoxic and hence have a negative effect on biocatalyst stability. Additionally, chemicals which increase the solubility of the substrate but do not sufficiently promote product export are not necessarily desirable, since the products themselves are typically cytotoxic (Alonso-Gutierrez et al., 2013; Lundemo et al., 2016). For example, applying dodecanoic acid dissolved in dimethyl sulfoxide led to reduced conversions and reaction rates relative to application of dodecanoic acid in solid form for *E. coli* expressing a CYP153A-CPR fusion construct (Lundemo et al., 2016), but the application of methyl- β -cyclodextrin as an alkane solubilising agent doubled product formation rates for *Pseudomonas putida* expressing CYP154A8 (Tieves et al., 2016). In the conversion of n-octane to 1-octanol by *E. coli* BL21(DE3) expressing CYP153A6, the addition of bis(2-ethylhexyl) phthalate (BEHP) had no significant effect on initial biotransformation rates, but enhanced biocatalyst stability from 24 h onwards, resulting in higher final titres after 60 h biotransformations, and allowing biotransformation to continue for up to 120 h. By contrast, application of hexadecene substantially decreased final titres (Olaofe et al., 2013). The presence of BEHP has also been known to improve the activity and cell viability of resting-cell *Mycobacteria* biocatalysts by acting as a product sink (de Carvalho, 2017), and could reduce the explosion risk posed by volatile organics in large-scale operation (Bühler and Schmid, 2004).

2.5 Potential Bottlenecks Affecting Whole-Cell Alkane Hydroxylation

The expression and stability of heterologous enzymes is central to the development of a cytochrome P450-based process. However, as discussed in Section 2.4.2, researchers working across a variety of systems have noted that high levels of enzyme expression do not correlate with high activity in whole-cell biotransformations. Jiang and Morgan (2004) found that *in vitro* activity of the plant P450 ferulate 5-hydroxylase was closely correlated with enzyme concentration, but when the enzyme was expressed in *Saccharomyces cerevisiae*, the activity of the whole cell biocatalyst did not increase at higher levels of heterologous expression. Kadisch et al. (2016) found that a reduction in the expression of AlkB in *Escherichia coli* actually improved product yields in the oxyfunctionalisation of dodecanoic

acid methyl ester. In the conversion of limonene to perillyl alcohol by growing *Pseudomonas putida* cultures, Cornelissen et al. (2011) found that concentrations of CYP153A6 as low as $0.12 \mu\text{mol.g}_{\text{DCW}}^{-1}$ were still not rate-limiting. In whole-cell biotransformations with *E. coli* BL21(DE3) expressing CYP153A6 and its redox partners, increasing the P450 concentration beyond $0.06 \mu\text{mol.g}_{\text{DCW}}^{-1}$ produced no improvements in 1-octanol titres (Olaofe et al., 2013), and cultures containing $0.76 \mu\text{mol.g}_{\text{DCW}}^{-1}$ have been shown to reach lower maximum titres than cultures containing $0.53 \mu\text{mol.g}_{\text{DCW}}^{-1}$ (Gudimich et al., 2012). Furthermore, as mentioned in Section 2.2.3, the highest reported activities reached by resting whole-cell *E. coli* expressing CYP153A6 for alkane hydroxylation have not exceeded $22 \text{ mol}_{\text{product}}.\text{mol}_{\text{enzyme}}^{-1}.\text{min}^{-1}$, despite *in vitro* activities of over $70 \text{ mol}_{\text{substrate}}.\text{mol}_{\text{enzyme}}^{-1}.\text{min}^{-1}$ having been observed for this enzyme, and a maximum theoretical activity of $90 \text{ mol}_{\text{substrate}}.\text{mol}_{\text{enzyme}}^{-1}.\text{min}^{-1}$ being postulated (Arnold et al., 2012; Funhoff et al., 2006).

When rates of alkane hydroxylation by whole cells do not increase as intracellular concentration of P450 increases, it signals that another factor must be limiting the biotransformation. Potential bottlenecks can be identified by considering the simplified reaction scheme shown in Figure 2.5. CYP153A6 receives electrons from ferredoxin (Fdx), which in turn must interact with ferredoxin reductase (FdxR), which obtains reducing equivalents from the nicotinamide cofactor NADH. Biocatalyst activity therefore requires a steady supply of NADH. CYP153A6 must also have sufficient access to its reaction substrates: alkane and molecular oxygen.

The following sections of the Literature Review will focus on those process engineering factors identified, through synthesis of the literature in this study, as key for efficient whole-cell biocatalysis for alkane oxidation: the availability of alkanes and removal of alcohols, including issues of solubility, toxicity, substrate and product inhibition, product over-oxidation, and cross-membrane transport; as well as the availability, efficient use, and regeneration of NADH cofactor. Oxygen supply will also be briefly discussed. Previous studies have examined all these factors independently for their contribution to the performance of various oxidoreductase enzymes. However, there is a gap in the literature when it comes to considering how these factors might influence one another. The balancing of substrates and cofactors is a central theme of the research presented in this thesis, and there are many hints throughout the literature that this is a key issue, not only for the CYP153A6 system, but also for the broader oxidoreductase space.

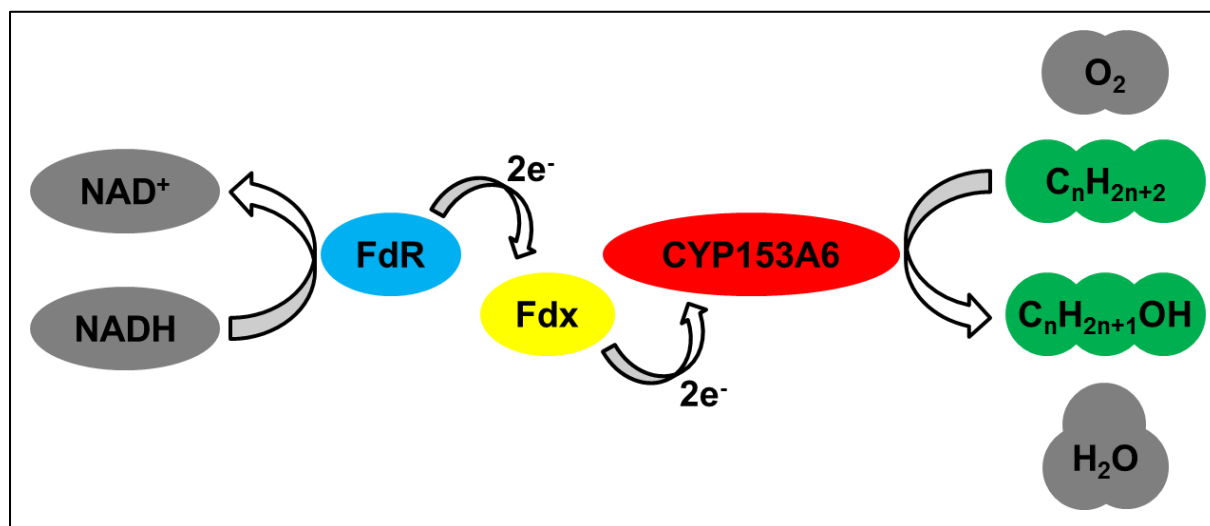


Figure 2.5: Simplified reaction scheme for aliphatic alkane activation by CYP153A6

2.5.1 Balancing enhancement of hydrocarbon transport with host cell viability

Poor transport of hydrocarbons is considered a key limitation in the conversion of organics by whole cells; in many cases, once transport is improved, product titres and reaction rates increase and become dependent on catalytic enzyme concentration (Alonso-Gutierrez et al., 2013; Lundemo and Woodley, 2015; Pennec et al., 2015). Furthermore, where cellular machinery is sensitive to product inhibition and by-product formation, rapid product efflux is key to the production of commodity chemicals (Boyarskiy

and Tullman-Ercek, 2015). However, toxicity tolerance is also a key issue when producing alcohols in whole-cell systems (Becker and Wittmann, 2015). Achieving sustained operation in the presence of toxic compounds is extremely challenging, because the toxicity induces cellular stress responses, causing a reduction in rates of growth or biocatalysis (Bühler et al., 2008). In general, host growth rates have been noted to decrease with increasing hydroxylation rates of medium chain-length alkanes, indicating inhibitory effects of substrates and products on cell metabolism (Bosetti et al., 1992). There is thus a trade-off between maximising concentrations of substrate and product, and minimising detrimental effects on cell physiology.

Transport of hydrocarbons between the aqueous phase and the cytoplasm

In whole-cell systems, alkane molecules must move from the bulk organic phase into the cytoplasm, which can occur either via direct interstitial contact, in which cells adhere to alkane droplets, or via solubilisation of alkane molecules into the aqueous phase, followed by diffusion into the cell. Of the numerous bacterial species known to metabolise alkanes, many accept alkanes that are solid at room temperature, and many are found in the microaqueous environments of various sediments. Therefore, it makes sense for them to engage in direct interstitial uptake. However, most alkane-degrading bacteria also produce some form of biosurfactant, which assists in the solubilisation or emulsification of the hydrocarbon substrate (Abbasian et al., 2015; Bouchez-Naitali et al., 1999; Rojo, 2009; Wentzel et al., 2007). When the genes responsible for alkane metabolism in *Pseudomonas putida* are heterologously expressed in *Escherichia coli*, the host cells have been observed to excrete an emulsifier during growth on n-octane or n-dodecane (Favre-Bulle et al., 1991; Grant et al., 2012).

Many heterologous hosts cannot rely on natural biosurfactants to improve substrate transport. Nevertheless, evidence suggests that even in heterologous hosts, alkane uptake may be taking place via direct interstitial contact with alkane droplets: increasing the substrate excess has been shown to boost initial biocatalytic activities, even when the original concentration already exceeds the solubility limit (Pennec et al., 2015), and the addition of emulsifiers has been shown to improve final titres (Grant et al., 2012). Furthermore, transfer rates between aqueous and organic phase of up to 64 mmol.L⁻¹.h⁻¹ have been reported for n-octane (Lundemo and Woodley, 2015), and transfer rates in the range of 15 – 45 mmol.L⁻¹.h⁻¹ are typical for alkanes with a chain length of C₇ – C₁₀, at alkane concentrations of 20% (v/v). Even for larger molecules such as C₁₁ or C₁₂ linear alkanes, rates of up to 15 mmol.L⁻¹.h⁻¹ have been reported (Schmid et al., 1998). Yet volumetric rates of alkane hydroxylation in whole-cell systems can be over two orders of magnitude lower. It is worth noting that transfer rates in systems at the millilitre scale will not necessarily match those observed in shake flasks or stirred tanks, since vials are not always set up to achieve thorough interphase mixing. However, there is sufficient evidence to suggest that poor diffusion of alkanes into the aqueous phase may not be the main source of transport limitations.

Cells have natural barriers that attempt to block the entry of toxic compounds, and efficient efflux mechanisms to eject toxins that enter the cell. Rates of passive diffusion through the cell membrane are determined by a molecule's partition coefficient into the lipid bilayer, which is higher for more hydrophobic molecules, and its diffusion coefficient, which is higher for smaller molecules. Medium chain length alkanes are extremely hydrophobic, but also fairly bulky. Their alcohol products are similarly bulky as well as being amphiphilic. Consequently, transport of substrate and product between the bulk medium and the cytoplasm is a significant limitation in whole-cell biotransformation of alkanes (Alonso-Gutierrez et al., 2013; Bordeaux et al., 2012; Boyarskiy and Tullman-Ercek, 2015; Grant et al., 2014).

Product inhibition and over-oxidation

Besides limitations in substrate availability, there are studies suggesting that alkane hydroxylases are vulnerable to competitive product inhibition. The apparent substrate affinity of a mutant of CYP102A1 decreased by an order of magnitude in cell free extracts spiked with low concentrations of α -pinene oxidation products (1 mM α -pinene oxide, 0.5 mM verbenol, and 0.5 mM myrtenol), suggesting that these compounds were competing with the substrate for access to the enzymes' active sites (Schewe et al., 2008). The initial dodecanoic acid hydroxylation rates of purified CYP153-based fusion constructs

were found to decrease in proportion to the amount of 12-hydroxydodecanoic acid included in the reaction mixture; a similar effect was observed when the enzymes were used in resting cultures of *Escherichia coli* BL21 (Lundemo et al., 2016).

The competition between substrate and product for active sites can result in product over-oxidation, which represents an inefficient use of oxygen and cofactor, and complicates downstream processing (Lundemo and Woodley, 2015). Across multiple systems, this competition has been shown to be exacerbated by unfavourable ratios between enzyme, substrate, and product. In the conversion of n-dodecane to 1-dodecanol by *E. coli* GEC137 expressing AlkB, over-oxidation of product decreased when AlkB expression was reduced by lowering inducer concentrations (Grant et al., 2011), or when the mass transfer of substrate was improved via the addition of emulsifying agents such as DMSO; but over-oxidation increased when the concentration of DMSO became high enough to suppress *in situ* product extraction (Grant et al., 2012). Similarly, *E. coli* JM101 demonstrated increased over-oxidation of hydroxydodecanoic acid methyl ester by AlkB when cells were expressing higher levels of the membrane protein AlkL, which is hypothesised to boost substrate import but not product export. Over-oxidation of product was also more prevalent in orbitally shaken vials than in stirred tanks, presumably due to poor substrate transport leading to low intracellular substrate concentrations that were insufficient to saturate the AlkB active sites (Kadisich et al., 2016). Meanwhile, the over-oxidation of cyclooctanol by *E. coli* BL21-Gold(DE3) expressing a CYP102A1 mutant decreased when the concentration of cyclooctane was increased from 0.4% (v/v) to 20% (v/v), creating a second liquid phase which would have provided *in situ* product extraction (Pennec et al., 2015). Finally, the selectivity of CYP153A6 for terminal hydroxylation of n-octane was 94% in *E. coli* cultures with a biomass concentration of 7 g_{DCW}.L⁻¹, but only 91% when cell density was 28 g_{DCW}.L⁻¹, despite higher volumetric titres at the lower biomass loading; reduced mass transfer in the high-density system could have limited substrate availability and product export (Gudiminch et al., 2012). Ensuring adequate transport of substrate and product therefore plays a key role in ensuring biocatalyst selectivity.

Substrate and product toxicity

Organic compounds with an n-octanol/water partition coefficient (logP_{OW}) between 1 and 5 partition readily into cell membranes. Increased exposure to alkanes and alcohols induces changes in the ratio of saturated and unsaturated fatty acids, and causes the membrane to become more fluid (Abbasian et al., 2015; Berla et al., 2015). These structural alterations interfere with homeostasis, disrupt the proton gradient that drives ATP generation, and prompt upregulation of toxin efflux mechanisms. As a result, the metabolic load on the cell increases substantially (Bühler et al., 2008; de Carvalho, 2017; Favre-Bulle et al., 1991; Kadisich et al., 2017; Schrewe et al., 2013a). Within the cytoplasm, organic compounds may also reduce the stability of enzymes by damaging protein structure, irreversibly binding to the active site, interfering with the hydration shell, or locking the protein conformation (Kadisich et al., 2017).

Smaller and more apolar molecules have increased diffusivity, but are also more likely to be toxic (Bühler and Schmid, 2004). Studies with *Pseudomonas putida* sp. 8141 found that cultures could grow on C₇ – C₁₂ linear alkanes at concentrations of up to 20% (v/v), but could not grow on 20% (v/v) n-hexane, which has a logP_{OW} of less than 4 (Bosetti et al., 1992). Similarly, *Escherichia coli* exhibited a consistent maximum growth rate in the presence of bulk amounts of solvents with a logP_{OW} of at least 4, but would not grow at all in the presence of solvents with a logP_{OW} of 3.7 or less (Favre-Bulle et al., 1991). *E. coli* expressing the CYP102A1 mutant 139-3C1NADH have also been found to suffer from substrate inhibition when hydroxylating cyclohexane, but not when hydroxylating cycloheptane or cyclooctane (Pennec et al., 2015).

However, while n-octane has been shown to have no significant effect on *E. coli* W3110 during exponential growth in two-liquid-phase systems, cells in stationary phase rapidly lost viability as octane was incorporated into the outer membrane, disrupting its structure in a manner comparable to the action of the surfactant Polymyxin B, and eventually resulting in membrane disintegration (Favre-Bulle et al., 1991). Meanwhile, in the hydroxylation of n-octane by CYP153A6 in *E. coli*, concentration of active P450 dropped 74% during the first 24 h of biotransformation, compared to 22% for cells in the absence of n-octane (Gudiminch et al., 2012). Furthermore, octanol has a logP_{OW} of 2.9, indicating an

amphiphilic nature which makes it particularly toxic to cells. In the conversion of n-octane to 1-octanol by resting *E. coli* BL21(DE3) cells expressing CYP153A6, final titres decreased when the reaction medium was spiked with 1-octanol, with complete inhibition of the biocatalyst when approximately 40 mM – equivalent to $1.25 \text{ g}_{\text{octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$ – was added (Olaofe et al., 2013).

2.5.2 Overcoming the transport limitations associated with cell membranes

Despite the dangers of increased exposure of cells to alkanes and alcohols, biocatalytic systems cannot be optimised without addressing limitations in hydrocarbon transport. In this regard, the cell membrane is the chief barrier to movement of substrate and product. However, the membrane also plays a key role in maintaining homeostasis and metabolic activity, which in turn affect enzyme stability and cofactor availability, both of which are essential for successful cytochrome P450 biocatalysis. A balance must therefore be struck between increasing cross-membrane transport and protecting the cell. Potential solutions include increasing the general permeability of the membrane via chemicals, physical treatment, or structural alterations at the genetic level; or introducing additional heterologous proteins that specifically target hydrocarbon transport.

Physical treatment to enhance cross-membrane transport

It is possible to disrupt the structure of the membrane without the application of chemicals. Cell disruption via freezing and subsequent rapid thawing has been shown to lead to increased coupling efficiencies for CYP101 enzymes expressed in *Escherichia coli* (Kaluzna et al., 2016). However, freeze-thaw treatment had a detrimental effect on the hydroxylation of dodecanoic acid by *Escherichia coli* expressing a CYP153 fusion construct; sonication of cells also led to decreased product formation (Lundemo et al., 2016). This once again highlights the difficulty of achieving the right amount of cell disruption to improve transport without compromising cell physiology.

Although not strictly a form of physical treatment, membrane fluidity can also be engineered through the adaptation of cell lines (de Carvalho, 2017) or through alterations to the culture medium. Magnesium starvation (Chubukov and Sauer, 2014) or increased availability of oleic or linoleic acid (Hama et al., 2004) during growth will produce cells with more fluidised membranes.

Application of chemicals for substrate emulsification and membrane permeabilization

A variety of organic solvents, detergents, antibiotics, and chelating agents are known to partition into cell membranes, increasing their phospholipid mobility and altering their structure. This can increase membrane fluidity, induce cells to alter their morphology, or result in cell lysis. The chemically-treated membranes will present less of a barrier to substrate and product transport, but their capacity to perform their physiological functions will be reduced. The extent of membrane modification is proportional to the concentration, temperature, and length of exposure to the chemical (Lounatmaa and Nanninga, 1976; Posokhov and Kyrychenko, 2013; Ruff et al., 2016).

Thus, a particular chemical may improve biocatalyst performance when applied in one system, but be detrimental when applied in another, as shown in Table 2.2 below. If applied to the biotransformation reaction mixture, these chemicals may also encourage or discourage the formation of organic/aqueous emulsions, which will affect the biotransformation (Grant et al., 2012).

Table 2.2: Attempts to facilitate biotransformation via treatment of *Escherichia coli**, *Schizosaccharomyces pombe***, or *Pseudomonas putida**** whole cells with emulsifying chemicals and permeabilising agents

Biocatalytic enzyme	Chemical treatment	Type of action	Observed effect	Reference
Carnitine racemase*	Acetone	Membrane permeabilization	Pre-treatment with 20% (v/v) for 10 minutes at up to 37°C improved L-carnitine yields by 10 – 20%	Canovas et al. (2005)
CYP153A6*			Initial n-octane hydroxylation rates doubled, but > 5% acetone caused cell aggregation and complete loss of activity	Olaofe et al. (2013)
CYP105A1*	Cetrimonium bromide	Membrane permeabilization	Reduced conversion of abietic acid	Janocha and Bernhardt (2013)
AlkB*	DMSO	Hydrocarbon emulsification	Two-fold improvements in specific activity on n-dodecane and eight-fold improvements in product formation per cell when applied at 4% (v/v), but high concentrations decreased selectivity	Grant et al. (2012)
CYP105A1*	EDTA	Membrane permeabilization	15-hydroxyabietic acid titres after 24 h increased by a factor of 2.7	Janocha and Bernhardt (2013)
Carnitine racemase*	Ethanol	Membrane permeabilization	Pre-treatment with 20% (v/v) for 10 minutes at up to 30°C improved L-carnitine yields by 17 – 25%	Canovas et al. (2005)
CYP21**	Hexadodecyl trimethyl ammonium bromide	Membrane permeabilization	Complete activity loss	Zehentgruber et al. (2010)
Carnitine racemase*	Isopropyl alcohol	Membrane permeabilization	Pre-treatment with 20% (v/v) for 10 minutes at up to 20°C improved L-carnitine yields by 5 – 15%	Canovas et al. (2005)
CYP154A8***	Methyl- β -cyclodextrin	Membrane permeabilization Hydrocarbon emulsification	Increased rates of n-alkane hydroxylation	Tieves et al. (2016)
AlkB*	Polyethyleneimine	Membrane permeabilization Hydrocarbon emulsification	None	Grant et al. (2012)
Carnitine racemase*			Pre-treatment with 0.1% (v/v) for 10 minutes at up to 40°C improved L-carnitine yields by 20 – 44%	Canovas et al. (2005)
CYP153A6*	Polymyxin B	Membrane permeabilization	Increased biocatalytic activity on n-octane and n-dodecane	Pennec et al. (2015)
CYP105A1*			15-hydroxyabietic acid titres after 24 h increased by a factor of 5	Janocha and Bernhardt (2013)
CYP153A6*	Toluene	Membrane permeabilization	Cell aggregation and complete loss of activity	Olaofe et al. (2013)
CYP105A1*			15-hydroxyabietic acid titres after 24 h increased by a factor of 2.5	Janocha and Bernhardt (2013)
Carnitine racemase*			Pre-treatment with 2% (v/v) for 10 minutes at up to 30°C improved L-carnitine yields by 10 – 22%	Canovas et al. (2005)
Carnitine racemase*	Triton X-100	Membrane permeabilization Hydrocarbon emulsification	Pre-treatment with 20% (v/v) for 10 minutes at up to 40°C improved L-carnitine yields by 15 – 35%	Canovas et al. (2005)
AlkB*			Specific activity on n-dodecane tripled	Grant et al. (2012)
CYP105A1*	Tween 20	Membrane permeabilization Anti-foaming	Reduced conversion of abietic acid	Janocha and Bernhardt (2013)
Carnitine racemase*			Pre-treatment with 2% (v/v) for 10 minutes at up to 40°C improved L-carnitine yields by 10 – 35%	Canovas et al. (2005)
CYP21*	Tween 80		50% increased activity on steroids	Zehentgruber et al. (2010)

Altering membrane structure via genetic engineering

Even though the outer membrane of gram-negative bacteria is known to present a major barrier to the passage of alkanes, bacterial strains with alkane hydroxylase activity are more likely to be gram-negative than gram-positive. These strains ensure sufficient substrate transport by expressing relevant transporter proteins and through the production of biosurfactants (Abbasian et al., 2015; Van Beilen et al., 2005). When carrying out biotransformations in heterologous hosts, the cell's hydrocarbon uptake capacity can be improved by co-expressing transporter proteins from a hydrocarbonoclastic species, or by genetically modifying or upregulating the host's own transport proteins. However, as with all membrane disruptions, overexpression of these proteins can interfere with translocation or with the cell's ability to maintain a potential gradient across the membrane, thus reducing the cell's metabolic capacity and making it more vulnerable to toxicity (Boyarskiy and Tullman-Ercek, 2015; Kadisch et al., 2017; Schrewe et al., 2013a). Furthermore, membrane pre-proteins must be exported to the outer membrane of *Escherichia coli* by an ATP-driven translocation system, and the successful expression of heterologous membrane proteins may therefore be more difficult to achieve than the expression of soluble heterologous proteins, and may impose a higher metabolic burden on the host (Baneyx, 1999; Kadisch et al., 2017).

Transporter proteins can be active (consuming ATP to force molecules across membranes) or passive (simply offering molecules wider channels for diffusion). Importer proteins tend to be more specific than exporters; hence the expression ratio of importers to exporters tends to be high. In bacteria, transporters may cross both the inner and outer membranes, but some cross only one and thereafter rely on passive diffusion (Boyarskiy and Tullman-Ercek, 2015). *E. coli* contain at least two native transporter proteins, OmpW and FadL, which can bind to small hydrophobic molecules in the environment and release them into the periplasm. These transporters are intended for the import of aliphatic fatty acids, but are also capable of transporting medium chain length alkanes (Call et al., 2016). Proteins similar to OmpW and FadL are found in most gram-negative bacteria, although they are generally not well characterised. They cross the outer membrane but not the cytoplasmic membrane, and contain a hydrophobic core which is unusual among outer-membrane proteins (Grant et al., 2014).

Meanwhile, the native *E. coli* efflux system AcrAB-TolC is able to export n-octane, as indicated by large reductions in cell growth in the presence n-octane when AcrB is deleted. Enhancement of octane efflux via directed evolution also led to improved growth in the presence of 1-octanol, as well as enhancing efflux rates of α -pinene, suggesting that the same transporter proteins which handle alkanes are also targeting other toxic hydrocarbons (Foo and Leong, 2013). *E. coli* tolerance towards decane, undecane, and isooctane can be increased by boosting the expression of efflux pumps (Dai and Nielsen, 2015).

Expression of heterologous transporter proteins has proven to be a successful strategy in several cytochrome P450-based biotransformations. In one case, the heterologous expression of the cross-membrane protein FhuA, genetically altered to act as a wide passive diffusion channel, resulted in a five-fold improvement in the transport of 7-benzoxo-3-carboxy-coumarin ethyl ester into *E. coli*. In the biotransformation of aromatics and terpenes, expression of this channel protein increased specific product formation rates by 50–150%, and decreased the rate of product over-oxidation. The improvements became more pronounced for more sterically demanding substrates (Ruff et al., 2016). Similarly, Call et al. (2016) observed a 4.5-fold increase in the rate of alkane import when FadL was over-expressed in *E. coli*. However, for cells expressing AlkB, heterologous expression of the *Pseudomonas oleovorans* transporter protein AlkL (Kok et al., 1989) was more effective than over-expression of either FadL or OmpW when it came to boosting specific activity on n-octane and increasing selectivity towards 1-octanol.

Table 2.3 summarises a number of studies where AlkL was expressed in *E. coli*. Julsing et al. (2012) found that when AlkL was co-expressed alongside AlkB in *E. coli*, the maximum specific activity of the biocatalyst increased by 23% on nonanoic acid methyl ester (NAME), 28-fold on dodecanoic acid methyl ester (DAME), 4-fold on n-octane, and 40-fold on n-nonane. Van Nuland et al. (2016) found that AlkL co-expression could improve initial activities on NAME by a factor of 1.7, although it was less beneficial for substrates with shorter chain lengths or lower logP_{ow} values. Ladkau et al. (2016) expressed both AlkB and ω -transaminase CV2025 in *E. coli*, and found that specific activity on DAME could be increased seven-fold by also expressing AlkL; the formation of by-products also decreased.

Unfortunately, however, AlkL appeared to increase the susceptibility of the host cells to substrate toxicity. Kadisch et al. (2016) noted that besides improving the maximum specific activities on n-octane or DAME by up to an order of magnitude, the presence of AlkL reduced the over-oxidation of 1-octanol by AlkB. In this case, however, high AlkL expression levels had a detrimental effect on overall space time yield, since activities fell off rapidly as the biotransformation progressed. This was not related to expression of the protein itself – in the absence of DAME, *E. coli* expressing high levels of AlkL exhibited similar growth rates to strains lacking AlkL. The negative effects were therefore associated with exposure to a greater quantity of toxic substrate inside the cytoplasm. Similarly, AlkL expression proved to be essential for the hydroxylation of dodecane, tetradecane, and hexadecane, but was found to reduce the formation of 1-octanol, due to biocatalyst instability (Grant et al., 2014). Meanwhile, in cytochrome P450 systems, the hydroxylation of limonene by *E. coli* whole cells has also been improved via AlkL co-expression (Cornelissen et al., 2013). Overall, the literature shows that AlkL expression represents a trade-off between activity and stability in AlkB-based biocatalysts, and suggests that the usefulness of AlkL is modulated by other factors specific to individual systems. It is therefore worth exploring whether AlkL can improve n-octane hydroxylation by CYP153A6 in *E. coli*.

Table 2.3: Expression of AlkL in *Escherichia coli* whole cells expressing an alkane hydroxylase for biotransformation

Substrate(s)	Observed effect	Reference
n-octane	4-fold increase in maximum specific activity	Julsing et al. (2012)
	3.5-fold decrease in yield on biocatalyst	Grant et al. (2014)
n-nonane	40-fold increase in maximum specific activity	Julsing et al. (2012)
n-dodecane	No product formation was observed when AlkL was not expressed	Grant et al. (2014)
n-tetradecane		
n-hexadecane		
NAME	23% increase in maximum specific activity	Julsing et al. (2012)
	1.7-fold increase in maximum specific activity	Van Nuland et al. (2016)
DAME	28-fold increase in maximum specific activity	Julsing et al. (2012)
	16-fold increase in maximum specific activity	Kadisch et al. (2016)
	7-fold increase in maximum specific activity Reduced by-product formation	Ladkau et al. (2016)
Limonene	2.5-fold increase in space time yield	Cornelissen et al. (2013)

2.5.3 Ensuring sufficient supply of NADH cofactor

Nicotinamide adenine dinucleotide (NADH) is the fuel that drives a CYP153A6-based biotransformation; it functions as a source of reducing equivalents for the redox partners that service the P450. Adequate supply of NADH is therefore crucial for sustained biotransformation. The addition of purified cofactor represents an unsustainable process cost, and is one of the key reasons why P450 biotransformations are typically carried out in whole cells, as discussed in Section 2.4.1. Biocatalyst performance can potentially be enhanced by improving the coupling efficiency of the P450 or boosting the host cell's capacity to regenerate cofactor.

The cofactor pool

The cellular cofactors NADH/NAD⁺ (nicotinamide adenine dinucleotide) and NADPH/NADP⁺ (nicotinamide adenine dinucleotide phosphate) are both capable of transferring reducing equivalents in the form of hydride ions. Although they carry out the same mechanistic function, the presence or absence of the phosphate atom has led many enzymes to evolve a preference for a particular type of nicotinamide cofactor. Although there are exceptions, the enzymes involved in anabolic processes tend to prefer NADPH, while those involved in catabolism tend to prefer NADH. The two cofactor pools are thus able to function somewhat independently of one another, each maintaining its own redox poise and flux; this separation helps to prevent futile metabolic cycles (Hollmann et al., 2011; King and Feist, 2014; Sellés Vidal et al., 2018).

The cofactor pool is highly responsive to environmental factors, as cells must balance their anabolic and catabolic processes to meet the requirements imposed by their environment. Oxygen-rich conditions favour ATP generation over NAD(P)H generation, and thus the action of the respiratory chain shifts cofactor pools towards NAD(P)⁺ (Bailey et al., 1996; Sellés Vidal et al., 2018). Furthermore, as mentioned in Section 2.4.3, the biocatalytic performance of whole cells is affected by the prevailing conditions during growth and induction, which among other things determines the state of the starting cofactor pool. Gudiminchí et al. (2011, 2012) found that *Escherichia coli* cells harvested after 20 h of cultivation had low NADH/NAD⁺ ratios regardless of whether CYP153A6 was expressed via continuous induction on lactose or point induction using IPTG. However, after 25 h, cultures under point induction had shifted their cofactor pools towards a higher concentration of NADH, while cultures under continuous induction had not.

Cofactor pools in *E. coli* demonstrate a high degree of stability. New cofactors are generated mainly to maintain pool size following cell division; during stages of non-growth, the pool size does not vary significantly. However, the rate at which nicotinamide cofactors go through the redox cycle is extremely high for growing cells. NADH/NAD⁺ also has a T₅₀ of 28 ± 1 minutes, meaning that roughly half of the carbon atoms in the cofactor pool are being replaced every half an hour. This suggests that cofactor is being damaged or partially consumed in certain metabolic reactions, and then repaired; although the core niacin moiety appears to be extremely stable (Hartl et al., 2017).

NADH demands associated with maintenance and biotransformation

Cofactor-dependent heterologous enzymes can upset the natural cofactor balance of the cell. Redox potential plays a key role in directing metabolic flux; a severe redox imbalance can seriously impair cellular functioning, including protein synthesis, intracellular transport and signalling, and even transcriptional regulation (Berla et al., 2015; Bühler and Schmid, 2004; Su et al., 2015; Wang et al., 2017). *Escherichia coli* cells can generate up to 2.5 molecules of ATP from the oxidation of one molecule of NADH as part of respiratory processes, so NADH-dependent biocatalysis could be expected to significantly decrease the energy available for cellular upkeep (Wu et al., 2016).

However, evidence suggests that cofactor pools exist in excess of the cell's minimum requirements. Cells continue to grow after their ability to synthesise fresh cofactors is removed, even though cofactor concentrations per cell must be decreasing with each round of cell division (Hartl et al., 2017). Furthermore, models have suggested that growing *E. coli* cultures can produce enough cofactor to support biotransformation activities of 575 – 720 $\mu\text{mol}_{\text{substrate}} \cdot \text{g}_{\text{DCW}}^{-1} \cdot \text{min}^{-1}$. In resting cultures, where cells require an estimated 8 – 10 $\text{mmol}_{\text{ATP}} \cdot \text{g}_{\text{DCW}}^{-1} \cdot \text{min}^{-1}$ for maintenance requirements (Chubukov and Sauer, 2014), maximum biocatalytic activities of 305 – 367 $\mu\text{mol}_{\text{substrate}} \cdot \text{g}_{\text{DCW}}^{-1} \cdot \text{min}^{-1}$ should still be attainable. However, real systems underperform these theoretical maximums by as much as four-fold (Blank et al., 2008).

For example, even though *Pseudomonas putida* strains have been estimated to require less than 1% of a cell's theoretically available NADH reserves in order to carry out biotransformation of n-octane (Vallon et al., 2013), *P. putida* sp. 8141 could not maintain production of alcohols after cells entered stationary phase, presumably because NADH could no longer be regenerated due to depletion of the pyruvate growth substrate (Bosetti et al., 1992). Meanwhile, the transfer of resting cells to fresh medium has been shown to renew the activity of whole-cell *E. coli* biocatalysts expressing CYP102A1 mutants for propane hydroxylation (Fasan et al., 2007). It has also been shown that 1-octanol formation rates improve significantly when *E. coli* expressing CYP153A6 are supplied with 10 g.L⁻¹ glucose as opposed to 5 g.L⁻¹, although increasing the concentration further to 16 or 22 g.L⁻¹ leads to stability loss (Gudiminchí et al., 2012). More directly, in the terminal hydroxylation of dodecanoic acid by a CYP153-based fusion construct, supplying resting cell suspensions with 1.5 – 3 mM NADPH resulted in increased initial rates and substantially improved final titres (Lundemo et al., 2016). NADPH availability has also been confirmed to limit the epoxidation of styrene once activities exceed 21 $\mu\text{mol}_{\text{substrate}} \cdot \text{g}_{\text{DCW}}^{-1} \cdot \text{min}^{-1}$ (Bühler et al., 2008), which is an order of magnitude lower than the maximum theoretical rates suggested by Blank et al. (2008).

Negative impact of uncoupled reactions

One hypothesis is that cytochromes P450 are consuming large amounts of cofactor, but it is not being channelled into biotransformation. As shown previously in Figure 2.3, cytochromes P450 are capable of diverting cofactor to uncoupled reactions. Before being inserted into the hydrocarbon substrate, oxygen can escape in the form of water or reactive oxygen species, namely superoxide and hydrogen peroxide. Reactive oxygen species are cytotoxic and hence impact negatively on the stability of the cell. Hydrogen peroxide may also induce oxidative deterioration of the haem group's porphyrin ring. Moreover, uncoupling is an inefficient use of resources, to the extent that total turnover numbers have been shown to correlate more closely with coupling efficiency than with catalytic rate in some systems (Bernhardt, 2006; Fasan et al., 2007; Holtmann and Hollmann, 2016; Julsing et al., 2008; Kadisch et al., 2017; Kaluzna et al., 2016; Lundemo and Woodley, 2015; Schrewe et al., 2013a).

Coupling efficiencies are commonly reported in the literature in studies of genetically engineered enzyme systems, but are less consistently reported for wild-type enzymes with their natural redox partners. Although coupling efficiencies of over 98% have been observed, especially in the case of enzymes modified with the specific aim of reducing uncoupling, coupling efficiencies below 50% are often reported (Bernhardt, 2006; Holtmann and Hollmann, 2016; Soussan et al., 2016). It is a particular issue in the biotransformation of non-natural substrates, since a common cause of uncoupling is sub-optimal substrate binding in the active site (Bühler and Schmid, 2004; Fasan et al., 2007; Lundemo and Woodley, 2015; Sazykin et al., 2019). Coupling efficiencies may also decrease with increasing extent of reaction, as has been shown for some flavin-based monooxygenases (Bühler et al., 2008; Holtmann and Hollmann, 2016). This may be related to the strong correlation between coupling efficiency and substrate availability (Bühler and Schmid, 2004; Kadisch et al., 2017). For example, the coupling efficiency of decoy molecule-mediated CYP102A1 converting gaseous alkanes could be increased by increasing system pressure, which increases the concentration of substrate in solution (Kawakami et al., 2015).

Redox partners can have a significant impact on coupling efficiency, and may also participate in uncoupling reactions themselves, producing hydrogen peroxide from oxygen (Holtmann and Hollmann, 2016). NADH consumption by CYP153A6 in the absence of substrate was shown to be directly proportional to ferredoxin concentration (Arnold et al., 2012). In cell free extracts containing CYP153A6, ferredoxin, and ferredoxin reductase, system stability decreased if the ratio of ferredoxin reductase to P450 was increased beyond 1:1, and although initial turnover frequencies and total turnover numbers increased as the ratio of P450 to ferredoxin was increased up to 1:30, further increases also had a detrimental effect on enzyme stability (Kochius et al., 2018). Meanwhile, the high biocatalytic activities demonstrated by CYP102A1, which exceed those of any other known wild-type P450, as well as its high coupling efficiencies of 93 – 96%, are commonly attributed to the natural fusion of its monooxygenase and reductase subunits (Fasan et al., 2007; Guengerich and Munro, 2013; Van Beilen and Funhoff, 2005). Fusion chimeras have been generated in an attempt to mimic this monomeric nature and associated high coupling efficiency (Grogan, 2011). For example, the coupling efficiency of CYP153A35 in the *in vitro* hydroxylation of palmitic acid could be increased by a factor of four via fusion to the reductase domain of CYP102A1 (Jung et al., 2016). In general, however, artificial fusion proteins tend to exhibit reductions in catalytic efficiency and stability (Girvan and Munro, 2016).

A coupling efficiency of 98% has been reported for purified CYP153A6, ferredoxin, and ferredoxin reductase. Even in the absence of substrate, the enzymes reached a maximum NADH consumption rate of only $15 \text{ mol}_{\text{NADH}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$, which was well below the $90 \text{ mol}_{\text{NADH}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$ demonstrated in the presence of n-octane. This suggests that the purified CYP153A6 system is relatively immune to uncoupling (Arnold et al., 2012). However, in whole-cell systems, the intracellular accumulation of product encourages uncoupling, as product molecules block the active site and prompt the enzyme to donate electrons and protons to oxygen instead (Holtmann and Hollmann, 2016).

Cofactor Regeneration Strategies

Whether reducing equivalents are lost to uncoupled reactions or to cellular metabolism, boosting their availability within the cytoplasm has been shown to improve biocatalytic performance (Bosetti et al., 1992; Bühler et al., 2008; Fasan et al., 2007; Gudimich et al., 2012; Lundemo et al., 2016). Since the

application of purified cofactor would be prohibitively expensive, cofactor regeneration systems must be devised (Hummel and Gröger, 2014).

The cofactor balance can be controlled via genetic engineering strategies, including up- or downregulating genes for endogenous cofactor-dependent proteins, replacing native oxidoreductases with heterologous versions having different cofactor specificity, expressing heterologous cofactor regeneration enzymes, expressing transhydrogenases which can interconvert NADH and NADPH, or engineering the biocatalytic enzyme to alter its cofactor preference. Such strategies have led to production increases of anywhere between 10% and 7-fold; their efficacy seems to depend on multiple factors, including host species, oxygen presence and uptake rate, substrate type and uptake rate, and growth rate (Chen et al., 2014; King and Feist, 2014; Sellés Vidal et al., 2018; Wang et al., 2017).

Such systems engineering approaches are laborious, time-consuming, and strain-specific. Therefore, most strategies to improve cofactor supply focus solely on the regeneration of NAD(P)⁺. The most common strategy involves the application of co-enzymes and co-substrates, discussed in detail in the next section. However, researchers are also looking for ways to avoid the use of nicotinamide cofactors entirely. These approaches are usually associated with *in vitro* operation, and include exploitation of the peroxide shunt pathway, application of artificial chemical mediators, photochemical regeneration, and electrochemical recycling.

The peroxide shunt is less efficient than oxygen-based catalysis, and the addition of peroxides to whole-cell systems poses numerous challenges. Although this is being tackled via genetic engineering of monooxygenases and characterisation of naturally-occurring peroxidases, instability remains a major issue (Holtmann and Hollmann, 2016; Jelsing et al., 2008; Soussan et al., 2016).

Artificial mediators such as deazaflavins or transition metal complexes are able to transfer electrons from cheap chemical sources such as iodosylbenzene, sodium diethyldithiocarbamate, or sodium formate. Diols are a particularly interesting source of non-natural reducing equivalents, since they produce potentially valuable lactones as a by-product. CYP102A1 has also been engineered to accept electrons from substrates other than NADPH. Unfortunately, coupling efficiencies of these systems are low due to incorrect or insufficient binding interactions between the artificial mediators and the cytochrome P450, and biocatalytic activities can be reduced by several orders of magnitude (Grogan, 2011; Holtmann et al., 2014; Holtmann and Hollmann, 2016; Kara et al., 2014; Soussan et al., 2016; Wachtmeister and Rother, 2016).

Rather than accepting reducing equivalents in the form of hydrides from NAD(P)H, flavin-based proteins can accept electrons directly from a cathode. Meanwhile, the anode recycles H₂O back into molecular oxygen. However, this strategy tends to result in lower turnover numbers than enzymatic regeneration, and currently requires the addition of costly mediators to boost efficiency. This defeats the purpose of moving away from NAD(P)H in the first place. Photochemical regeneration of redox partners is also being explored, but cannot yet match the effectiveness of utilising NADH (Hollmann et al., 2011; Holtmann et al., 2014; Holtmann and Hollmann, 2016; Kara et al., 2014; Soussan et al., 2016; Rauch et al., 2020).

The use of dehydrogenases and sacrificial substrates for cofactor renewal

In a pared-down version of systems engineering, biocatalytic enzymes that obtain reducing equivalents from NAD(P)H can be coupled with additional heterologous enzymes that reduce NAD(P)⁺. In the ideal case, a multi-step reaction cascade from substrate to product is designed to be redox neutral overall (Holtmann et al., 2014; Hummel and Gröger, 2014; Kara et al., 2014). Unfortunately, not all products lend themselves to this approach. Thus, a more common strategy is to carry out cofactor regeneration in parallel to the biotransformation. Typically, a cytochrome P450 will be combined with a dehydrogenase, which regenerates NAD(P)H by oxidising a “sacrificial” substrate such as an alcohol, formate, glucose, glucose-6-phosphate, glycerol, or phosphite. Of these, systems based on formate, glucose, and glycerol are the most commonly applied; their relative strengths and weaknesses are outlined in Table 2.4. Other co-enzymes that have been utilised include transhydrogenase, mitochondrial NADH kinase, mitochondrial alternative oxidase, and cytoplasmic NADH oxidase (Ceccoli et al., 2014; Holtmann et al., 2014; Hummel and Gröger, 2014; Kara et al., 2014; Wachtmeister and

Rother, 2016; Wang et al., 2017). In certain cases, the enzyme responsible for the biotransformation is also able to act on a sacrificial substrate, with the combination of the two reactions being redox neutral. This avoids the need to express additional heterologous enzymes. However, many cytochromes P450 either do not have this capacity, or else the conditions that are favourable for the biotransformation reaction are not optimal for the regeneration reaction. Furthermore, the two substrates must compete with one another for access to enzyme, which can limit the rate of biotransformation (Hummel and Gröger, 2014; Kara et al., 2014). More generally, potential drawbacks of dehydrogenase-based regeneration systems stem from the need to include a sacrificial substrate, and the associated generation of by-products. This can lead to increased process costs, additional complications in downstream processing, and interference with biocatalyst activity or stability (Hummel and Gröger, 2014; Kara et al., 2014; Soussan et al., 2016).

Table 2.4: Comparison of formate, glucose, and glycerol for use in dehydrogenase-based cofactor regeneration systems

Sacrificial carbon source	Advantages	Disadvantages	Reference(s)
Formate	Well-established; has been applied industrially Cheap and non-harmful Irreversible reaction, resulting in strong driving force	Low specific activity Carbon dioxide co-product is environmentally unfriendly	Hummel and Gröger, 2014
Glucose	High specific activity Cheap	Causes acidification of medium Gluconate co-product complicates downstream processing	Hummel and Gröger, 2014 Soussan et al., 2016 Kaluzna et al., 2016
Glycerol	Minimises acetate production Offers a higher yield of reducing equivalents relative to glucose Dihydroacetone co-product is potentially valuable	Reduced biotransformation activity relative to glucose	Bordeaux et al., 2014 Chen et al., 2014 Hummel and Gröger, 2014 Tieves et al., 2016

Dehydrogenases have been applied for cofactor regeneration in a number of P450 systems with varying results. For example, Pennec et al. (2015) found that glucose dehydrogenase had no significant effect on the hydroxylation of C₆ – C₁₀ cyclic alkanes by *Escherichia coli* expressing CYP153A6, Tieves et al. (2016) found that glucose dehydrogenase led to increased initial hydroxylation rates of n-octane by CYP154A8, but did not improve the productivity of extended biotransformations, and Kochius et al. (2018) found that the application of glucose dehydrogenase to cell free extracts containing CYP153A6 led to two-fold higher titres after 7 h, but significantly reduced titres after 17.5 h, following loss of biocatalyst stability due to acidification of the medium.

On the other hand, *E. coli* JM109, expressing CYP105A1 for abietic acid hydroxylation, exhibited a 10% increase in product titres following heterologous expression of alcohol dehydrogenase (Janocha and Bernhardt, 2013). Co-expression of glucose dehydrogenase has been shown to double the activity of aldehyde reductase on 4-chloro-3-oxobutanoate (Kataoka et al., 1999), increase rates of α -pinene oxidation by CYP102A1 by up to 6.7-fold (Schewe et al., 2008), and enhance CYP102A1-based indigo synthesis (Julsing et al., 2008). Also, the conversion of camphor by CYP101, heterologously expressed in *E. coli*, could be increased by an order of magnitude through co-expression of glycerol dehydrogenase (Mouri et al., 2006).

NADH regeneration rates in *E. coli* BL21(DE3) have been shown to increase substantially following heterologous expression of glycerol dehydrogenase. However, the effects on hydroxylation of n-octane were mixed: some cultures showed substantial improvements in initial activity and maximum titres, while in others, biocatalyst performance was somewhat reduced. Glycerol dehydrogenase expression was also shown to decrease expression of CYP153A6 by almost 60% (Gudiminch et al., 2011).

2.5.4 Supplying oxygen for respiration and as a substrate

Cytochromes P450 suffer from what Holtmann and Hollmann (2016) referred to as the “oxygen dilemma”: all of the reactions that they carry out involve the insertion of an oxygen atom into a hydrocarbon molecule via highly reactive iron-oxo intermediates, yet these same intermediates can undergo spontaneous reduction into harmful reactive oxygen species. Thus, oxygen is simultaneously a key substrate and a serious threat to biocatalyst stability.

Oxygen demand in whole cells is closely linked to metabolism, and can vary widely depending on the bacterial strain and the levels of stress the cells are experiencing. Values as low as $0.1 \text{ mmolO}_2 \cdot \text{g}_{\text{DCW}}^{-1} \cdot \text{h}^{-1}$ (Farmer and Jones, 1976, for continuous culture undergoing glycerol-limited growth at 20°C) and as high as $21.2 \text{ mmolO}_2 \cdot \text{g}_{\text{DCW}}^{-1} \cdot \text{h}^{-1}$ (Andersen and Meyenburg, 1980, for batch culture growing on glycerol at 37°C) have been reported for *Escherichia coli*. Oxygen limitation prompts the cell to make substantial adjustments to its metabolic capacities, usually involving substantial reductions in its long-term metabolic output (Rosano and Ceccarelli, 2014). In the case of P450 biotransformations, however, too much oxygen can be as harmful as too little. Excess oxygen supply during cell growth has been shown to promote the incorrect folding of P450s (Julsing et al., 2008), and excess oxygen availability during biotransformation has been linked to decreases in regioselectivity and increases in product over-oxidation (Bühler and Schmid, 2004). In one case, *E. coli* expressing a CYP102A1 mutant, capable of hydroxylating propane, could maintain activity for longer periods when the oxygen/propane ratio was reduced (Fasan et al., 2007).

Although the stoichiometric oxygen requirements for P450 catalysis are well within the capacity of industrial-size fermenters, oxygenases typically have much higher Michaelis-Menten constants than proteins in the cell's electron transport chain, meaning that biotransformations can potentially become oxygen-limited as cells divert oxygen towards respiration (Lundemo and Woodley, 2015). Also, shake flasks typically demonstrate inferior mixing and mass transfer relative to stirred bioreactors, and vials demonstrate inferior mixing relative to shake flasks (Kadisich et al., 2016; Lundemo et al., 2016). For example, Meisner (2013) found that the K_{LA} values achieved in 60 mL vials, shaken orbitally, were almost 40% lower than the K_{LA} values which could be achieved in stirred tanks agitated at 860 rpm. However, Gudimich et al. (2012) found that, in the conversion of n-octane to 1-octanol by CYP153A6 in resting *E. coli* BL21(DE3), biotransformation could be carried out for 24 h at a cell density of $20 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$ without any noticeable reduction in product formation rates, provided the concentration of active P450 was relatively low. Furthermore, 1-octanol production did not level off until between 36 and 48 h, reaching final titres of over 50 mM. However, when the expression of CYP153A6 was increased to concentrations exceeding $0.5 \text{ } \mu\text{molP}_{450} \cdot \text{g}_{\text{DCW}}^{-1}$, cells appeared to become oxygen-limited, possibly because oxygen was being wasted on uncoupling reactions.

2.6 Summary

One of the great challenges of traditional chemistry is developing catalysts that can act on hydrocarbons in a regioselective and enantioselective manner. Nature, however, has already solved this problem. Organisms have evolved to synthesise a wide variety of complex hydrocarbons as part of their secondary metabolic processes, and other organisms have inevitably evolved mechanisms to degrade these hydrocarbons. A range of bacterial and fungal life forms have also evolved to take advantage of various hydrocarbons as a niche growth substrate. In fact, saturated alkanes, considered the most “recalcitrant” substrate due to their stable and indistinguishable covalent bonds, are readily metabolised by a large number of microorganisms.

The key enzyme in these metabolic processes is an alkane hydroxylase, usually an iron-based monooxygenase. Operating under mild process conditions, these enzymes take oxygen atoms, from O_2 available in air, and insert them into the alkane substrate. Hydroxylases such as the cytochrome P450 CYP153 family can perform this reaction with exceptional selectivity for the terminal carbon atom, producing primary alcohols. These enzymes represent a significant opportunity to produce platform chemicals from alkanes in processes that are more direct, selective, safe, and environmentally friendly than traditional chemical routes.

Cytochromes P450 are difficult to commercialise due to their multi-component and cofactor-dependent nature. The obvious answer to these challenges is the application of these enzymes within a whole-cell environment. However, such whole-cell biocatalysts have consistently demonstrated activities that are orders of magnitude lower than the known maxima of the P450s being expressed, and the titres and space time yields of these processes fail to meet minimum standards for commercialisation.

An examination of the literature offers conflicting explanations for this underperformance. Over-expression of the biocatalytic enzyme is resulting in metabolic burden, except when under-expression of the biocatalytic enzyme is limiting the biotransformation (see Section 2.4.2). Cells are over-exposed to substrate, leading to toxicity effects (Section 2.5.1), but cells are also under-exposed to substrate, lowering the overall reaction rate (Section 2.5.2). The enzyme suffers from a lack of NADH cofactor, or possibly from an excess of NADH being channelled into uncoupled reactions (Section 2.5.3). The supply of oxygen is too low to support maintenance metabolism, but the availability of oxygen is also leading to the formation of reactive oxygen species (Section 2.5.4). The synthesis of these literature reports leads to the clear indication of interdependencies between the variables highlighted. The only logical way to relate these findings to one another, is to assume that these factors are interacting in some way. The key lies, not in optimising any individual aspect of the biocatalytic reaction, but in optimising the balance between reaction components. Alkane hydroxylation influences host cell physiology, which influences alkane hydroxylation, in a series of interlocking cycles which have yet to be fully characterised.

2.7 Defining the Research Project

2.7.1 Problem Statement and objectives

The fundamental problem in most biotransformation research is the same: the enzyme of interest displays a property that chemical catalysts struggle to replicate – in the case of CYP153A6, the ability to hydroxylate alkanes with extreme selectivity for the terminal position – but the biocatalytic system is failing to reach the levels of performance necessary for commercial viability.

In the case of monooxygenase-based biotransformations, a synthesis of the literature reviewed points to the importance of *balancing* the key components of the biotransformation reaction. Low levels of enzyme, alkane, oxygen, or NADH cofactor will limit the rate of production of 1-alcohols. However, a relative excess of enzyme, oxygen, or NADH can result in formation of reactive oxygen species and over-oxidation of product, while an excess of alkane or alcohol can be toxic. In the case of whole-cell systems, the biotransformation is interacting with endogenous metabolic cycles, which can have implications for biocatalyst activity and stability.

This study sets out to address the following key questions:

How is the biotransformation of n-octane impacted by increasing levels of CYP153A6 expression, dehydrogenase activity, and substrate accessibility? In what ways do these factors interact?

The level of active CYP153A6 per cell was modified by varying the concentration of haem precursors (δ -aminolevulinic acid and ferric chloride) present during the cell growth and P450 expression stages, in order to test the effects of varied intracellular P450 at a constant culture density, and hence determine whether the conversion of n-octane was limited by a lack of biocatalytic active sites. A comparison of biotransformations carried out at different culture densities provided insight into the effects of P450 concentration per unit volume. This study formed part of a broader project, one aspect of which was an investigation into the concentrations of electron transport partners. The CYP153A6:FdR:Fdx ratio was therefore not considered in this work, although it was subsequently found to have a significant effect on alkane bioconversion (Kochius et al., 2018).

To determine whether the biotransformation was limited by a lack of NADH cofactor, strains co-expressing additional glycerol dehydrogenase for cofactor regeneration were compared to strains relying exclusively on their native cofactor regeneration capacity. The limitations presented by

cross-membrane transport of substrate and product were investigated in a variety of ways: the integrity of cell walls was compromised through the application of acetone, Polymyxin-B or Triton X-100; various degrees of physical membrane breakage were achieved via passage of cells through a pressure-based cell disruption system; and the membrane channel protein AlkL from *Pseudomonas oleovorans* was co-expressed alongside CYP153A6. Multiple combinations of glycerol dehydrogenase co-expression and membrane permeabilization were tested, in order to determine the effects of increasing cofactor and substrate supply simultaneously.

The ultimate goal of the study was to advance the development of an alkane hydroxylation process that meets industrial standards in terms of titre ($\text{mmol}_{\text{product}}\cdot\text{L}^{-1}$), space time yield ($\text{mmol}_{\text{product}}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$), and yield on biocatalyst ($\text{mmol}_{\text{product}}\cdot\text{g}_{\text{DCW}}^{-1}$), while maintaining specificity for terminal hydroxylation. While the study itself may not lead to an optimised whole-cell biocatalyst, it is aimed at identifying areas of interaction that offer potential for significant improvements in biocatalyst activity and stability.

2.7.2 Research hypotheses and key questions

The research hypotheses focus on potential limitations in enzyme, cofactor, and substrate – particularly the interaction between these limitations.

Hypothesis 1: The intracellular concentration of CYP153A6 will increase in proportion to the concentrations of haem precursors that are added to the medium during the growth and expression phase. However, due to limitations in substrate and cofactor availability, increasing concentrations of CYP153A6 will not improve biocatalyst activity. Meanwhile, the expression of additional proteins will increase the metabolic burden on the cell. Consequently, biocatalyst performance in terms of maximum titre, yield on biocatalyst, and space time yield will decrease with increasing CYP153A6 concentration above a critical value.

Hypothesis 2: When a heterologous glycerol dehydrogenase is co-expressed with CYP153A6, NADH regeneration rates will increase, but unaddressed transport limitations will prevent the biocatalyst from utilising the additional cofactor effectively. Meanwhile, the expression of additional proteins will increase the metabolic burden on the cell. Consequently, cells co-expressing additional glycerol dehydrogenase will not outperform intact cells expressing only CYP153A6 in terms of initial hydroxylation rate, and will underperform in terms of final titre, yield on biocatalyst, and space time yield.

Hypothesis 3: Membrane permeabilization techniques will allow for enhanced transport of both n-octane and 1-octanol, leading to higher initial hydroxylation rates. However, compromising membrane integrity will cause the cell to lose important components and make it more vulnerable to toxicity. Consequently, permeabilised cells will be unable to sustain their initial high rates, and will underperform relative to non-permeabilised cells, in terms of final titre, yield on biocatalyst, and space time yield.

Hypothesis 4: Permeabilised cells co-expressing glycerol dehydrogenase alongside CYP153A6 will experience increased metabolic burden as well as detrimental effects associated with loss of membrane integrity. However, they will have access to additional substrate as well as the cofactors necessary to activate it. Consequently, initial hydroxylation rates will increase, as will maximum space time yield, but the biocatalyst will be unable to accumulate high titres of 1-octanol due to loss of stability.

Hypothesis 5: The co-expression of the membrane transport protein AlkL alongside CYP153A6 will increase n-octane availability without compromising membrane integrity. Consequently, the initial hydroxylation rate will increase. However, as with other permeabilization techniques, the biocatalyst will not be able to sustain these hydroxylation rates due to insufficient regeneration of NADH cofactor. Consequently, cells co-expressing AlkL will not outperform cells expressing only CYP153A6 in terms of final titre, yield on biocatalyst, or average space time yield.

Hypothesis 6: The combination of CYP153A6, enhanced n-octane transport due to co-expression of AlkL, and enhanced NADH regeneration due to co-expression of glycerol dehydrogenase, will provide the biocatalyst with a continuous supply of both substrate and cofactor. These benefits will outweigh the detrimental effects of high metabolic burden associated with expressing multiple heterologous proteins. Consequently, the hydroxylation rate, final titre, yield on biocatalyst, and space time yield will increase relative to cells expressing only CYP153A6.

This gives rise to the following key questions:

- To what extent can the expression levels of active CYP153A6 be controlled by the concentrations of δ -aminolevulinic acid and ferric chloride?
- Will specific activity be dependent on specific P450 concentration?
- Will cells with higher specific concentrations of CYP153A6 demonstrate reduced stability?
- How will the co-expression of glycerol dehydrogenase affect the expression and stability of CYP153A6?
- Will the presence of additional dehydrogenase result in higher initial activities?
- Will the presence of additional dehydrogenase affect biocatalyst stability?
- Will the activity and stability of whole-cell biocatalysts be affected by cell density?
- Will these effects differ based on whether glycerol dehydrogenase is being co-expressed?
- Will membrane permeabilization result in higher initial activities?
- Will membrane permeabilization reduce biocatalyst stability?
- Does it matter which type of membrane permeabilization technique is applied, or do different forms of permeabilization provide similar results?
- Is the efficacy of membrane permeabilization affected by co-expression of glycerol dehydrogenase?
- How does the co-expression of AlkL affect the expression and stability of CYP153A6?
- Does AlkL co-expression improve specific activity?
- Does AlkL co-expression compromise biocatalyst stability?
- Can the host cell support simultaneous expression of CYP153A6, glycerol dehydrogenase, and AlkL?
- Does the co-expression of glycerol dehydrogenase enhance the effects of AlkL?

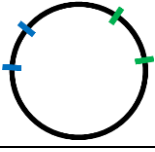
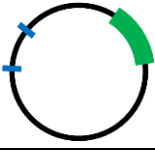
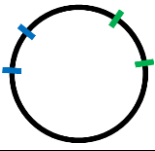
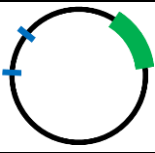
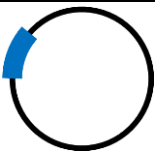
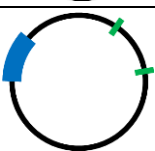
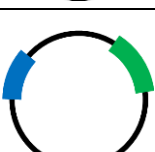
3 Methodology

3.1 Chemicals, bacterial strains, and plasmids

Unless stated otherwise, chemicals and antibiotics were obtained from Sigma-Aldrich/Merck at laboratory grade.

The descriptions and sources of plasmids used in this study are listed in Table 3.1. The CYP153A6 used in this study contains a mutation five residues downstream of the conserved haem-binding cysteine, in which the leucine residue was replaced with a proline residue (Gudimich et al., 2012).

Table 3.1: List of plasmids used to create biotransformation strains

Plasmid	Characteristics		Source
pET(CYP)	Vector pET28b+: T7 promoter/ <i>lac</i> operator, kanamycin resistance. Contains genes for the alkane hydroxylase operon from <i>Mycobacterium</i> sp. HXN-1500, consisting of CYP153A6 and its native ferredoxin reductase and ferredoxin.		Professor MS Smit Department of Microbial, Biochemical and Food Biotechnology University of the Free State
pACYC	Vector pACYCDuet-1: two multiple cloning sites (MCS), T7 promoter/ <i>lac</i> operator, chloramphenicol resistance		
pACYC(GLD)	Vector pACYCDuet-1, containing genes for glycerol dehydrogenase from <i>E. coli</i> in MCS-2 of pACYC		
pCDF	Vector pCDFDuet-1: two multiple cloning sites, T7 promoter/ <i>lac</i> operator, streptomycin resistance		
pCDF(GLD)	Vector pCDFDuet-1, containing genes for glycerol dehydrogenase from <i>E. coli</i> in MCS-2 of pCDF		
pBME(AlkL)	Contains AlkL gene construct synthesised based on the protein's GenBank sequence (accession number AJ245436.1) plus <i>Nco</i> I and <i>Hind</i> III binding sites, in vector pBME, T7 promoter, ampicillin resistance		Biomatik
pCDF(AlkL)	Vector pCDFDuet-1, containing aforementioned AlkL gene construct in MCS-1 of pCDF		This study
pCDF(AlkL+GLD)	Vector pCDFDuet-1, containing aforementioned AlkL gene construct in MCS-1, and glycerol dehydrogenase from <i>E. coli</i> in MCS-2		

In order to create pCDF(AlkL), pBME(AlkL) and pCDF were separately digested with NcoI and HindIII, fragments were separated via gel electrophoresis, and the AlkL fragment was ligated with the linearised pCDF. When pCDF(GLD) was digested with NcoI and HindIII, unexpected fragments appeared in the electrophoresis gel (see Figure 3.1 C and D). It transpired that the gene sequence for glycerol dehydrogenase contains code in two locations that matches that of restriction site NcoI, as well as code in two locations that matches that of restriction site HindIII. Hence it was necessary to pursue a more complex cloning strategy. Plasmid pCDF(AlkL) was digested with XbaI and NotI, allowing for selection of the fragment containing MCS-1, the T7 promoter and *lac* operator, and the *lacI* sequence. Plasmid pCDF(GLD) was digested with XbaI and NotI, allowing for selection of the fragment containing the CDF origin, the streptomycin resistance sequence, and MCS-2. These two fragments were then ligated to form pCDF(AlkL+GLD). Figure 3.2 presents a schematic illustration of the cloning strategy that was followed to create the AlkL-containing plasmids.

Plasmids pCDF, pCDF(GLD), pCDF(AlkL) and pCDF(AlkL+GLD) were transformed into *E. coli* BL21(DE3) and selected on Lysogeny Agar plates with appropriate antibiotics. A single colony was selected and grown overnight under antibiotic selection in 10 mL of Lysogeny Broth, in order to create stocks which were stored at -60°C.

Escherichia coli BL21(DE3) was used as the host species for all biotransformation experiments. Cells were transformed with various combinations of the plasmids described in Table 3.1, creating eight different strains which are described in Table 3.2.

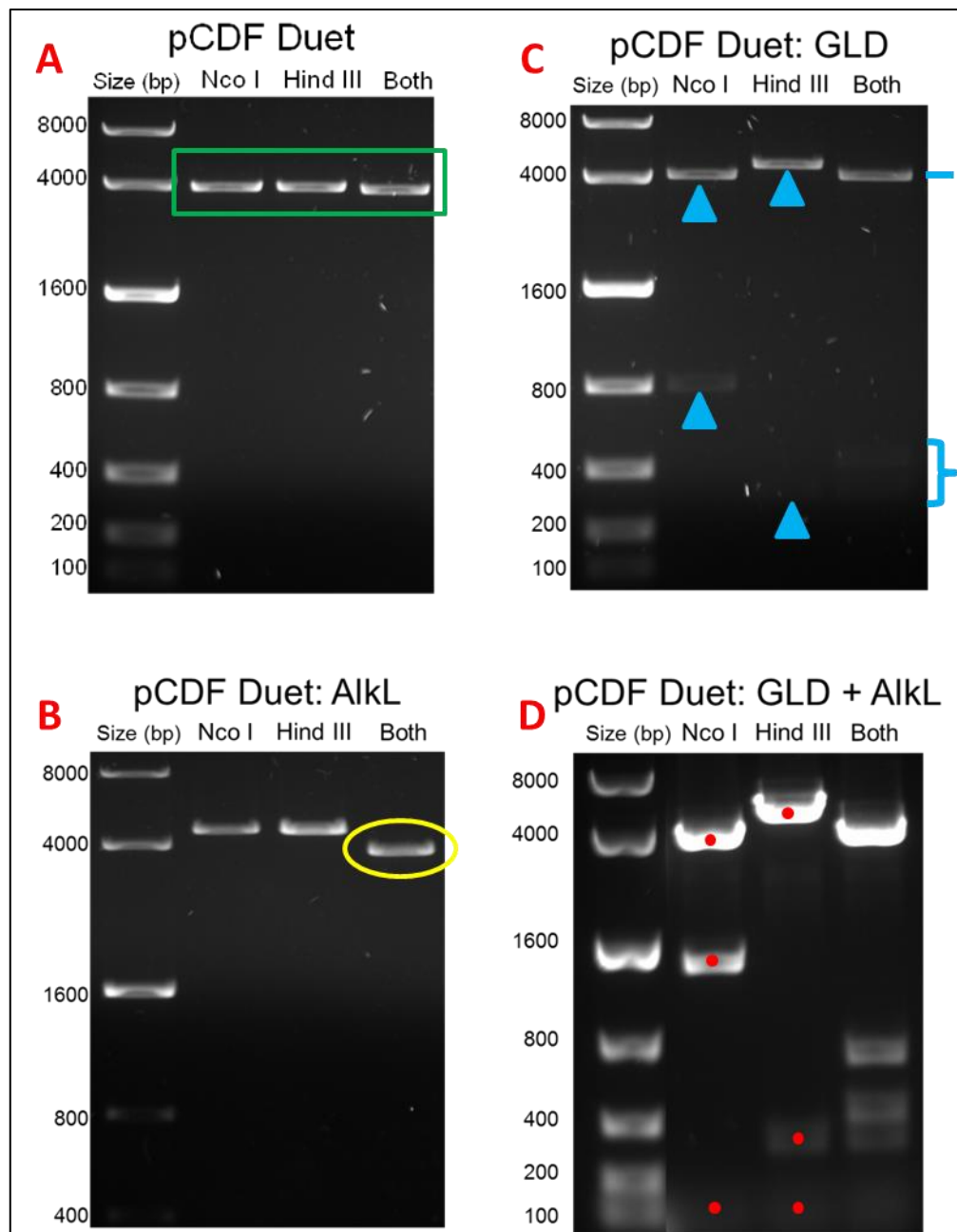


Figure 3.1: Gel fragment patterns resulting from restriction digests and subsequent electrophoresis of plasmids

A: The empty pCDFDuet-1 plasmid contains 3781 base pairs (bp). There are only 74 bp between restriction site NcoI and restriction site HindIII, hence there is a negligible difference in the sizes of the linearised fragments, regardless of whether one or both enzymes are used for digestion.

B: Cutting pCDF(AlkL) with either NcoI or HindIII results in a single linear fragment. Digestion with both enzymes allows for separation of the 711 bp AlkL fragment. The smaller fragment is not clearly visible in this image, but the decrease in size of the remaining fragment is obvious (yellow circle).

C: Because the sequence for glycerol dehydrogenase contains code in two locations that matches that of restriction site NcoI, as well as code in two locations that matches restriction site HindIII, digesting pCDF(GLD) with either enzyme individually results in two fragments appearing (of which two can be seen in this image – blue arrows); simultaneous digestion with both enzymes results in four fragments (blue brackets – the smallest fragment is only 68 bp and has run off the gel).

D: For the same reasons, digesting pCDF(AlkL+GLD) with NcoI or HindIII results in three fragments (red dots), but digestion with NcoI and HindIII together results in six fragments (again, the smallest has run off the gel).

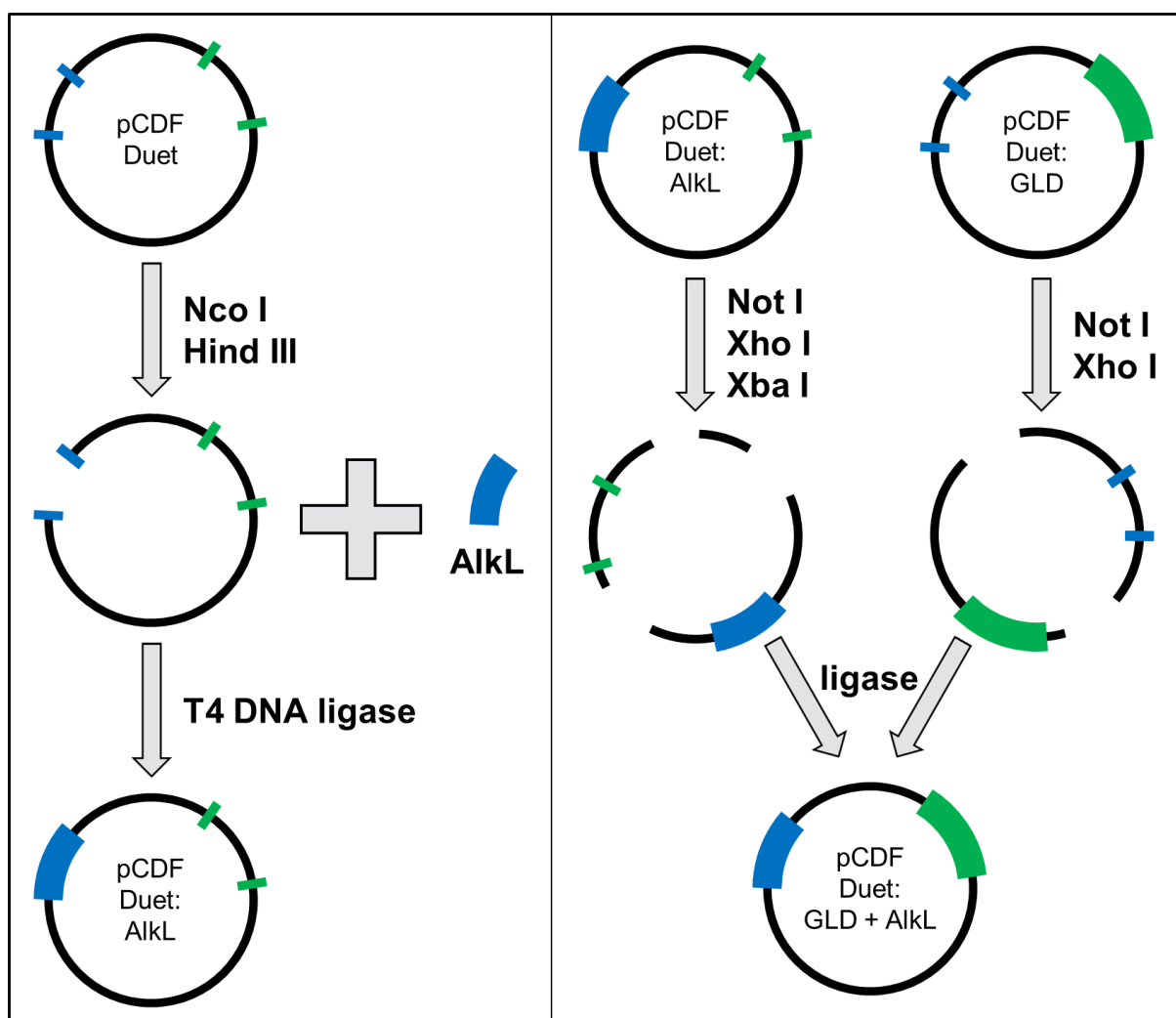
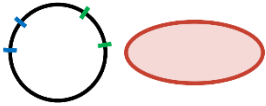
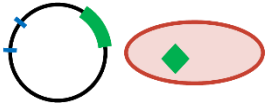
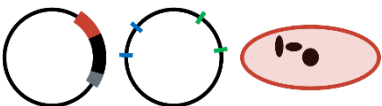
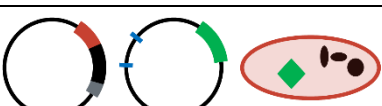
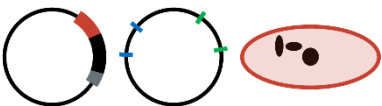
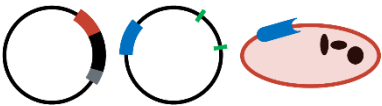
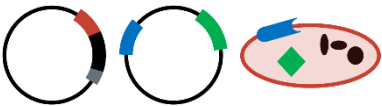


Figure 3.2: Schematic representation of the cloning process followed to create the plasmids pCDFDuet(AIkL) and pCDFDuet(GLD+AIkL)

Table 3.2: List of strains used in this study, indicating which aspects of work made use of each strain. The schematics in the second column indicate the relevant plasmids each strain contained (see Table 3.1) along with a schematic representation of the heterologous proteins that would be expressed following induction.

Strain description		Used to investigate*:	Source
<i>E. coli</i> BL21(DE3) containing pET(CYP)		Enzyme loading (4.1, 4.3) Oxygen supply (4.4) Hydrocarbon transport (5.1)	Professor MS Smit
<i>E. coli</i> BL21(DE3) containing pACYC		Hydrocarbon transport (5.1)	Department of Microbial, Biochemical and Food Biotechnology
<i>E. coli</i> BL21(DE3) containing pACYC(GLD)		Hydrocarbon transport (5.1)	
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pACYC		Cofactor regeneration and hydrocarbon transport (5.2.1, 5.3, 5.4.1)	University of the Free State
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pACYC(GLD)		Cofactor regeneration and hydrocarbon transport (5.1, 5.2.1, 5.3, 5.4.1)	
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF		Enzyme loading (4.2) Cofactor regeneration and hydrocarbon transport (5.2.2, 5.4.2) Expression of AlkL (5.7)	This study
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(GLD)		Enzyme loading (4.2) Cofactor regeneration and hydrocarbon transport (5.2.2, 5.4.2) Expression of AlkL (5.7)	
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(AlkL)		Enzyme loading (4.2) Expression of AlkL (5.7)	
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(AlkL+GLD)		Enzyme loading (4.2) Expression of AlkL (5.7)	

*Relevant sections of Results and Discussion are given in brackets

3.2 Cell growth and protein expression

Pre-cultures consisting of 5 – 10 mL Lysogeny Broth (10 g.L⁻¹ tryptone, 5 g.L⁻¹ yeast extract, 5 g.L⁻¹ NaCl) were inoculated from glycerol stocks (previously maintained below -60°C). Pre-cultures were supplemented with 30 µg.mL⁻¹ kanamycin and either 30 µg.mL⁻¹ chloramphenicol (for strains containing a plasmid based on the pACYCDuet vector) or 100 µg.mL⁻¹ streptomycin (for strains containing a plasmid based on the pCDFDuet vector). Pre-cultures were then grown overnight (12 – 18 h) at 37°C with shaking.

Auto-induction (AI) medium was prepared from sterile solutions and had a final composition as follows: 9.2 g.L⁻¹ tryptone, 4.6 g.L⁻¹ yeast extract, 5 g.L⁻¹ glycerol, 0.5 g.L⁻¹ glucose, 2 g.L⁻¹ α-lactose (for induction of heterologous expression), 3.3 g.L⁻¹ (NH₄)₂SO₄, 6.8 g.L⁻¹ KH₂PO₄, 7.1 g.L⁻¹ Na₂HPO₄, 0.24 g.L⁻¹ MgSO₄, and 30 µg.mL⁻¹ kanamycin and/or 30 µg.mL⁻¹ chloramphenicol and/or 100 µg.mL⁻¹ streptomycin (as required for plasmid maintenance). FeCl₃ and δ-aminolevulinic acid (δ-ALA) were added to the AI medium. Standard concentrations were 1 mM δ-ALA and 50 µM FeCl₃; these concentrations were applied for all experiments except those investigating enzyme loading (described in Chapter 4) and those investigating the effects of AlkL (described in Section 5.7).

Aliquots of AI medium were transferred to shake flasks; flask volume was either 250 mL, 500 mL, or 1000 mL, depending on the quantity of biomass desired for the experiment. A ratio of 2 mL liquid volume per 10 mL flask volume was applied. The medium was inoculated with 2% (v/v) pre-culture and incubated at 20°C and 200 rpm for 48 h.

3.3 Biocatalyst preparation

3.3.1 Harvesting

Following growth and enzyme expression, cells were centrifuged at 5000 – 7000 rpm and 4°C for 5 – 10 minutes. The cell pellets were resuspended in chilled 200 mM potassium phosphate buffer containing 200 mM glycerol. In early experiments, buffer at pH 8 was used to counter acidification of the reaction medium. However, when the medium was tested with pH strips following 72 h of biotransformation, significant acidification had not occurred. Therefore, in later experimental sets, the buffer pH was reduced to 7.4, which is closer to the preferred pH range of *E. coli* and CYP153A6.

3.3.2 Whole cells

High-density cultures were resuspended to a final biomass concentration of ± 200 g.L⁻¹ wet cell weight; low-density cultures were resuspended to ± 20 g.L⁻¹ wet cell weight. In high-density cultures, NADH was added to a final concentration of 0.25 mM.

3.3.3 Mechanically broken cells

Resuspended cells (± 400 g_{wcw}.L⁻¹) were homogenised using a one-shot cell disruptor (Constant Systems Ltd). The pressure was set to either 15 000 or 30 000 psi, and the resulting suspensions were diluted 1:1 with buffer containing 0.5 mM NADH (final concentration 0.25 mM).

To create cell free extract, a portion of resuspended cells (± 400 g_{wcw}.L⁻¹) was homogenised at 30 000 psi and then centrifuged at 7000 rpm and 4°C for 30 minutes; the resulting supernatant was diluted 1:1 with buffer containing 0.5 mM NADH (final concentration 0.25 mM).

3.3.4 Chemically permeabilised cells

For permeabilization by acetone, a portion of resuspended cells (± 400 g_{wcw}.L⁻¹) was mixed with 5% (v/v) acetone, vortexed for 3 minutes, and centrifuged at 7000 rpm and 4°C for 10 minutes. The cell pellet was washed with an equal volume of phosphate buffer, centrifuged again, and resuspended in the same buffer to ± 200 g_{wcw}.L⁻¹.

For permeabilization by Polymyxin B or Triton X-100, a portion of cells was diluted to a final biomass concentration of $\pm 200 \text{ g}_{\text{WCW}}\cdot\text{L}^{-1}$ in phosphate buffer containing either Polymyxin B to achieve a final concentration of $30 \mu\text{M}$ in the aqueous phase, or Triton X-100 to achieve a final concentration 0.25% (v/v) in the aqueous phase.

A subset of chemical permeabilization experiments were repeated with low-density cultures ($\pm 20 \text{ g}_{\text{WCW}}\cdot\text{L}^{-1}$); Polymyxin B or Triton X-100 was added as before.

3.4 Biotransformation

Biotransformations were carried out at 20°C and 200 rpm in 40 mL capped amber vials, except for the experiments described in Section 4.4, where 40 mL vials were compared to 60 mL vials. The vials had a diameter of 2.5 cm and a height of either 8 cm or 12 cm.

For experiments with high-density cultures, the vials contained 1.2 mL cell suspension and 0.2 mL n-octane. For experiments with low-density cultures, the vials contained 1 mL cell suspension, 0.2 mL n-octane and 0.1 mL bis(2-ethylhexyl)phthalate (BEHP).

For experiments in which the vial headspace was replenished, fresh air was added to the headspace as follows: each time vials were removed for sampling, a portion of the vials remaining in the incubator (those designated as “replenished” samples) had their caps removed one at a time, and replaced with a purpose-made cap, as shown in Figure 3.3. The cap was attached to a 60 mL syringe filled with air, which was injected into the vial. The regular cap was then screwed back on, and the vial was returned to the incubator.

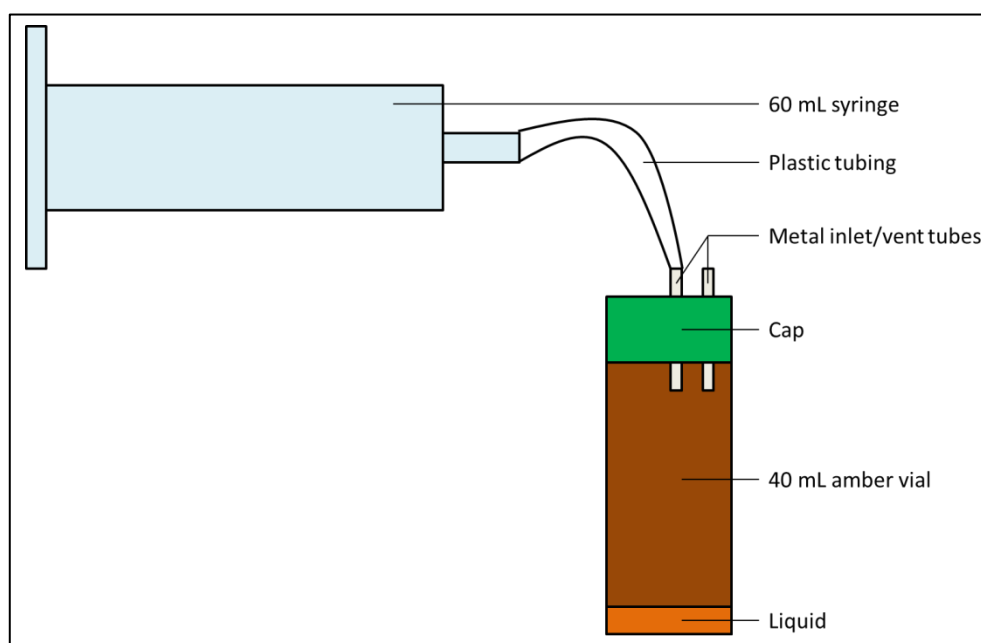


Figure 3.3: Apparatus for the replacement of vial headspace (not to scale)

3.5 Sampling and analysis

For each sample point, at least two vials were sacrificed. Wherever practical, vials and samples were kept on ice after being removed from the incubator.

3.5.1 Quantification of dry cell mass

A 1 – 2 mL sample aliquot was transferred to a pre-dried, pre-weighed 2 mL Eppendorf tube, and centrifuged at $\geq 13\,000$ rpm for 10 minutes at room temperature. The supernatant was discarded, and the cell pellets were dried at 80°C for ≥ 24 hours and cooled in a desiccator for ≥ 12 hours, before being weighed. Cell density was then calculated as follows:

$$\text{Cell density (g}_{\text{DCW}}\text{L}^{-1}) = \frac{\text{mass of cell pellet (g)}}{\text{volume of sample aliquot (L)}} \dots\dots\dots \text{Equation 3.1}$$

3.5.2 Quantification of CYP153A6

Determination of the concentrations of the active and inactive forms of CYP153A6 was based on the spectrophotometric assay described by Guengerich et al. (2009). The activity of cytochrome P450 enzymes depends on the haem moiety. Haemoproteins typically exhibit a spectral peak in the vicinity of 420 nm. However, a distinctive characteristic of all known cytochromes P450 is that the haem moiety is axially ligated by cysteine thiolate; when bound to carbon monoxide, the resulting complex produces a spectral peak at 450 nm rather than 420 nm. The height of the peak correlates with the concentration of active CYP153A6 in the sample, with an extinction coefficient of $91\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$. If the enzyme is incorrectly folded, the haem group will not be correctly ligated, which will render the enzyme inactive, but still able to contribute to the spectral peak at 420 nm. The amount of inactive enzyme can be estimated using an extinction coefficient of $110\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$, after correcting for the interference of the P450 form with the 420 nm peak.

In the case of measurements taken from flasks or from initial cell preparations, 1 mL of sample was diluted with 3 mL of the buffer used for resuspension, mixed with a small amount of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), and split equally between two 4 mL cuvettes. One cuvette was transferred to a fume hood, where carbon monoxide (CO) was bubbled directly into the liquid for 1 minute using a glass Pasteur pipette connected to a small CO cylinder. The second cuvette was not exposed to CO and served as the “blank”. A spectrophotometer (Genesys 10UV or Genesys 10s UV-Vis) was used to measure the absorbance of each well between 400 and 550 nm at 1 nm intervals, with the blank cuvette being used for automatic baseline correction. The concentrations of active and inactive CYP153A6 were calculated according to the following formulae:

$$P450 \text{ (active, } \mu\text{M}) = \frac{\Delta A_{450} - \Delta A_{490}}{0.091} \dots\dots\dots \text{Equation 3.2}$$

$$P420 \text{ (inactive, } \mu\text{M}) = \frac{1}{0.11} \left[(\Delta A_{420} - \Delta A_{490}) + \frac{0.041}{0.091} (\Delta A_{450} - \Delta A_{490}) \right] \dots\dots\dots \text{Equation 3.3}$$

In the case of measurements taken directly from vials, 200 μL of aqueous phase was drawn off, diluted 1:1 with the same buffer used for biotransformation, mixed with a few grains of sodium dithionite, and split equally between two wells of a flat-bottomed, clear, 96-well clip-in spectrophotometry plate (Greiner). One clip of wells was then exposed to CO for 5 minutes via incubation in a closed container continuously flushed with CO. The other clip of wells was not exposed to CO and served as the “blank”. A spectrophotometer (Spectramax M2 or Fluostar omega BMG) was used to measure the absorbance of each well between 360 and 500 nm at 2 nm intervals. The blank wells were used for manual baseline correction. Concentrations of active and inactive CYP153A6 were calculated using Equations 1 and 2 above, except that ΔA_{420} , ΔA_{450} and ΔA_{490} had to be calculated manually as follows:

$$\Delta A = A_{\text{CO treated}} - A_{\text{blank}} \dots\dots\dots \text{Equation 3.4}$$

3.5.3 Extraction and analysis of organic phase

The organic phase was extracted into 1 mL ethyl acetate containing 2 mM 1-decanol as an internal standard. Samples were analysed via gas chromatography (Shimadzu GC-2010 in the case of high-density experiments; Varian GC-3900 in the case of low-density experiments) using a FactorFour VF-5 ms column (95% dimethylpolysiloxane, 60 m × 0.32 mm). A significant unexpected peak was noticed in high-density experiments, and was identified as octyl acetate via GC–MS of selected samples. The concentration of octyl acetate was estimated by comparing the areas of the 1-octanol and octyl acetate peaks.

The run programme on the Shimadzu was as follows: 1 µL injection volume, split ratio of 50, 2.4 mL.min⁻¹ H₂, oven temperature held at 60°C for 9 min, ramped at 25°C.min⁻¹ to 180°C, held for 1.2 min. The run programme on the Varian was as follows: 0.5 µL injection volume, split ratio of 80, 1 mL.min⁻¹ N₂, oven temperature held at 60°C for 3 min, ramped at 30°C.min⁻¹ to 180°C, held for 7 min.

3.5.4 Key performance indicators for comparison of biotransformation experiments

The data collected at various time points over the course of each biotransformation was used to derive several key indicators that could be used for comparison of biocatalytic performance, as described in Table 3.3.

Table 3.3: Derivation of key performance indicators

Description	Units	Calculation
Cell density	g _{DCW} .L ⁻¹	See Section 3.5.1
Volumetric concentration of active CYP153A6, or P450 concentration	µmol _{P450} .L ⁻¹	See Section 3.5.2
Intracellular concentration of active CYP153A6	µmol _{P450} .g _{DCW} ⁻¹	(volumetric P450 concentration) ÷ (cell density)
Length of biotransformation period	h	Number of hours for which biotransformation was sustained
Titre	mmol _{product} .L ⁻¹	See Section 3.5.3
Yield on biocatalyst	mmol _{product} .g _{DCW} ⁻¹	(titre) ÷ (cell density)
Space time yield (STY)	mmol _{product} .L ⁻¹ .h ⁻¹	(titre) ÷ (biotransformation period)
Specific production rate	mmol _{product} .g _{DCW} ⁻¹ .h ⁻¹	(yield on biocatalyst) ÷ (biotransformation period)
Turnover number (TON)	mol _{product} .mol _{P450} ⁻¹	(titre × 1000) ÷ (P450 concentration)
Turnover frequency (TOF)	mol _{product} .mol _{P450} ⁻¹ .min ⁻¹	(TON) ÷ (biotransformation period × 60)
Period of maximum productivity	h	Number of hours for which the biocatalyst maintained its <i>maximum</i> specific production rate

3.6 Experimental Blocking

The experimental work can be divided into four distinct blocks, each of which investigated one or more of the potential limiting factors identified from a review of the literature.

Block A covers variations in enzyme loading, including the effect of haem precursor addition on P450 expression levels, the effect of co-expression of glycerol dehydrogenase and/or AlkL on P450 expression levels, and the effect of intracellular P450 concentration on biocatalyst performance. The results of Block A are covered in Chapter 4.

Block B consists of a short set of experiments investigating the role of oxygen supply in the biotransformation, including variations in biotransformation headspace volume, and replacement of the biotransformation headspace. These results are discussed in Section 4.4.

Block C covers the largest portion of the experimental work: the permeabilization of cell membranes in conjunction with varying expression levels of glycerol dehydrogenase. Cells permeabilised via mechanical breakage or chemical treatment were compared to untreated cells, in strains with or without additional glycerol dehydrogenase, in high-density and low-density cultures. The results of Block C are covered in Chapter 5.

Block D represents a continuation of the investigations undertaken in Block C. Instead of permeabilising the membrane prior to biotransformation, co-expression of the membrane channel protein AlkL was used to improve alkane transport. A brief set of experiments compared strains expressing AlkL, glycerol dehydrogenase, both, or neither. These results are discussed in Section 5.7.

4 Expression and Efficacy of CYP153A6

Literature relating to the heterologous expression of cytochrome P450s is discussed in Sections 2.4.3 and 2.4.4. A key aspect of the expression strategy is the addition of surplus iron and of the haem precursor δ -aminolevulinic acid (δ -ALA), to ensure that the cell generates sufficient haem moieties for incorporation into the active site.

This section of work investigated the relationship between the concentrations of ferric chloride (FeCl_3) and δ -ALA in the auto-induction medium and the intracellular concentration of active CYP153A6 at the end of the expression period.

The expression of multiple heterologous proteins is also known to impose a metabolic burden on the host cell. At a variety of precursor concentrations, strains expressing only CYP153A6, FdR, and Fdx were compared to strains which were also expressing glycerol dehydrogenase, the membrane channel protein AlkL, or both.

Finally, biotransformations at comparable cell densities were carried out to determine whether variation in the intracellular concentration of active CYP153A6 had any effect on the conversion of n-octane to 1-octanol.

4.1 Effect of precursor concentrations on P450 expression

Strains used in this section of work:



Cultures of *Escherichia coli* carrying genes for CYP153A6, FdR, and Fdx were grown and expressed in auto-induction medium (see Section 3.2). The key feature of this medium is that cells undergo diauxic growth on glucose (0.5 g.L^{-1}) followed by α -lactose (2 g.L^{-1}). The move to lactose metabolism induces the expression of heterologous proteins under the control of the *lac* operon (which was carried by all plasmids used in this study – see Table 3.1 for a full list of plasmids used). The growth and expression period was $48 \pm 2 \text{ h}$. Nine parallel cultures were grown, and the starting concentrations of δ -ALA and FeCl_3 in the auto-induction medium were varied at three levels each. The end-point concentrations of active CYP153A6 in the various cultures were determined via carbon monoxide difference spectrophotometry (see Section 3.5.2). Dry cell weights were also determined for each culture. Two such experiments were conducted; in the first, δ -ALA was added at concentrations of 0, 0.5 and 1 mmol.L^{-1} , while FeCl_3 was added at 0, 50 and $500 \mu\text{mol.L}^{-1}$; in the second experiment, δ -ALA was added at concentrations of 0, 0.6 and 1 mmol.L^{-1} , while FeCl_3 was added at 10, 50 and $100 \mu\text{mol.L}^{-1}$.

The results are presented graphically in Figure 4.1. In the first experiment (Fig. 4.1-A), in the culture where no additional δ -ALA or FeCl_3 was added to the medium, the concentration of active CYP153A6 was $0.10 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$. In the culture where the δ -ALA addition was increased to 0.5 mmol.L^{-1} , the CYP153A6 expression rose to $0.19 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$. However, a further increase to 1 mmol.L^{-1} δ -ALA addition resulted in a culture where only $0.13 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ was produced. In the culture where $50 \mu\text{mol.L}^{-1}$ FeCl_3 was added, but no δ -ALA was added, the cells produced $0.17 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$. However, in other cultures with $50 \mu\text{mol.L}^{-1}$ of FeCl_3 addition, the addition of 0.5 mmol.L^{-1} of δ -ALA resulted in $0.54 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ and 1 mmol.L^{-1} of δ -ALA resulted in $0.77 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$. The highest level of FeCl_3 addition, $500 \mu\text{mol.L}^{-1}$, resulted in $0.16 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ in the culture where no δ -ALA was added, $0.67 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ where 0.5 mmol.L^{-1} δ -ALA was added, and $1.08 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ where 1 mmol.L^{-1} δ -ALA was added.

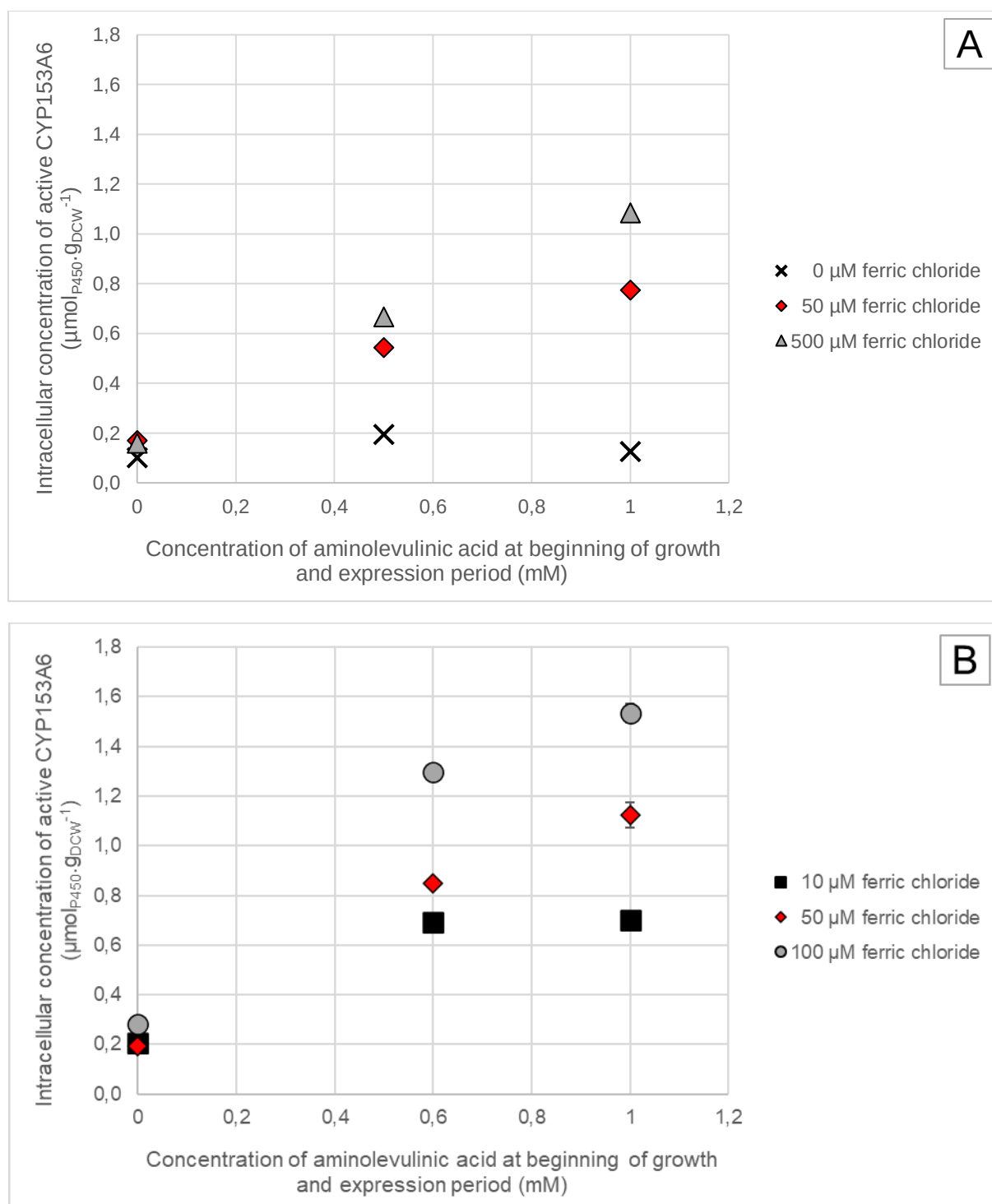


Figure 4.1: Effect of haem precursor concentration on expression of active CYP153A6. Cells were cultured on auto-induction medium for 48 ± 2 h at 20°C and 200 rpm. In each experiment, nine separate cultures were inoculated from the same preculture. The concentrations of aminolevulinic acid (δ -ALA) and ferric chloride (FeCl_3) added to each culture were varied. Dry cell weights and concentrations of active CYP153A6 were measured for each culture, and used to determine the average specific enzyme loading for cells in the culture. Error bars in Fig 1-B represent the average of two sample replicates.

The second experiment (Fig. 4.1-B) investigated a tighter band of FeCl_3 concentrations. When $10 \mu\text{mol} \cdot \text{L}^{-1}$ FeCl_3 was added to a culture, it produced active CYP153A6 to a final concentration of $0.21 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ when no δ -ALA was added, $0.69 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ when $0.6 \text{ mmol} \cdot \text{L}^{-1}$ δ -ALA was added, and $0.70 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ when $1 \text{ mmol} \cdot \text{L}^{-1}$ δ -ALA was added. In cultures where $50 \mu\text{mol} \cdot \text{L}^{-1}$ FeCl_3 was added, CYP153A6 expression was $0.19 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ when no δ -ALA was added,

0.85 $\mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ when 0.6 mmol.L^{-1} δ -ALA was added, and 1.13 $\mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ when 1 mmol.L^{-1} δ -ALA was added. In cultures where 100 $\mu\text{mol.L}^{-1}$ FeCl_3 was added, CYP153A6 expression was 0.28 $\mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ when no δ -ALA was added, 1.30 $\mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ when 0.6 mmol.L^{-1} δ -ALA was added, and 1.53 $\mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ when 1 mmol.L^{-1} δ -ALA was added.

If no δ -ALA was added to the growth medium, the addition of ferric chloride had no impact on the level of active CYP153A6 expressed. The effect of δ -ALA addition was similarly ineffectual in cultures with no added ferric chloride. However, in the presence of 0.5 mmol.L^{-1} δ -ALA, the intracellular P450 concentration could be increased by a factor of 2.8 or 3.4 by also adding 50 or 500 $\mu\text{mol.L}^{-1}$ FeCl_3 . When δ -ALA addition was 1 mmol.L^{-1} , an increase from 0 to 50 $\mu\text{mol.L}^{-1}$ FeCl_3 increased expression of active enzyme by a factor of 6.2; however, further increases from 50 to 100 $\mu\text{mol.L}^{-1}$ FeCl_3 or from 50 to 500 $\mu\text{mol.L}^{-1}$ FeCl_3 increased expression by only 1.4-fold. Similarly, when FeCl_3 concentration was 50 $\mu\text{mol.L}^{-1}$, a change from no added δ -ALA to 0.5 mmol.L^{-1} δ -ALA increased expression by a factor of 3 to 4, but a further doubling of the δ -ALA concentration from 0.5 to 1.0 mmol.L^{-1} increased expression by a factor of only 1.3 or 1.4. Similar trends have been noted by researchers expressing the human cytochrome P450s CYP1A2 and CYP2A13. For these microsomal P450s, Fe^{3+} was found to be a less effective precursor than δ -ALA when only one chemical was added, but the simultaneous addition of both components was found to be the most effective strategy for high P450 expression (Lu et al., 2010, 2013; Sudhamsu et al., 2010).

As the normalised data in Figure 4.2 shows, when concentrations of FeCl_3 were very low (0 – 10 μM), then doubling the initial δ -ALA concentration from 0.5 or 0.6 mmol.L^{-1} to 1 mmol.L^{-1} had no effect on the concentration of active CYP153A expressed. In contrast, the doubling of δ -ALA concentration had a substantial effect on final P450 concentration when the initial FeCl_3 concentration was 50 μM . At a FeCl_3 concentration of 100 μM , the same change in δ -ALA concentration had less of an impact on P450 concentration, although it was still beneficial. This is consistent with the hypothesis that availability of δ -ALA constitutes the rate-limiting step in haem formation, with iron availability as a secondary limitation that comes into play when δ -ALA is available in excess.

Increasing the initial FeCl_3 from 100 to 500 $\mu\text{mol.L}^{-1}$ did not improve expression of active CYP153A6, and may even have depressed it slightly. One possible reason for this is that free haem can be toxic to cells because of its ability to bind O_2 and thus facilitate the formation of hydrogen peroxide (Michener et al., 2012; Muraki et al., 2015). Therefore, at the highest concentrations of δ -ALA and FeCl_3 , the cells may have generated haem more rapidly than it was needed for incorporation into P450, leading to increased concentrations of reactive oxygen species, and hence impacting the health of the cell and its ability to synthesise proteins. It is also possible that an excess of free haem led to its incorporation into incompletely or incorrectly folded CYP153A6, producing inactive protein.

Optimising the concentration of expensive precursors is essential for creating a cost-effective process. Furthermore, avoiding unnecessary heterologous expression could potentially result in more robust whole-cell biocatalysts. These results show precursor addition is subject to diminishing returns, especially if applied in an unbalanced way. Understanding the interactive effects of δ -ALA and FeCl_3 on CYP153A6 expression allows for improved control of the expression process.

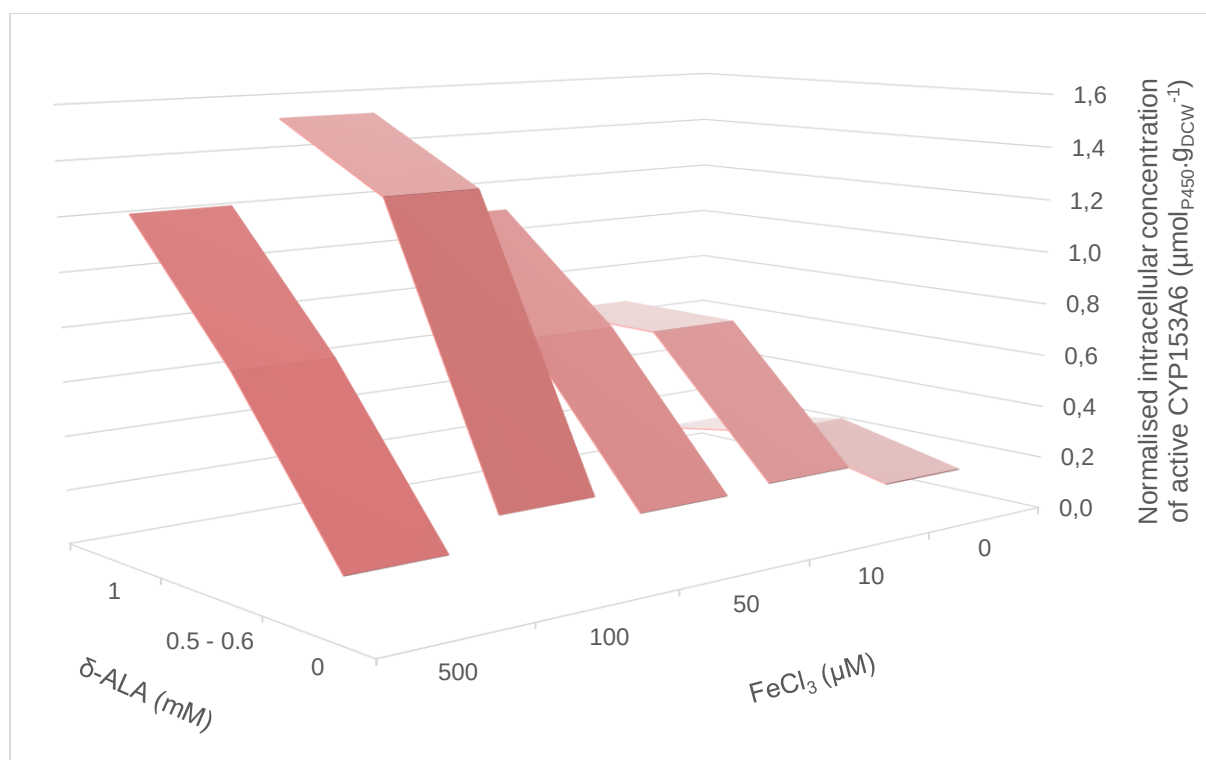


Figure 4.2: Data from both experiments depicted in Figure 4.1, normalised based on P450 concentrations at the common condition of 50 μM added ferric chloride and no added aminolevulinic acid. Cultures at a δ-ALA concentration of 0.5 or 0.6 mM were handled as a single data set. The data point at 50 μM FeCl₃ and 0.5 – 0.6 mM δ-ALA is an aggregate of the data points from both experiments.

4.2 Effect of additional heterologous expression on P450 expression

Strains used in this section of work:

<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF		C
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(GLD)		CG
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(AIKL)		CA
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(AIKL+GLD)		CAG

In addition to the expression of the CYP operon (CYP153A6 and its helper proteins, ferredoxin and ferredoxin reductase) some of the strains used in this study were expressing a heterologous glycerol dehydrogenase, the membrane channel protein AIKL, or both. Previous studies on the CYP153A6 system found that the expression of heterologous glycerol dehydrogenase could reduce active CYP153A6 concentration by up to 58%, with effects being more severe in the case of continuous induction as opposed to point induction (Gudiminch et al., 2011). Therefore, preliminary growth and expression experiments were carried out with strains designated as C, CG, CA and CAG. All strains contained the CYP operon on pET28b+; in addition, the following heterologous genes and plasmids were present:

- strain C contained the empty pCDFDuet vector,
- strain CG contained glycerol dehydrogenase on pCDFDuet,
- strain CA contained AlkL on pCDFDuet, and
- strain CAG contained both glycerol dehydrogenase and AlkL on pCDFDuet.

Carbon monoxide difference spectrophotometry was used to determine the concentrations of the active (P450) and inactive (P420) forms of CYP153A6, indicated by the absorbance at 450 nm and 420 nm, respectively (see Section 3.5.2). When the four strains were grown using the same auto-induction medium under the same conditions, the intracellular P450 concentration in the different cultures varied considerably, as shown in Figure 4.3.

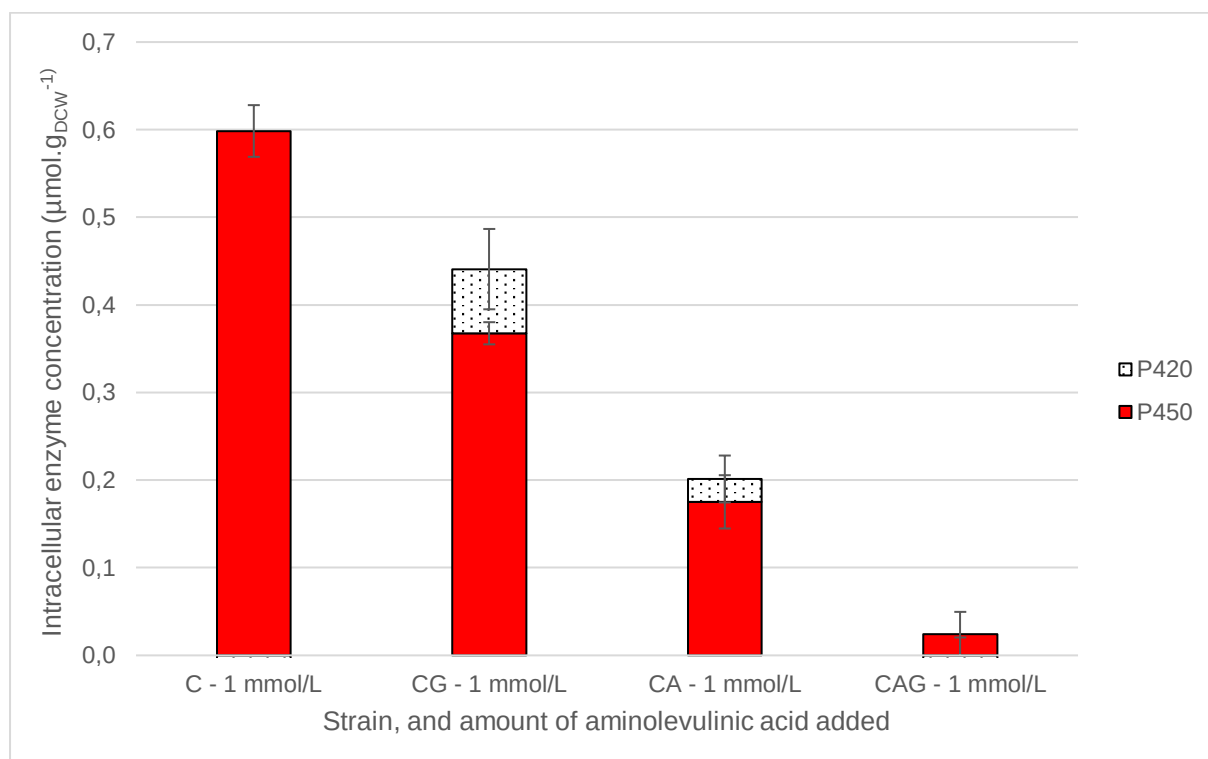


Figure 4.3: Concentration of CYP153A6 in strains expressing various additional heterologous proteins, as determined by CO difference spectrophotometry. P450 (solid red bars) refers to the active form of the enzyme; P420 (black dotted bars) refers to the inactive form. Cultures were incubated for 48 h on auto-induction medium at 20°C and 200 rpm. The auto-induction medium contained 50 $\mu\text{mol.L}^{-1}$ ferric chloride (FeCl_3) and 1 mmol.L^{-1} δ -aminolevulinic acid (δ -ALA). Error bars represent the standard deviation of three sample replicates. C: CYP operon on pET28b+ along with empty pCDFDuet. CG: CYP operon on pET28b+ along with glycerol dehydrogenase on pCDFDuet. CA: CYP operon on pET28b+ along with AlkL on pCDFDuet. CAG: CYP operon on pET28b+ along with glycerol dehydrogenase and AlkL on pCDFDuet.

Thus, strains C, CG, and CA were grown in the presence of different levels of δ -aminolevulinic acid (δ -ALA) and 50 $\mu\text{mol.L}^{-1}$ ferric chloride. The aim was to control the CYP153A6 expression at levels comparable to those of the lowest-expression strain, CAG. The results are summarised in Table 4.1 and presented graphically in Figure 4.4 through Figure 4.6. The CAG strain, with 1 mmol.L^{-1} δ -ALA added to the auto-induction medium, was used as a control across the three experiments. CAG produced an average intracellular P450 concentration of $0.146 \pm 0.022 \mu\text{mol.g}_{\text{DCW}}^{-1}$. The average intracellular P420 concentration was $0.229 \pm 0.096 \mu\text{mol.g}_{\text{DCW}}^{-1}$, meaning that only 40% of the total CYP153A6 produced by strain CAG was in an active form.

Table 4.1: Intracellular concentrations of CYP153A6 for four different strains, exposed to various δ -ALA starting concentrations in the growth and expression medium. 50 $\mu\text{mol.L}^{-1}$ FeCl_3 was added in all cases. C: CYP operon on pET28b+ along with empty pCDFDuet. CG: CYP operon on pET28b+ along with glycerol dehydrogenase on pCDFDuet. CA: CYP operon on pET28b+ along with AlkL on pCDFDuet. CAG: CYP operon on pET28b+ along with glycerol dehydrogenase and AlkL on pCDFDuet.

Accompanying figure	Strain	Amount of aminolevulinic acid added (mmol.L^{-1})	Intracellular concentrations of CYP153A6 ($\mu\text{mol.g}_{\text{DCW}}^{-1}$)	
			Active (P450)	Inactive (P420)
4.3	C	1.00	0.599 ± 0.030	-0.050 ± 0.023
	CG		0.368 ± 0.013	0.073 ± 0.046
	CA		0.175 ± 0.030	0.026 ± 0.027
	CAG		0.024 ± 0.026	-0.015 ± 0.035
4.4 – 4.6	C	0.05	0.207 ± 0.034	0.006 ± 0.011
		0.10	0.306 ± 0.035	0.030 ± 0.020
		0.25	0.411 ± 0.062	0.185 ± 0.021
	CG	0.10	0.219 ± 0.028	0.110 ± 0.027
		0.25	0.247 ± 0.013	0.249 ± 0.055
		0.50	0.254 ± 0.021	0.266 ± 0.041
	CA	0.50	0.277 ± 0.013	0.261 ± 0.139
		0.77	0.232 ± 0.024	0.266 ± 0.118
		1.00	0.227 ± 0.021	0.251 ± 0.140
	CAG	1.00	0.146 ± 0.022	0.229 ± 0.096
4.7	C	0.05	0.178 ± 0.012	-0.013 ± 0.016
	CG	0.09	0.138 ± 0.013	0.031 ± 0.008
	CA	0.10	0.088 ± 0.020	0.084 ± 0.044
	CAG	1.00	0.063 ± 0.012	0.258 ± 0.095

Strain C exceeded the maximum P450 expression levels of the CAG control by 1.6-fold, even at 0.05 mmol.L⁻¹ δ -ALA addition. Specific P450 concentration in the strain C culture increased from $0.207 \pm 0.034 \mu\text{mol.g}_{\text{DCW}}^{-1}$ at 0.05 mmol.L⁻¹ δ -ALA addition, to $0.306 \pm 0.035 \mu\text{mol.g}_{\text{DCW}}^{-1}$ at 0.10 mmol.L⁻¹ δ -ALA addition, to $0.411 \pm 0.062 \mu\text{mol.g}_{\text{DCW}}^{-1}$ at 0.25 mmol.L⁻¹ δ -ALA addition. However, the fraction of total protein in the active form decreased from 97% to 91% to 69% as the δ -ALA addition increased.

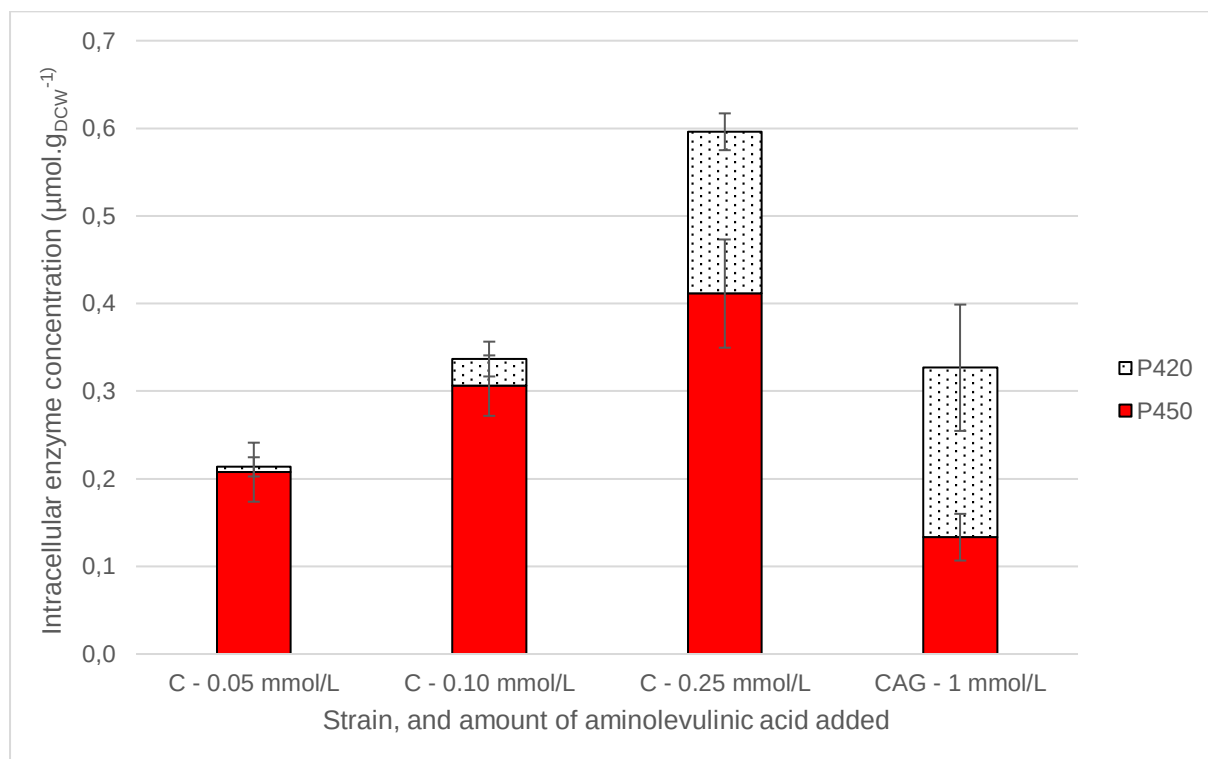


Figure 4.4: Concentration of active and inactive CYP153A6 in cells expressing A6/FdR/Fdx, at various levels of δ -ALA addition. P450 (solid red bars) refers to the active form of the enzyme; P420 (black dotted bars) refers to the inactive form. Cultures were incubated for 48 h at 20°C and 200 rpm, on auto-induction medium containing 50 $\mu\text{mol.L}^{-1}$ ferric chloride (FeCl_3); δ -aminolevulinic acid (δ -ALA) was added at the concentrations specified. Error bars represent the standard deviation of three biological replicates (grown from the same pre-culture), each of which included two sample replicates. C: CYP operon on pET28b+ along with empty pCDFDuet. CAG: CYP operon on pET28b+ along with glycerol dehydrogenase and AlkL on pCDFDuet.

Strain CG exceeded the maximum P450 expression levels of the CAG control by 1.4-fold at a δ -ALA addition of 0.10 mmol.L⁻¹. Active protein made up 67% of the total CYP153A6 concentration. When δ -ALA addition was increased to 0.25 mmol.L⁻¹, the P450 concentration did not increase significantly, but the proportion of active protein declined from 67% to 50%. A further doubling of δ -ALA addition to 0.50 mmol.L⁻¹ produced no significant changes.

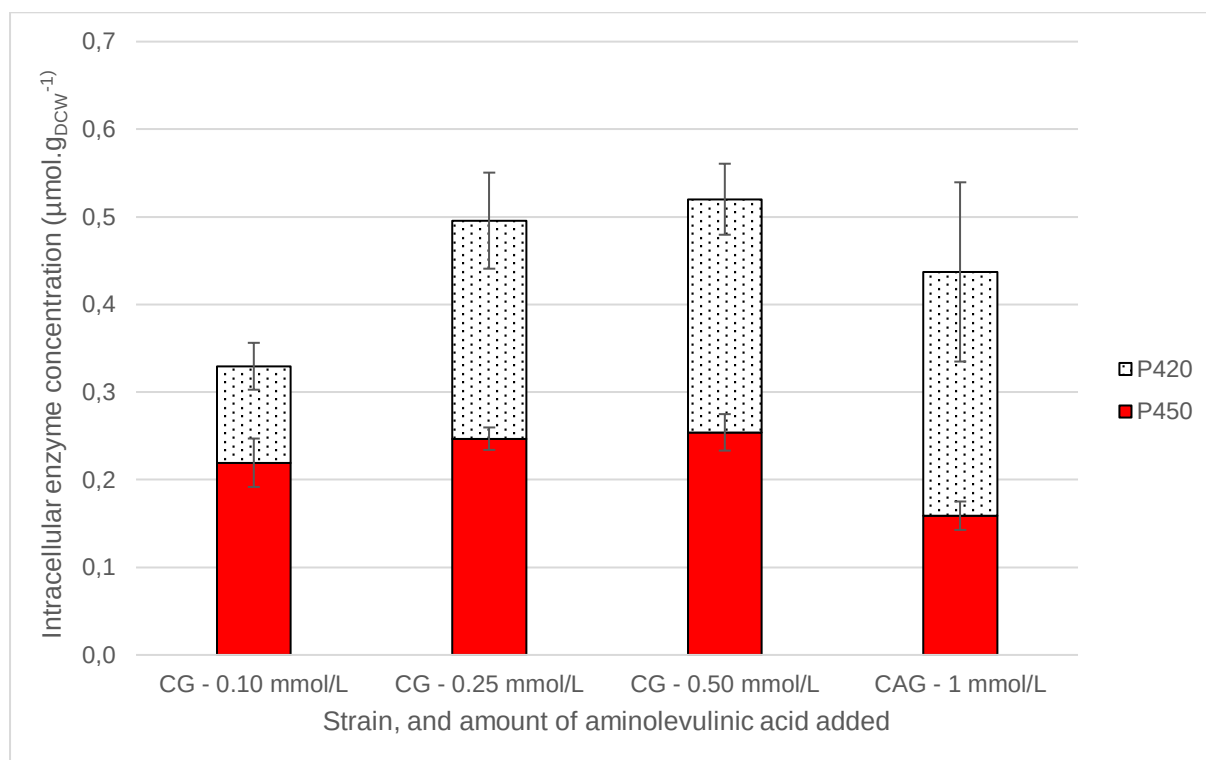


Figure 4.5: Concentration of active and inactive CYP153A6 in cells expressing A6/FdR/Fdx and co-expressing glycerol dehydrogenase, at various levels of δ -ALA addition. P450 (solid red bars) refers to the active form of the enzyme; P420 (black dotted bars) refers to the inactive form. Cultures were incubated for 48 h at 20°C and 200 rpm, on auto-induction medium containing 50 μ mol.L⁻¹ ferric chloride (FeCl₃); δ -aminolevulinic acid (δ -ALA) was added at the concentrations specified. Error bars represent the standard deviation of three biological replicates (grown from the same pre-culture), each of which included two sample replicates. CG: CYP operon on pET28b+ along with glycerol dehydrogenase on pCDFDuet. CAG: CYP operon on pET28b+ along with glycerol dehydrogenase and AlkL on pCDFDuet.

At a δ -ALA addition of 0.50 mmol.L^{-1} , strain CA produced 1.5-fold more P450 than the CAG control, with a comparable ratio of active protein at 46% of the total. Increasing the δ -ALA addition to 0.75 or 1 mmol.L^{-1} had no significant effect on the production of either the active or inactive form of CYP153A6.

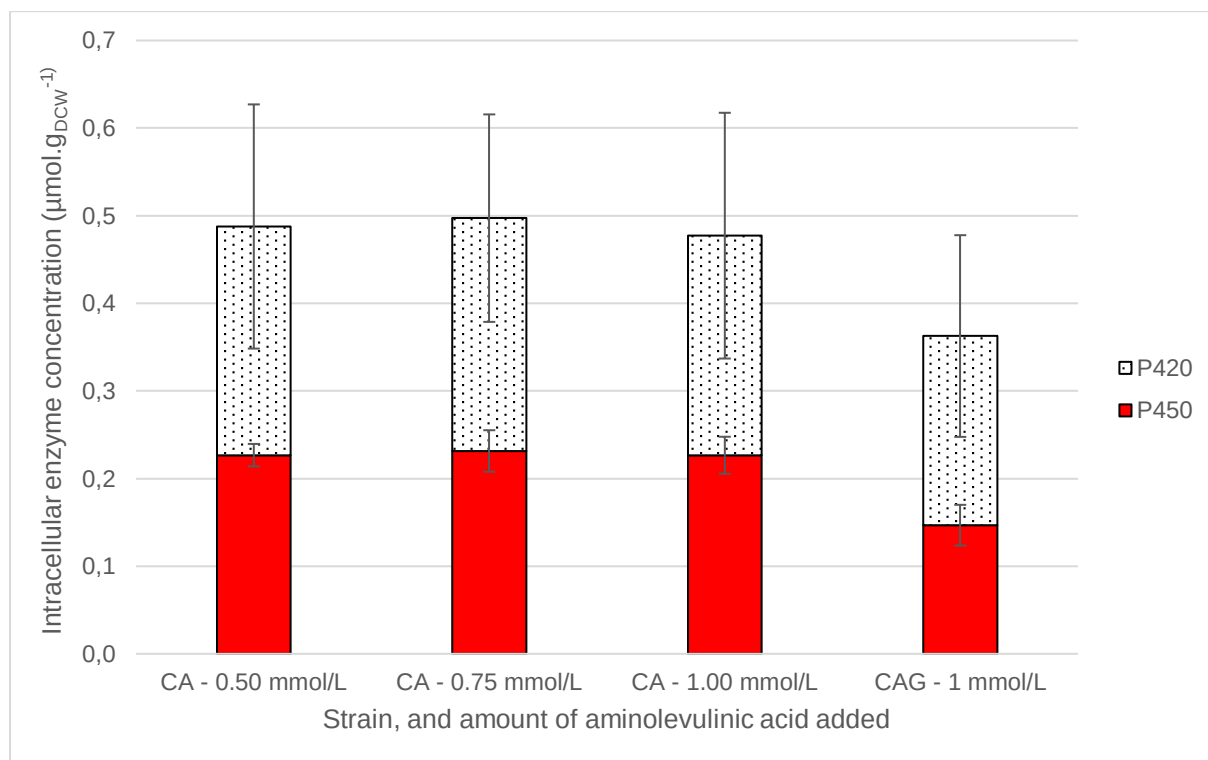


Figure 4.6: Concentration of active and inactive CYP153A6 in cells expressing A6/FdR/Fdx and co-expressing AlkL, at various levels of δ -ALA addition. P450 (solid red bars) refers to the active form of the enzyme; P420 (black dotted bars) refers to the inactive form. Cultures were incubated for 48 h on auto-induction medium at 20°C and 200 rpm. The auto-induction medium contained $50 \mu\text{mol.L}^{-1}$ ferric chloride (FeCl_3); δ -aminolevulinic acid (δ -ALA) was added at the concentrations specified. Error bars represent the standard deviation of three biological replicates (grown from the same pre-culture), each of which included two sample replicates. CA: CYP operon on pET28b+ along with AlkL on pCDFDuet. CAG: CYP operon on pET28b+ along with glycerol dehydrogenase and AlkL on pCDFDuet.

Based on these results, all four strains were cultured simultaneously on auto-induction media containing $50 \mu\text{mol.L}^{-1}$ ferric chloride and varying concentrations of δ -ALA. Figure 4.7 shows the average outcome of four biological replicates. Strain C was cultured in medium containing 0.05 mmol.L^{-1} δ -ALA addition, and produced $0.178 \pm 0.012 \mu\text{mol.g}_{\text{DCW}}^{-1}$ active CYP153A6, with no inactive protein being detected. Strain CG was cultured in medium with 0.10 mmol.L^{-1} δ -ALA addition (except for one replicate which was tested at 0.05 mmol.L^{-1} δ -ALA), and produced $0.138 \pm 0.013 \mu\text{mol.g}_{\text{DCW}}^{-1}$ active CYP153A6, with the active form constituting 82% of the total CYP153A6. Strain CA was also cultured in medium with 0.10 mmol.L^{-1} δ -ALA, and produced $0.088 \pm 0.020 \mu\text{mol.g}_{\text{DCW}}^{-1}$ active CYP153A6, with the active form constituting 51% of the total CYP153A6. Strain CAG was cultured in medium with 1 mmol.L^{-1} δ -ALA addition, yet it produced only $0.063 \pm 0.012 \mu\text{mol.g}_{\text{DCW}}^{-1}$ active CYP153A6, and the active form constituted only 20% of the total CYP153A6.

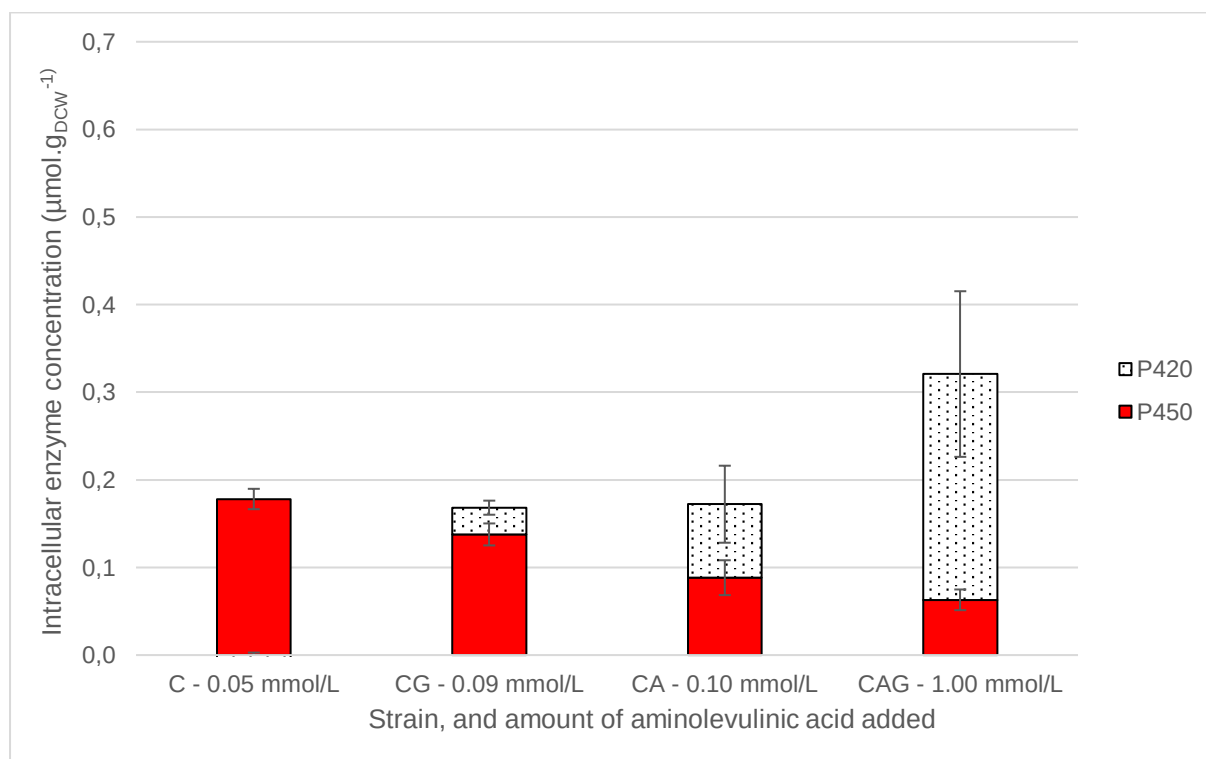


Figure 4.7: Application of varying levels of δ -ALA addition in order to moderate the expression of active CYP153A6 across cultures expressing various additional heterologous proteins. P450 (solid red bars) refers to the active form of the enzyme; P420 (black dotted bars) refers to the inactive form. Cultures were incubated for 48 h on auto-induction medium at 20°C and 200 rpm. The auto-induction medium contained $50 \mu\text{mol.L}^{-1}$ ferric chloride; δ -aminolevulinic acid was added to the concentrations specified. Error bars represent the standard deviation of four biological replicates, each of which included two sample replicates. C: CYP operon on pET28b+ along with empty pCDFDuet. CG: CYP operon on pET28b+ along with glycerol dehydrogenase on pCDFDuet. CA: CYP operon on pET28b+ along with AlkL on pCDFDuet. CAG: CYP operon on pET28b+ along with glycerol dehydrogenase and AlkL on pCDFDuet.

The expression of additional heterologous proteins was detrimental to CYP153A6 expression. Expression of either glycerol dehydrogenase or the membrane channel protein AlkL led to reduced concentrations of total CYP153A6 relative to the strain carrying the empty pCDFDuet vector, while also increasing the relative quantity of inactive protein formation. This resulted in intracellular concentrations of active CYP153A6 being reduced by roughly one third when glycerol dehydrogenase was co-expressed, and by roughly two thirds when AlkL was co-expressed. Co-expressing the CYP operon, glycerol dehydrogenase, and AlkL simultaneously led to a 25-fold reduction in the intracellular concentration of active CYP153A6. The negative effects of AlkL expression on CYP153A6 expression are thus more substantial than the effects of dehydrogenase expression, with the expression of both glycerol dehydrogenase and AlkL leading to very large quantities of inactive protein being formed, in both relative and absolute terms.

Increasing δ -ALA addition appears to boost CYP153A6 expression up to a concentration of 0.25 mmol.L^{-1} , after which its addition ceases to affect expression of the protein in either its active or inactive form; this is compatible with the results discussed in Section 4.1. For the CG and CA strains, the ratio of inactive to active form remains relatively constant after the limiting δ -ALA concentration is exceeded; this suggests that the formation of inactive enzyme is not due to a lack of haem for incorporation into the active site, but must be related to translation errors, improper protein folding, or a failure to incorporate key residues into the active site. Maintaining and expressing heterologous genes is known to impose a significant metabolic burden on the cell, because it consumes cofactors and cellular components that would otherwise be directed to the production of homologous proteins. This burden is exacerbated if the heterologous protein requires codons or amino acids that the cell produces only in limiting quantities (Alonso-Gutierrez et al., 2013; Baneyx, 1999; Becker and Wittmann, 2015; Kadisch et al., 2017). This would explain why the formation of inactive protein is lowest for cells carrying an empty pCDFDuet plasmid and highest for cells expressing two proteins from the pCDFDuet plasmid. It is also in line with the observation that cells expressing glycerol dehydrogenase (which originates from *E. coli*) are able to produce slightly more active CYP153A6 than cells expressing the membrane transport protein (which originates from a different bacterial family, *Pseudomonas*).

Although expression of active CYP153A6 could not be controlled to completely equivalent levels between strains, expression was nevertheless at more comparable levels when δ -ALA addition was modified on a strain-by-strain basis.

4.3 Effect of enzyme loading on biotransformation

Strains used in this section of work:



It has been shown that varying the δ -aminolevulinic acid and ferric chloride concentrations in auto-induction media will result in varied CYP153A6 expression in *Escherichia coli*. This strategy was used to investigate the effects of intracellular P450 concentration on whole-cell biotransformations. Manipulation of δ -ALA and FeCl_3 concentrations was used to generate cultures of *E. coli* expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid, with a range of P450 expression levels. These cells, suspended to the same cell density in phosphate buffer, were used to carry out n-octane biotransformations on the millilitre scale over a 69 h period.

As shown in Table 4.2 and Figure 4.8, the maximum intracellular concentration of active CYP153A6 varied from $1.15 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ (for the strain grown without any additional precursors) to $3.13 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ (for the two strains exposed to at least 0.25 mM δ -ALA and $50 \mu\text{M}$ FeCl_3 simultaneously). Cultures had lost 40 – 80% of their P450 content after 69 h; average intracellular P450 concentration over the course of the biotransformation ranged from $0.26 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ (for the strain grown without any additional precursors) to $2.44 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ (for the strain exposed to 0.5 mM δ -ALA and $100 \mu\text{M}$ FeCl_3). At any given sampling point, the variation between the highest and lowest intracellular P450 concentrations was at least 6.5-fold.

Table 4.2: Concentration of active CYP153A6 in *E. coli* containing the A6 along with its electron transfer proteins, FdR and Fdx, on the pET28b+ plasmid. Growth and expression were carried out for 48 h at 20 °C and 200 rpm. Aminolevulinic acid and ferric chloride was added to the growth and expression medium at the concentrations specified.

δ -ALA (mM)	FeCl ₃ (μ M)	Intracellular P450 concentration ($\mu\text{mol}_{\text{P450}}\cdot\text{g}_{\text{DCW}}^{-1}$)		
		Initial	Maximum	Average*
0.00	0	1.15 ± 0.12	1.15 ± 0.12	0.26
0.00	50	1.18 ± 0.23	1.18 ± 0.23	0.49
0.25	0	2.09 ± 0.54	2.09 ± 0.54	0.72
0.10	25	1.54 ± 0.14	1.54 ± 0.14	0.45
0.25	50	1.02 ± 0.48	3.13 ± 0.10	1.73
0.50	100	0.31 ± 0.37	3.13 ± 0.39	2.44

* Time-weighted average

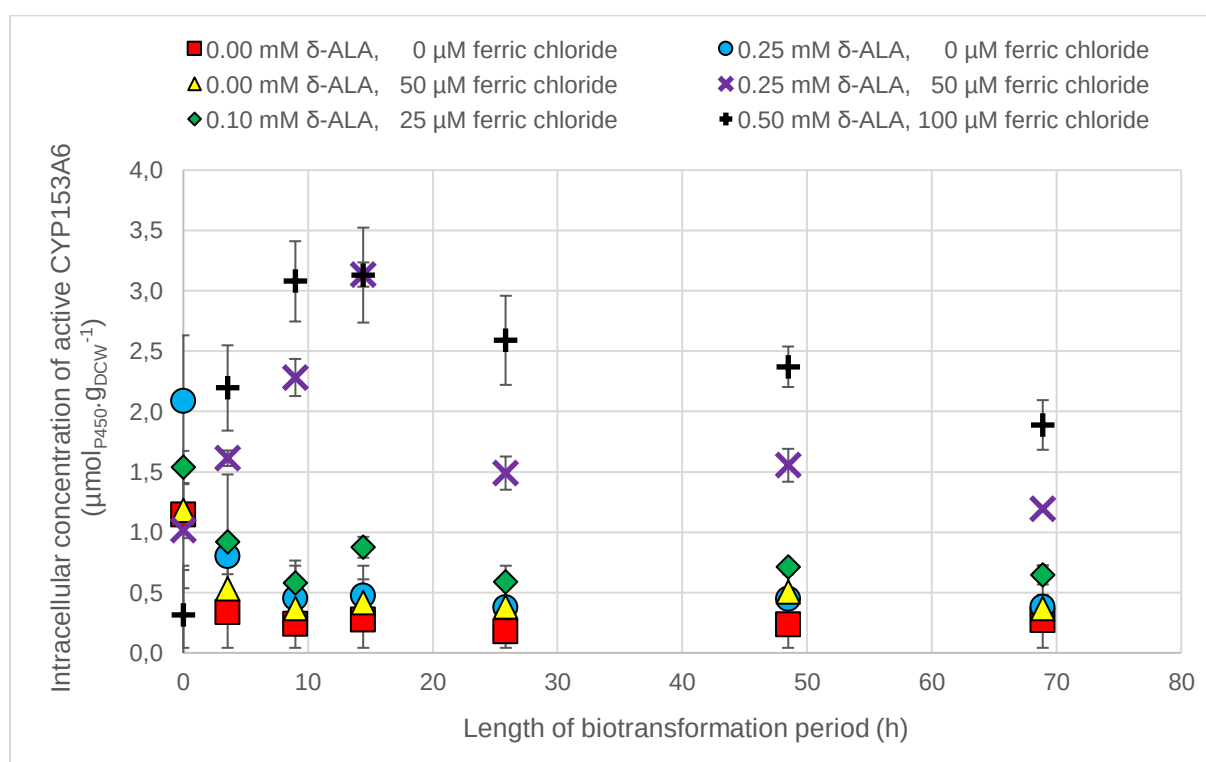


Figure 4.8: Intracellular concentration of active CYP153A6 during biotransformations with cultures grown and expressed with different concentrations of δ -ALA and FeCl₃. All cultures consisted of *E. coli* containing CYP153A6 along with its electron transfer proteins, FdR and Fdx, on the pET28b+ plasmid (strain no.1 in Table 3.2). Following expression, cells were resuspended in phosphate buffer to a cell density of $24 \pm 2 \text{ g}_{\text{DCW}}\cdot\text{L}^{-1}$. Biotransformations were carried out at 20°C and 200 rpm for a total of 69 h. The biotransformation reaction mixture consisted of 1 mL cell suspension, 0.2 mL n-octane, and 0.1 mL BEHP. Biotransformations were carried out in 40 mL amber vials; vials were sacrificed at each time point for analysis. Concentration of active CYP153A6 was determined via CO difference absorbance assay.

Cultures with lower CYP153A6 expression levels (maximum concentration $< 3 \mu\text{mol}_{\text{P450}}\cdot\text{g}_{\text{DCW}}^{-1}$) followed a similar pattern with regards to enzyme stability. Their P450 levels were highest at the beginning of biotransformation, and decreased steadily over the first 9 h, before stabilising at a level of $0.2 - 0.9 \mu\text{mol}_{\text{P450}}\cdot\text{g}_{\text{DCW}}^{-1}$. In contrast, the two cultures with the highest expression levels (maximum concentration $3.13 \pm 0.39 \mu\text{mol}_{\text{P450}}\cdot\text{g}_{\text{DCW}}^{-1}$) followed an unusual pattern: P450 concentrations measured at the start of biotransformation were much lower than expected, but later readings suggested that the intracellular P450 concentration had risen during the first 9 – 14 h of biotransformation. After reaching their

maximum P450 concentration, these cultures began to lose P450 at a rate similar to that of lower-expression cultures. (The matter of CYP153A6 stability is discussed further in Section 5.5.1)

The apparent turnover frequencies (TOF) after 4 h of biotransformation are presented in Figure 4.9. The only biotransformation product produced at detectable concentrations was 1-octanol, which was used to calculate apparent turnover frequencies, since the addition of n-octane in excess made it difficult to accurately detect small changes in substrate concentration. The maximum TOF observed was $0.90 \pm 0.23 \text{ mol}_{1\text{-octanol}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$. This TOF was exhibited by cells with the lowest intracellular P450 concentration of $0.34 \pm 0.06 \text{ } \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$. The TOF decreased consistently as the intracellular P450 concentration rose, reaching $0.09 \pm 0.02 \text{ mol}_{1\text{-octanol}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$ for cultures containing $2.20 \pm 0.35 \text{ } \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$.

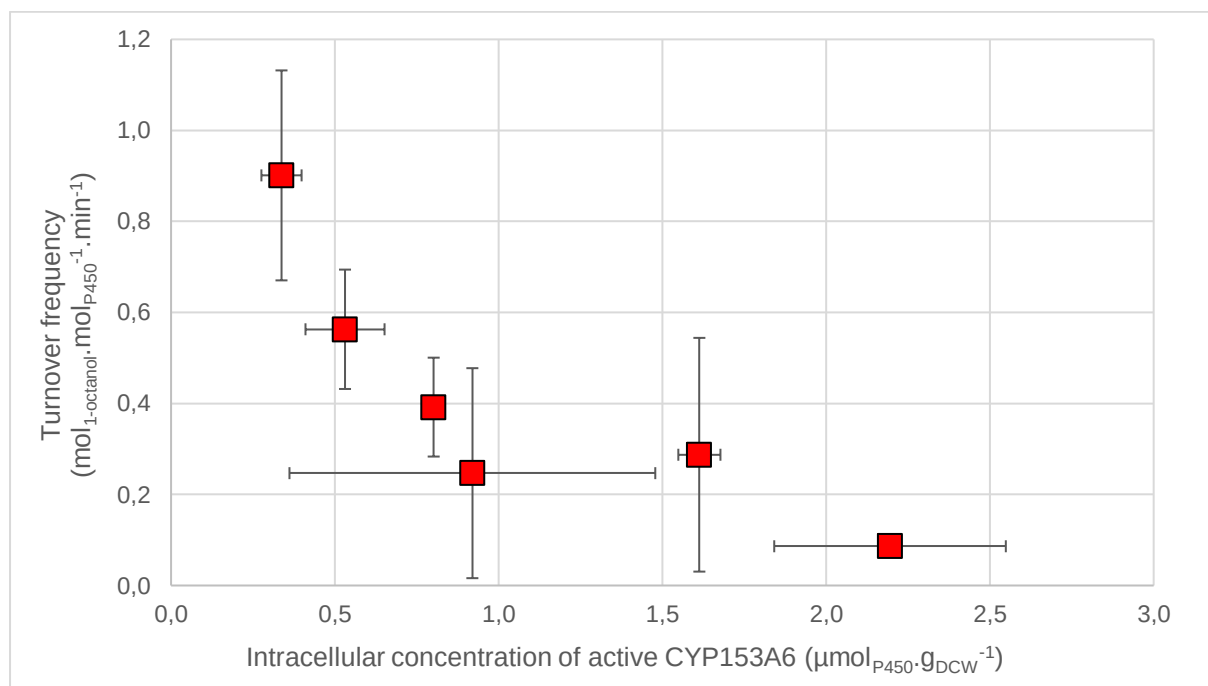


Figure 4.9: Relationship between the concentration of active CYP153A6 per cell and the apparent average turnover frequency after 4 h of biotransformation at 20°C and 200 rpm. The experiment involved six separate cultures, each having a different intracellular P450 concentration. The biotransformation reaction mixture consisted of 1 mL whole cells suspended in phosphate buffer to a cell concentration of $24 \pm 2 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$, 0.2 mL n-octane, and 0.1 mL BEHP. Biotransformations were carried out in 40 mL amber vials; vials were sacrificed at each time point for analysis. Concentrations of 1-octanol were measured using gas chromatography following extraction of organics into ethyl acetate containing 2 mM 1-decanol as internal standard; error bars represent the standard deviation over two sample replicates (i.e. two vials). Concentration of active CYP153A6 was determined via CO difference absorbance assay; error bars represent standard deviations of two sample replicates.

As Figure 4.10 shows, apparent TOF throughout the biotransformation followed a similar trend to the TOF at 4 h. The highest TOF recorded overall was $2.89 \pm 1.01 \text{ mol}_{1\text{-octanol}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$ for cultures containing $0.18 \pm 0.01 \text{ } \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ after 26 h of biotransformation. The lowest TOF recorded overall was $0.04 \pm 0.01 \text{ mol}_{1\text{-octanol}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$ for cultures containing $3.08 \pm 0.33 \text{ } \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ after 9 h of biotransformation.

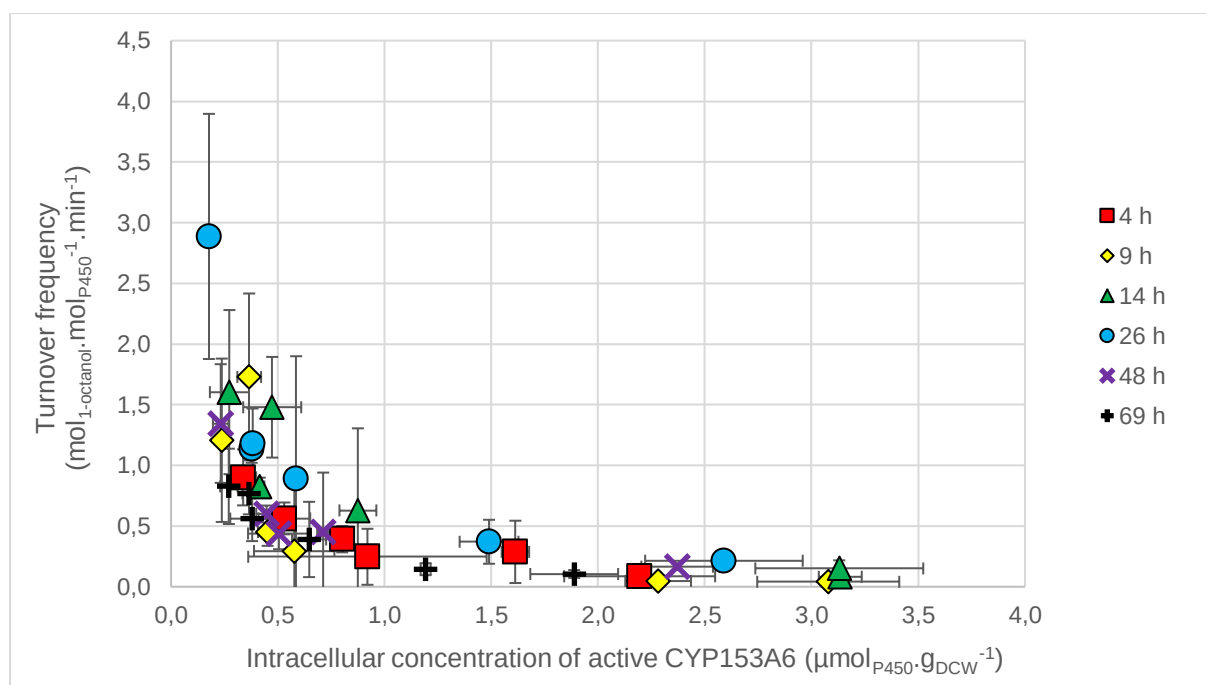


Figure 4.10: Relationship between the concentration of CYP153A6 per cell and the apparent average turnover frequency at various points in the biotransformation. The experiment involved six separate cultures, each having a different intracellular P450 concentration. The biotransformation reaction mixture consisted of 1 mL whole cells suspended in phosphate buffer to a cell concentration of $24 \pm 2 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$, 0.2 mL n-octane, and 0.1 mL BEHP. Biotransformations were carried out in 40 mL amber vials; vials were sacrificed at each time point for analysis. Concentrations of 1-octanol were measured using gas chromatography following extraction of organics into ethyl acetate containing 2 mM 1-decanol as internal standard; error bars represent the standard deviation over two sample replicates (i.e. two vials). Concentration of active CYP153A6 was determined via CO difference absorbance assay; error bars represent standard deviations of two sample replicates.

In cases where a biotransformation was limited by the concentration of active enzyme, each individual enzyme could be expected to be working as fast as it can; hence, increasing the enzyme concentration would not change the amount of substrate converted per unit of enzyme over a set period – turnover frequency would remain constant with enzyme concentration, at some value determined by enzyme kinetics. Once some limiting concentration of active enzyme had been exceeded, the biotransformation would become limited by other factors. Each individual enzyme would convert less substrate, on average, as a surplus of enzymes competed for the limiting component – the apparent turnover frequency would decrease as enzyme concentration increased. This concept is illustrated schematically in Figure 4.11.

In this study, apparent TOF did not plateau as the concentration of active CYP153A6 decreased, suggesting that concentrations as low as $0.18 \pm 0.01 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ are still above the limiting point for the unmodified whole-cell system. A previous study on CYP153A6 in *E. coli* found that an increase from 0.02 to 0.06 $\mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ almost doubled the 1-octanol produced in a 70 h biotransformation (Olaofe, 2013), suggesting that the limiting concentration for intact *E. coli* cell systems is somewhere between 0.06 and 0.18 $\mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$.

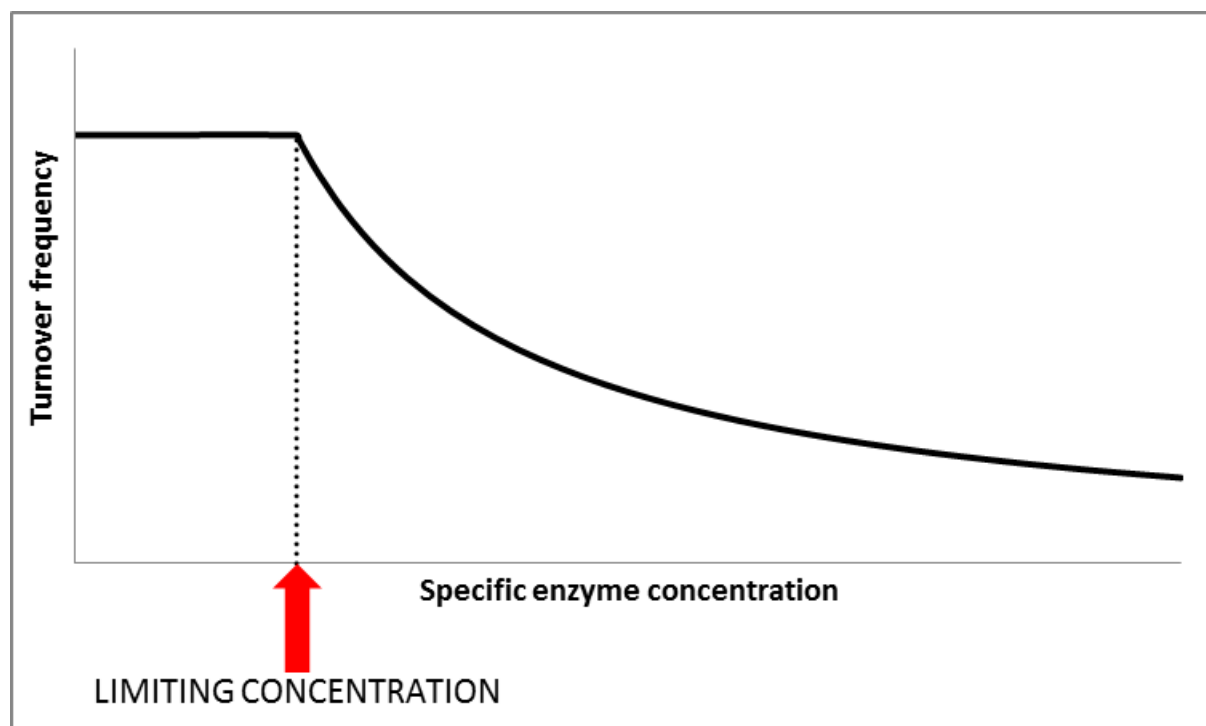


Figure 4.11: Schematic showing a theoretical relationship between the concentration of biocatalytic enzyme per cell and the apparent turnover frequency (moles substrate converted per mole enzyme per unit time). The limiting specific concentration is marked with a dotted line and indicated by a red arrow. Below the limiting concentration, the biotransformation is limited by enzyme availability, and the turnover frequency (the amount of substrate converted by each enzyme per unit time) remains constant at its maximum value. Above the limiting concentration, the biotransformation becomes limited by factors other than enzyme availability, and thus the average amount of substrate converted per enzyme per unit time decreases as enzyme concentration increases.

The time course for production of 1-octanol is shown in Figure 4.12. Maximum titres of 1-octanol ranged from 19.9 ± 6.9 mM for cultures with the lowest intracellular P450 concentrations, to 30.2 ± 4.3 mM for cultures with the highest intracellular P450 concentrations. The average concentration of active CYP153A6 varied by a factor of 5.8 between these cultures; this variation only changed 1-octanol production by a factor of 1.5.

Furthermore, 1-octanol production was also considered on a cell-specific basis, since the experimental protocol involved resuspending all cultures in phosphate buffer to the same wet cell weight (see Section 3.3.2), resulting in dry cell weights that varied by up to 10%. Figure 4.12-B shows that after correcting for cell density, the variation in P450 concentration did not result in significant differences in 1-octanol formation. The maximum yield on biomass ranged from $0.86 \pm 0.41 \mu\text{mol}_{1\text{-octanol}}.\text{g}_{\text{DCW}}^{-1}$ (for cultures where P450 varied from $3.13 - 1.02 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$) to $1.16 \pm 0.24 \mu\text{mol}_{1\text{-octanol}}.\text{g}_{\text{DCW}}^{-1}$ (for cultures where P450 varied from $1.18 - 0.36 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$).

In general, the time course of the biotransformations could be described as an S-curve: the rate of 1-octanol production was low during the early stages of biotransformation, picked up at or around the 9 h mark, then began to level off between 14 h and 26 h. Cultures containing an average of $0.7 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ (an intermediate enzyme loading) displayed the highest rate of product formation overall, but began levelling off sooner than cultures with higher or lower enzyme loadings. The cultures with the highest enzyme loading (1.8 or $2.2 \mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ on average) did appear to perform slightly better between 12 h and 48 h, but measurements at the 69 h timepoint produced titres and yields on biomass at lower levels than the cultures with lower enzyme loadings.

All of these variations were within the margin of experimental error. Ultimately, the intracellular P450 concentration had minimal impact on the final amount of 1-octanol produced, and minimal impact on the time course of the reaction, under the conditions tested. This further supports the hypothesis that active CYP153A6 is not a limiting component for unmodified whole cells.

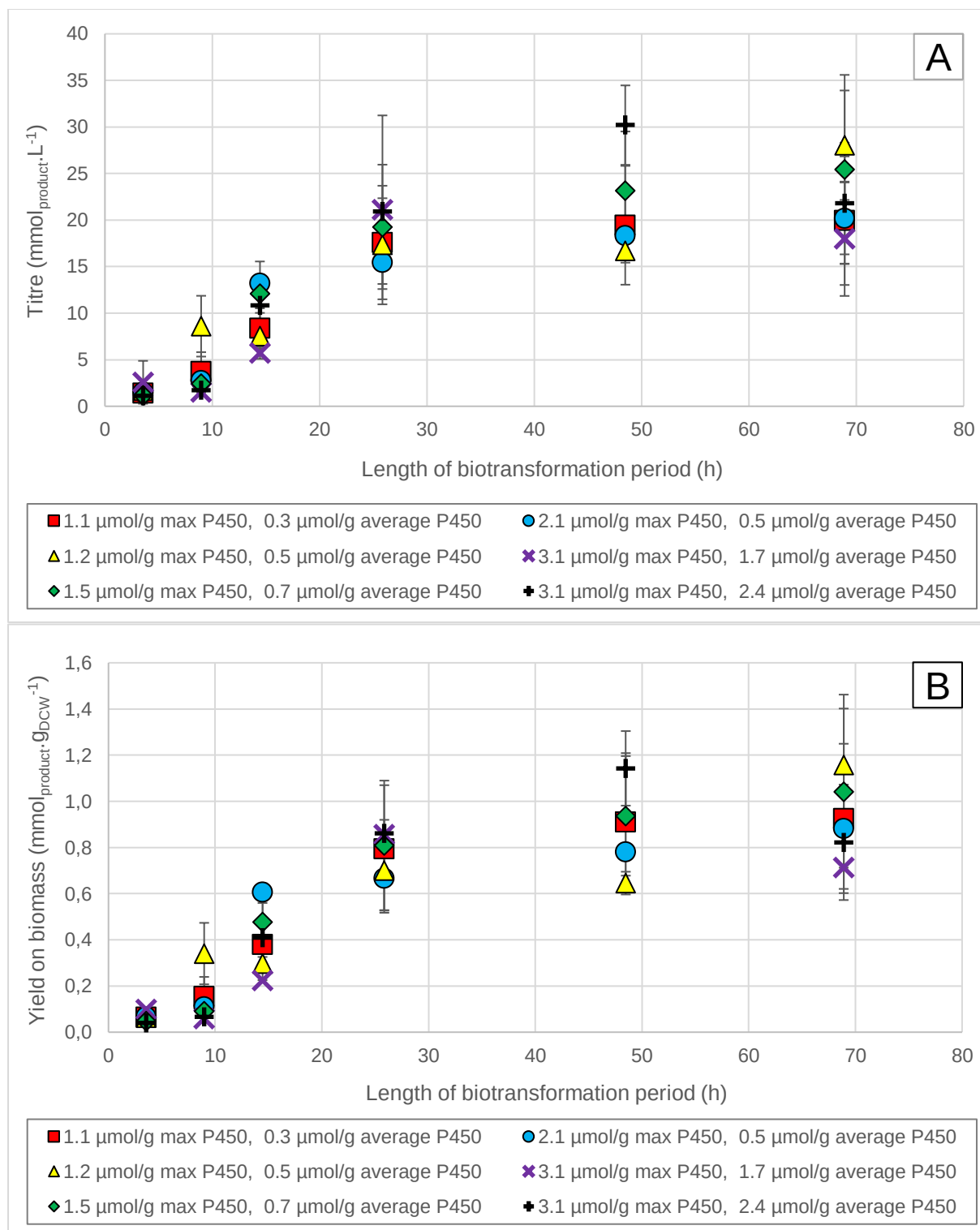


Figure 4.12: Effect of active CYP153A6 concentration on 1-octanol production in six separate cultures, each having a different intracellular P450 concentration. The biotransformation reaction mixture consisted of 1 mL whole cells suspended in phosphate buffer to a cell concentration of 24 ± 2 g_{DCW} · L⁻¹, 0.2 mL n-octane, and 0.1 mL BEHP. Biotransformations were carried out in 40 mL amber vials; vials were sacrificed at each time point for analysis. Concentrations of 1-octanol were measured using gas chromatography following extraction of organics into ethyl acetate containing 2 mM 1-decanol as internal standard; error bars represent the standard deviation over two sample replicates (i.e. two vials).

Figure 4.13 shows the space time yields (STY) for the various cultures, calculated based on the concentration of 1-octanol detected at each sampling point. Most cultures offered optimum yields after either 14 h or 26 h. The highest STY was $0.96 \pm 0.36 \text{ mmol.L}^{-1}.\text{h}^{-1}$ after 9 h for cultures containing an average of $0.5 \mu\text{molP}_{450}.\text{g}_{\text{DCW}}^{-1}$; the next highest were $0.92 \pm 0.01 \text{ mmol.L}^{-1}.\text{h}^{-1}$ after 14 h for cultures containing an average of $0.8 \mu\text{molP}_{450}.\text{g}_{\text{DCW}}^{-1}$, and $0.84 \pm 0.14 \text{ mmol.L}^{-1}.\text{h}^{-1}$ after 26 h for cultures containing $2.31 \pm 0.25 \mu\text{molP}_{450}.\text{g}_{\text{DCW}}^{-1}$. From 26 h onwards, STY did not vary substantially between cultures; after 69 h, all values fell within the range of $0.26 - 0.41 \text{ mmol.L}^{-1}.\text{h}^{-1}$. This is further evidence that this system was not limited by low expression of active CYP153A6. At a biomass concentration of $24 \pm 2 \text{ g}_{\text{DCW}}.\text{L}^{-1}$, these space time yields equate to between 0.13 and $0.67 \mu\text{mol}_{1\text{-octanol}}.\text{g}_{\text{DCW}}^{-1}.\text{min}^{-1}$. CYP153A6 has been shown to reach turnover frequencies of between 50 and $90 \mu\text{mol}_{1\text{-octanol}}.\mu\text{molP}_{450}^{-1}.\text{min}^{-1}$ (Arnold et al., 2012; Funhoff et al., 2006). If the enzymes in this experiment were achieving similar TOF values, an intracellular P450 concentration of between 0.001 and $0.013 \mu\text{molP}_{450}.\text{g}_{\text{DCW}}^{-1}$ would have been sufficient to produce the space time yields observed. This confirms that the individual enzymes are not operating “to capacity” – another factor is limiting their performance.

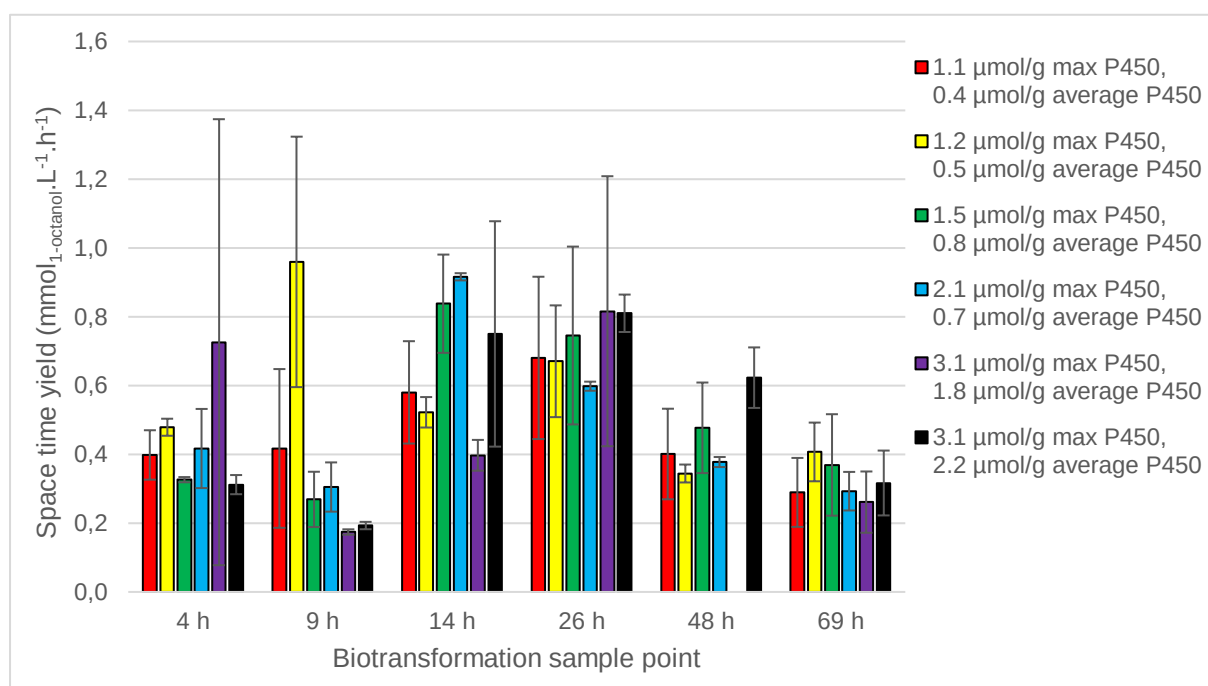


Figure 4.13: Effect of active CYP153A6 concentration on space time yields over the course of a 69 h biotransformation. The experiment involved six separate cultures, each having a different intracellular P450 concentration. The biotransformation reaction mixture consisted of 1 mL whole cells suspended in phosphate buffer to a cell concentration of $24 \pm 2 \text{ g}_{\text{DCW}}.\text{L}^{-1}$, 0.2 mL n-octane, and 0.1 mL BEHP. Biotransformations were carried out in 40 mL amber vials; vials were sacrificed at each time point for analysis. Concentrations of 1-octanol were measured using gas chromatography following extraction of organics into ethyl acetate containing 2 mM 1-decanol as internal standard; error bars represent the standard deviation over two sample replicates (i.e. two vials).

4.4 Oxygen supply

Strains used in this section of work:

<i>E. coli</i> BL21(DE3) containing pET(CYP)		
--	--	---

The biotransformation of n-octane to 1-octanol requires molecular oxygen, at a stoichiometric ratio of 1:1. Furthermore, although resting cells are known to have a lower oxygen demand than growing cells (Bühler et al., 2008; Kadisch et al., 2017; Willrodt et al., 2016), some oxygen is nevertheless required to support maintenance metabolism, and would have to be diverted away from the biotransformation. If the reaction were limited by a lack of oxygen, it could explain why increasing the concentration of active CYP153A6 was not beneficial.

Oxygen limitation was investigated in two ways: by varying the size of the biotransformation vial from 40 mL to 60 mL (keeping cross-sectional area constant); and by carrying out biotransformations in 40 mL vials, half of which had their headspace replenished at regular intervals using a 60 mL syringe filled with air (see Figure 3.3). If cells were rapidly consuming the available oxygen, causing the biotransformation to become stoichiometrically limited, then increasing the headspace volume ought to extend the biotransformation period. Regular replacement of the headspace would also remove the stoichiometric limitation; additionally, it would maintain a higher concentration of oxygen in the headspace, thus maintaining a higher driving force for oxygen mass transfer.

Figure 4.14 shows the results of four sets of biotransformations, each carried out with cell suspension from a single growth and expression culture, which was added to either 40 mL or 60 mL vials. One pair of experiments was carried out over two days (Figure 4.14-A) and one pair was extended to three days (Figure 4.14-B).

In the two-day experiments, increased headspace volume had minimal effect on the biotransformation. The amount of 1-octanol produced per gram biomass in the 60 mL vials was frequently within a standard deviation of the amount produced by cells in 40 mL vials. In the first experiment (replicate I) the yield on biomass after 52 h was 20% higher for cells in 40 mL vials: $3.97 \pm 0.20 \text{ mmol}_{1\text{-octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$, compared to $3.31 \pm 0.38 \text{ mmol}_{1\text{-octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$ for cells in 60 mL vials. In the second experiment (replicate II) the yield on biomass after 52 h was independent of headspace volume: $4.49 \pm 0.69 \text{ mmol}_{1\text{-octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$ for both sets of cells.

However, trends were different in the three-day experiments. In the first three-day experiment (replicate III), the yield on biomass was consistently higher for cells in 60 mL vials. After 48 h, cells in 40 mL vials had produced $2.30 \pm 0.30 \text{ mmol}_{1\text{-octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$, while cells in 60 mL vials had produced $3.27 \pm 0.41 \text{ mmol}_{1\text{-octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$ (an improvement of 42%). The yields on biomass after 72 h were $3.04 \pm 0.28 \text{ mmol}_{1\text{-octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$ in 40 mL vials, and $4.56 \pm 0.33 \text{ mmol}_{1\text{-octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$ in 60 mL vials (an improvement of 50%). In the second three-day experiment (replicate IV), cells in 40 mL vials appeared to cease biotransformation entirely between 24 h and 48 h, depressing yields on biomass. Meanwhile, yield on biomass after 48 h was $7.38 \pm 0.50 \text{ mmol}_{1\text{-octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$ for cells in 60 mL vials; a three-fold improvement on the $2.32 \pm 0.24 \text{ mmol}_{1\text{-octanol}} \cdot \text{g}_{\text{DCW}}^{-1}$ produced by cells in 40 mL vials. However, the cells in 40 mL vials resumed production of 1-octanol between 48 h and 72 h, and did so at an apparent rate which exceeded that of the cultures in 60 mL vials over the same period – a result that would not be expected from a system in which all of the oxygen had been depleted. Replicate IV was unusual in another way: in both 40 mL and 60 mL vials, cells displayed an initial rate of product formation that was substantially higher than initial rates observed in the previous three experiments. It is not clear what caused these cells to behave so differently to the other cultures tested. The cell density and intracellular P450 concentrations of this culture were not abnormal, and biotransformation conditions were the same as previous experiments.

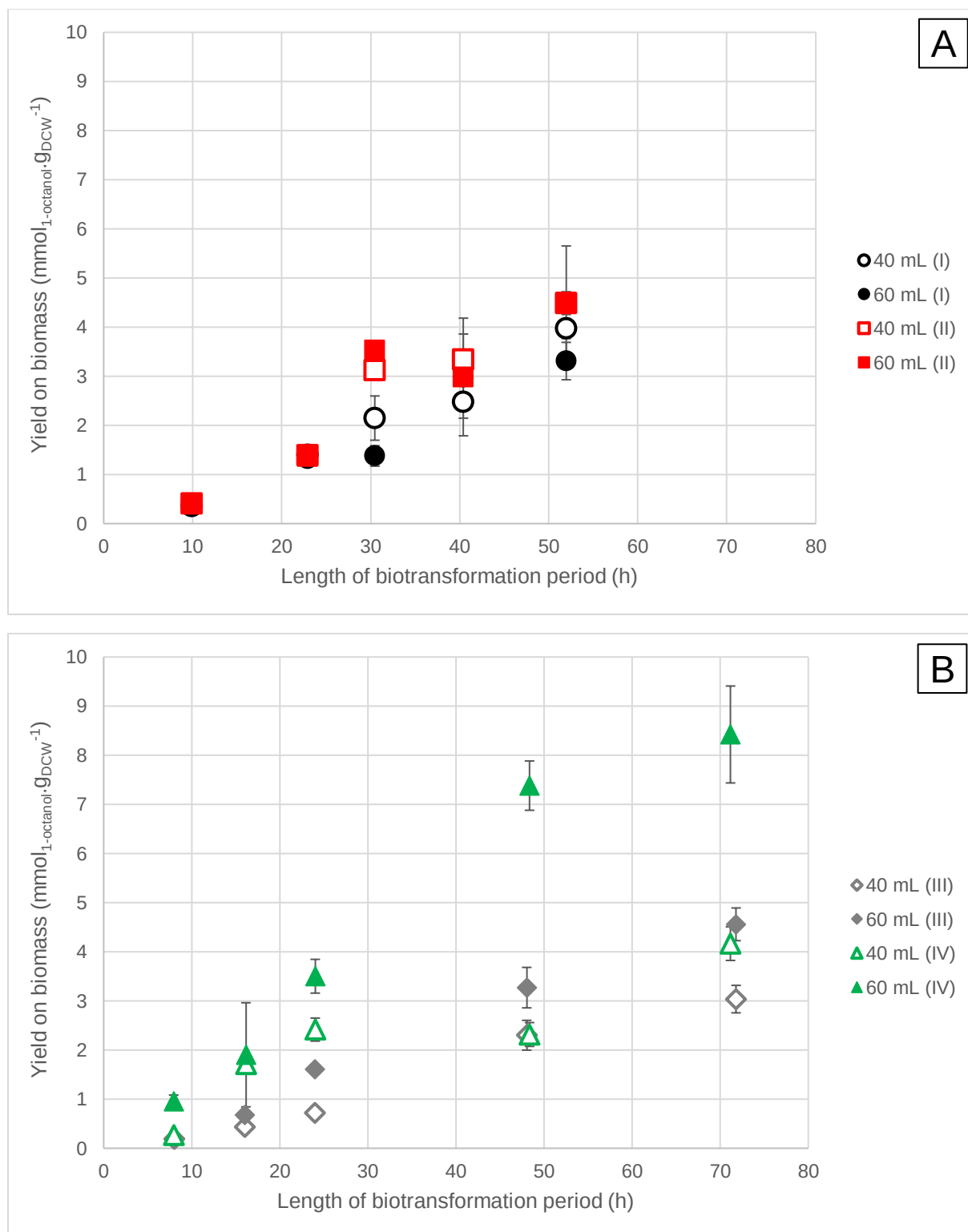


Figure 4.14: Four biological replicates, designated I – IV, investigating the effects of headspace volume on the production of 1-octanol per gram of dry cell weight. 1 mL suspensions of whole cells in phosphate buffer (average cell density $4.45 \pm 0.15 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$) were added to either 40 mL or 60 mL vials, along with 0.2 mL n-octane and 0.1 mL BEHP. Biotransformations were carried out for either two days (replicates I, II) or three days (replicates III, IV). Vials were sacrificed for sampling at each time point. Clear shapes represent results from 40 mL vials and filled shapes represent results from 60 mL vials. Error bars represent the standard deviation over three sample replicates (i.e. three vials).

The effect of headspace volume on the space time yields of the various biotransformations is shown in Figure 4.15. After accounting for experimental error, relatively small differences in STY were observed between cells in 40 mL vials and cells in 60 mL vials, although cells in 60 mL vials generally outperformed their counterparts in 40 mL vials. However, large improvements in STY were observed only in replicate IV, in which the production of 1-octanol plateaued in the 40 mL vials, leading to substantially higher STY for cells in 60 mL vials after 48 h or 72 h. Furthermore, a comparison between biological replicates reveals that a culture from a 40 mL vial was matching or outperforming a culture from a 60 mL vial at almost every timepoint.

Overall, a 50% increase in headspace volume appeared to boost biocatalyst performance slightly, especially once biotransformations were extended past two days. However, even in 40 mL vials, cultures were capable of sustaining biotransformations for periods extending to 72 h. Furthermore, small variations arising between biological replicates appeared to exert a far bigger impact on 1-octanol production than the total quantity of oxygen available to the cells.

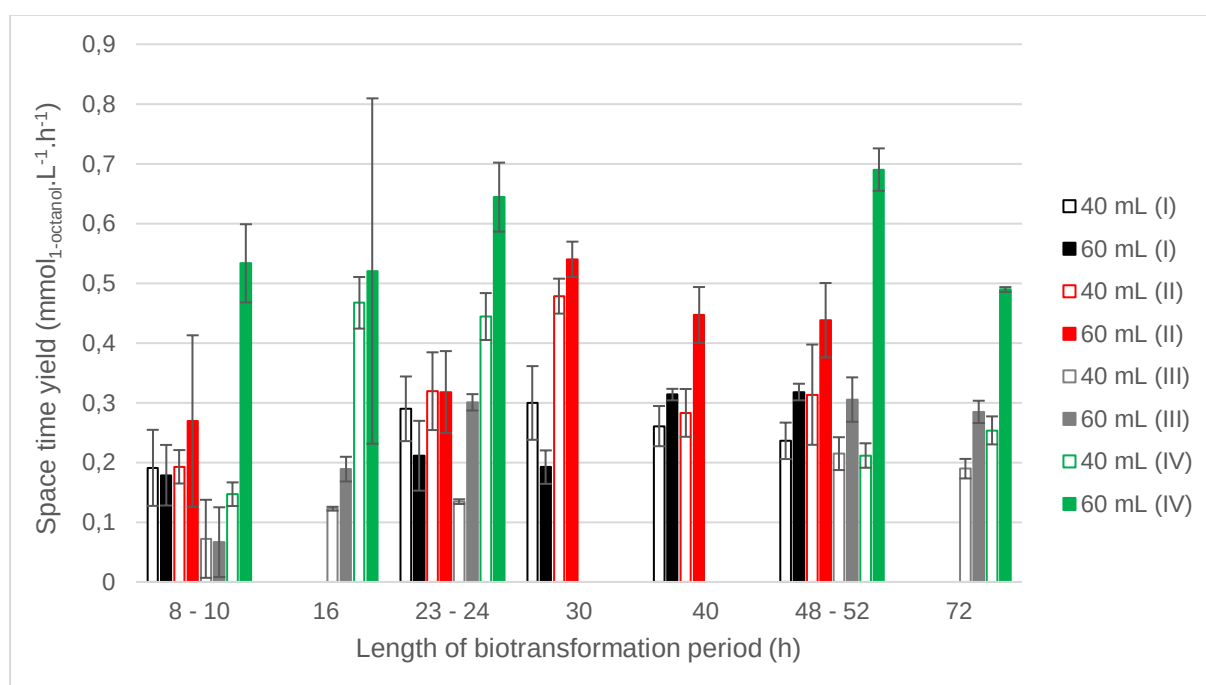


Figure 4.15: Effect of headspace volume on the space time yields of four biological replicates, designated I – IV. 1 mL suspensions of whole cells in phosphate buffer (average cell density 4.45 ± 0.15 g_{pcw}·L⁻¹) were added to either 40 mL or 60 mL vials, along with 0.2 mL n-octane and 0.1 mL BEHP. Biotransformations were carried out for either two days (replicates I, II) or three days (replicates III, IV). Vials were sacrificed for sampling at each time point. Clear shapes represent results from 40 mL vials and filled shapes represent results from 60 mL vials. Error bars represent the standard deviation over three sample replicates (i.e. three vials).

The potential for oxygen limitation was further investigated in three sets of extended biotransformations, each of which was carried out using cell suspension from a single growth and expression culture. All vials had the same dimensions (with a volume of 40 mL), but half were used to carry out biotransformations according to standard protocol, while the other half had their headspace replenished every 24 h, as described in Section 3.4.

As Figure 4.16 shows, 1-octanol production per gram of cells varied between biological replicates, but within each culture, the regular replacement of the headspace did not improve the yield on biomass at any of the timepoints sampled. For biotransformations carried out over three days (replicate I), replacement of the headspace actually appeared to depress the yield on biomass. In two subsequent biotransformations, each extended over five days (replicates II and III), headspace replacement made no significant difference to the yield of 1-octanol on biomass. Cells stopped producing 1-octanol somewhere between the 73 h and 96 h timepoints, regardless of whether the headspace was replenished or not.

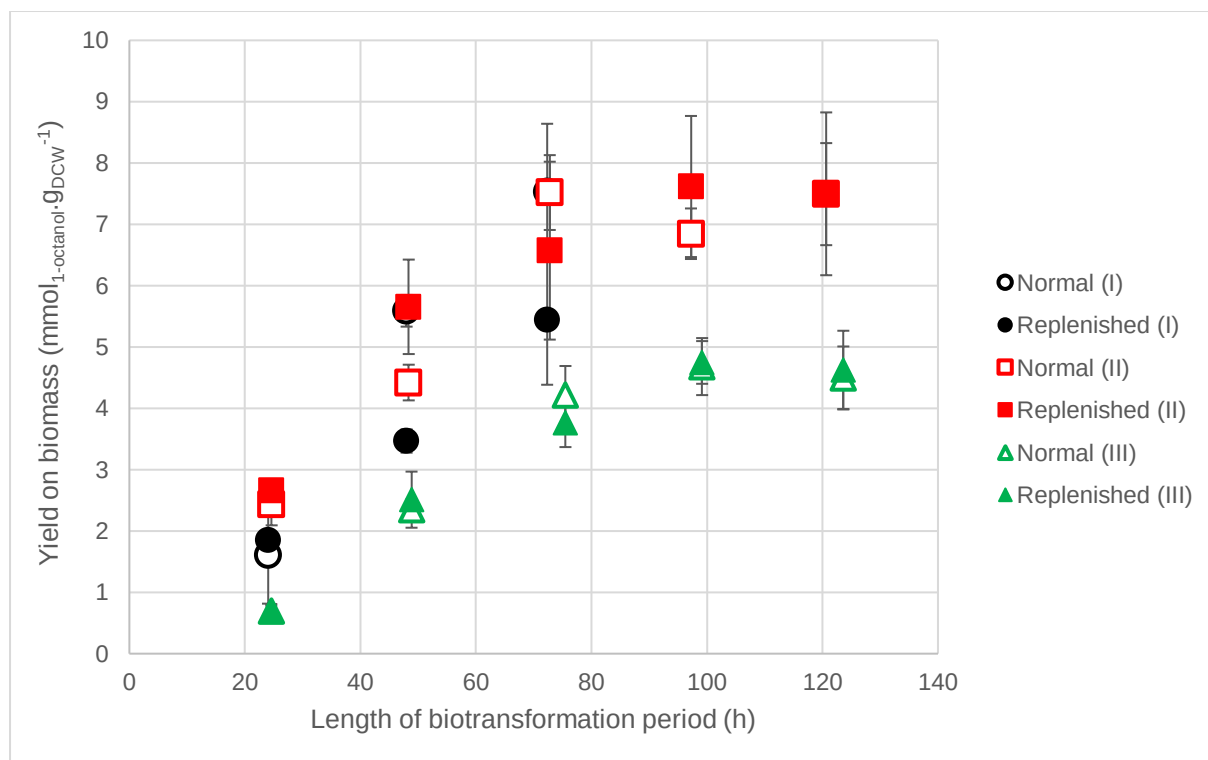


Figure 4.16: Three biological replicates, designated I – III, investigating the effects of headspace replenishment on the production of 1-octanol per gram of dry cell weight. 1 mL suspensions of whole cells in phosphate buffer (average cell density $4.72 \pm 0.06 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$) were added to 40 mL vials, along with 0.2 mL n-octane and 0.1 mL BEHP. Biotransformations were carried out for three to five days. Vials were sacrificed for sampling at each time point. Clear shapes represent results from vials in which the headspace was *not* replenished. Filled shapes represent results from vials for which the headspace was replaced every 24 h. Error bars represent the standard deviation over three sample replicates (i.e. three vials).

Limitations in oxygen supply could be expected to arise from two sources: a stoichiometric limitation due to oxygen depletion, or a kinetic limitation due to inadequate mass transfer. At the beginning of the biotransformation, the amount of oxygen in the headspace is approximately 8 mL (in a 40 mL vial) or 12 mL (in a 60 mL vial) – 0.333 mmol or 0.499 mmol respectively, at 20°C and atmospheric pressure. Based on reaction stoichiometry, where one molecule of oxygen is consumed per molecule of n-alkane oxidised, most biotransformations utilised less than 30 μmol ; the maximum utilisation of oxygen as biotransformation substrate was $48.4 \pm 5.3 \mu\text{mol}$. Therefore, the incorporation of oxygen into the substrate could not be resulting in stoichiometric oxygen limitation.

Like other enzymes in the cytochrome P450 family, CYP153A6 is vulnerable to uncoupling reactions, wherein either dioxygen molecules bound to the haem group are doubly protonated to form hydrogen peroxide (rather than cleaved to form water and a bound reactive intermediate) or the reduced form of the mediator protein donates electrons to the bound reactive intermediate, causing the oxygen atom to be converted into a water molecule (rather than inserted into the alkane substrate), as outlined in Sections 2.2.3 and 2.5.3. Since each uncoupling reaction consumes a molecule of dioxygen, uncoupling could potentially impose a significant oxygen demand on the culture. However, the coupling efficiency of CYP153A6 on octane has been reported to be 98% in the case of purified enzymes (Arnold et al., 2012); hence uncoupling is not expected to be a significant contributor to the oxygen demand.

Cellular maintenance could therefore be expected to account for most of the system's oxygen demand. Oxygen demand in *Escherichia coli* cultures can vary considerably, as outlined in Table 4.3.

Table 4.3: Metabolic oxygen demand estimated from values reported in literature for various aerobic cultures.

Source	<i>E. coli</i> strain	Metabolic state of cells in culture	Temperature (°C)	Oxygen demand (mL.g _{DCW} ⁻¹ .h ⁻¹)	Maximum time for which culture could be sustained in 40 mL vials, at either low or high cell density (h)	
					4.5 g _{DCW} .L ⁻¹	35 g _{DCW} .L ⁻¹
Carneiro De Melo et al. (1996)	NCIMB 9485	Resting cells (potassium phosphate buffer with glucose)	37	9	207	26
		Resting cells (potassium phosphate buffer with glucose and nisin)		25	66	8
Andersen and Meyenburg (1980)	B/r NF790	Growing cells (glucose-limited batch culture)	37	504	4	< 1
		Growing cells (glycerol-limited batch culture)		539	3	< 1
Rice and Hempfling (1978)	B	Resting cells (potassium phosphate buffer with succinate)	37	16	111	14
		Growing cells (oxygen-limited continuous culture with succinate)		87	20	3
(Farmer and Jones, 1976)	W	Growing cells (glycerol-limited continuous culture)	20	2	739	95
			30	14	123	16
			40	31	58	7
Mainzer and Hempfling (1976)	B	Growing cells (glucose-limited continuous culture)	17.5	42	42	5
			23.5	83	21	3
			30.0	105	17	2
			32.0	112	16	2
			34.5	58	31	4
			42.0	313	6	< 1
Hempfling and Mainzer (1975)	B	Growing cells (glucose-limited continuous culture)	37	23	78	10
		Growing cells (glycerol-limited continuous culture)		254	7	< 1
		Growing cells (succinate-limited continuous culture)		308	6	< 1

Carbon source has been shown to play a significant role in determining oxygen demand in continuously growing cultures: at 37°C cells metabolising glycerol were shown to consume eleven times as much oxygen for maintenance compared to cells metabolising glucose (Hempfling and Mainzer, 1975). However, it appears that this ratio is affected by the culture temperature as well as the identity of the bacterial strain: above 40°C *E. coli* B requires an order of magnitude more oxygen on glucose than *E. coli* W does on glycerol (Farmer and Jones, 1976; Mainzer and Hempfling, 1976). It is likely that temperature plays the stronger role, as it has been shown to cause maintenance oxygen demand to fluctuate from 0.9 mmol.g_{DCW}⁻¹.h⁻¹ at 37°C to 12.1 mmol.g_{DCW}⁻¹.h⁻¹ at 42°C during growth on glucose (Mainzer and Hempfling, 1976) or from 0.1 mmol.g_{DCW}⁻¹.h⁻¹ at 20°C to 1.2 mmol.g_{DCW}⁻¹.h⁻¹ at 40°C during growth on glycerol (Farmer and Jones, 1976). However, most of the oxygen demand in growing cultures is linked to biomass formation. The maintenance oxygen demand of resting cells is substantially lower than their growing counterparts, and a 40 mL vial should contain enough oxygen to sustain a low-density resting culture for several days. Cells exposed to high concentrations of hydrocarbons risk losing membrane integrity, which may increase maintenance oxygen demand; Carneiro De Melo et al.

(1996) observed a 22-fold increase in oxygen demand for resting *E. coli* treated with the bacteriocin nisin. On the other hand, cultures at low temperatures appear to have very low maintenance oxygen demands when glycerol is used as the carbon source. Given the significant variations outlined above, the oxygen requirements for cellular maintenance in the CYP153A6 system are not easy to estimate based on literature values. By comparing Carneiro De Melo et al. (1996) with Hempfling and Mainzer (1975) or Anderson and Meyerburg (1980), or by comparing the resting and growing cultures studied by Rice and Hempfling (1978), it could be assumed that resting cells require between 2.6 and 56 times less oxygen than growing cells; thus a maintenance oxygen demand of $2 \text{ mL.g}_{\text{DCW}}^{-1}.\text{h}^{-1}$ for glycerol-limited growth at 20°C (Farmer and Jones, 1976) would imply that resting cells at 20°C require less than $1 \text{ mL.g}_{\text{DCW}}^{-1}.\text{h}^{-1}$. If so, then 1 mL of resting culture with a cell density of $4.5 \text{ g}_{\text{DCW}}.\text{L}^{-1}$ could support cellular maintenance with only $4.5 \text{ }\mu\text{L}$ of oxygen per hour, or $108 \text{ }\mu\text{L}$ per day, or 0.5 mL per five days. At such a low rate of consumption, the oxygen available in a 40 mL vial would last for up to 75 days. Cellular maintenance could not be causing oxygen depletion under such conditions.

The experimental results confirm that oxygen depletion is not limiting biotransformations under the current system conditions. Biotransformations in 40 mL vials, in which the headspace of some vials was regularly replenished, did not show any improvements in 1-octanol production. Even after four days, cells were achieving comparable yields of 1-octanol on biomass regardless of whether the headspace was regularly replenished or not. These results are in line with previous work on cytochromes P450 in *E. coli*. In the production of 1-octanol from n-octane by CYP153A6, vials were opened every six to eight hours to allow circulation of air in the headspace, but this did not prevent 1-octanol production from levelling off (Olaofe, 2013). In the production of 2-octanol from n-octane over 24 h by CYP154A8, $50 \text{ g}_{\text{WCW}}/\text{L}$ was found to be the optimal biomass concentration for 10 mL cultures in 100 mL flasks (Tieves et al., 2016); at the same ratio of oxygen to biomass, the cultures used in this study would have required only 4 mL of headspace.

This leaves limitations in gas-liquid mass transfer. For aqueous-organic mixtures matching the volumes used in this study, oxygen transfer rates have been estimated at $77 \text{ mmol.L}^{-1}.\text{h}^{-1}$ (Meissner, 2013) for 60 mL vials with the same diameter as the 40 mL vials. This is two orders of magnitude above the range of space time yields observed in these experiments (see Figure 4.15), suggesting that oxygen mass transfer is unlikely to be the factor which limits these biotransformations.

Overall, literature suggests that 40 mL vials should provide oxygen in excess of the combined stoichiometric requirements of biotransformation and resting cell maintenance. The vials should also provide sufficiently high rates of oxygen transfer. This was confirmed by the experimental results, in which variations in the supply of oxygen had no significant effect on space time yields or yields on biomass. Oxygen limitation is not the primary factor limiting biotransformation, under the conditions tested.

4.5 Summary of investigations into P450 expression and oxygen limitations

CYP153A6 was expressed in *Escherichia coli*, along with its natural redox partner proteins, ferredoxin reductase (FdxR) and ferredoxin (Fdx). Expression of active CYP153A6 could be improved by adding aminolevulinic acid (a precursor in the haem formation pathway) and by fortifying the growth and expression medium with additional iron (in the form of ferric chloride). As per the results described in Section 4.1, if either of these chemicals was lacking, then expression of active CYP153A6 was poor. The best results were obtained by increasing the concentration of both chemicals in tandem. Increasing the concentration of δ -ALA was not particularly effective if FeCl_3 concentration was reduced from 50 to $10 \text{ }\mu\text{M}$, while increasing the FeCl_3 concentration from 100 to $500 \text{ }\mu\text{M}$ did not lead to substantially enhanced expression, even at the highest δ -ALA concentrations tested. A combination of $100 \text{ }\mu\text{M}$ FeCl_3 and 1 mM δ -ALA produced the highest intracellular concentration of active CYP153A6 ($1.53 \text{ }\mu\text{molP}_{450}.\text{g}_{\text{DCW}}^{-1}$), but arguably the most efficient combination was $100 \text{ }\mu\text{M}$ FeCl_3 and 0.6 mM δ -ALA, which achieved 85% of the maximum P450 concentration ($1.30 \text{ }\mu\text{molP}_{450}.\text{g}_{\text{DCW}}^{-1}$) with 40% less δ -ALA.

As Section 4.2 showed, the expression of additional heterologous proteins depressed the intracellular concentration of active CYP153A6 that could be achieved, both by decreasing the total quantity of enzyme that was expressed, and by increasing the percentage that was expressed in an inactive form. The expression of AlkL from the pCDFDuet plasmid was more detrimental to P450 concentrations than the expression of glycerol dehydrogenase from the same plasmid. Impact was most severe in cells expressing glycerol dehydrogenase and AlkL simultaneously, alongside the A6/FdR/Fdx. The addition of δ -ALA and FeCl_3 was only moderately beneficial in these systems: it was possible to increase the total amount of CYP153A6 being expressed, but this also led to increases in the amount of inactive haemoprotein detected.

However, CYP153A6 expression levels were found not to be a limiting factor in whole-cell n-octane bioconversions under the operating conditions used in this study, as demonstrated in Section 4.3. The same conclusion has been drawn regarding other P450 systems: CYP154A8 expression levels did not limit the conversion of n-octane to 2-octanol in *Pseudomonas putida* (Tieves et al., 2016), and intracellular concentration of CYP153A35 had no effect on the rate of hydroxylation of palmitic acid in *E. coli* (Jung et al., 2016).

It is clear that individual CYP153A6 enzymes are not performing as well in whole cells as they have been shown to do in *in vitro* studies. Other limitations on biocatalyst performance must therefore be identified and addressed. As discussed in Section 4.4, this study did not explicitly consider oxygen mass transfer, but oxygen depletion was investigated and rejected as a potential limitation. The work presented in Chapter 5 therefore investigated two other potential limiting factors: regeneration of NADH cofactor, and transfer of n-octane across the cell membrane.

5 Cofactor regeneration and hydrocarbon transport

As outlined in Section 2.5.3, several whole-cell biocatalyst systems have shown improved hydrocarbon conversion when cofactor supply was enhanced through artificial cofactor addition (Lundemo et al., 2016; Lundemo and Woodley, 2015). This suggests that the availability of cofactor can be a limiting factor in whole-cell biotransformation. However, increasing the rate of cofactor regeneration, via the heterologous expression of dehydrogenases, has yielded mixed results depending on the system in which it has been applied (Gudimich et al., 2011; Pennec et al., 2015; Schewe et al., 2008; Tieves et al., 2016).

On the other hand, as outlined in Section 2.5.2, the transport of hydrophobic substrates has emerged as a key limitation in many whole-cell biocatalytic systems (Alonso-Gutierrez et al., 2013; Bordeaux et al., 2012; Boyarskiy and Tullman-Ercek, 2015; Grant et al., 2014). The application of solubilising chemicals or membrane permeabilization techniques has often, but not always, improved biocatalyst performance (see Table 2.2 and Table 2.3 for examples and references).

It was hypothesised that cofactor supply and n-octane supply are both rate-controlling in the CYP153A6 resting cell system; hence the improvement of cofactor regeneration rates would not be beneficial unless transport limitations were overcome. To test this hypothesis, the capacity of resting *Escherichia coli* cells to regenerate cofactor was enhanced through the heterologous expression of glycerol dehydrogenase (GLD), combined with a supply of glycerol co-substrate in the biotransformation buffer. The effects of this enhanced cofactor regeneration capacity were tested in biotransformation systems that had undergone a range of interventions intended to facilitate n-octane transport:

- Intact cells
- Cells washed in acetone
- Cells exposed to a solubilising/permeabilising agent (Polymyxin B or Triton X-100)
- Cells ruptured under moderate pressure
- Cells disintegrated under high pressure
- Cell free extract
- Cells co-expressing of the membrane channel protein AlkL

The methods by which cells were prepared are described in detail in Section 3.3. The biocatalytic performance of the cells was assessed in resting-cell biotransformations which lasted between 24 h and five days.

Chapter 5 presents results from multiple investigations into cofactor regeneration and cross-membrane transport. Section 5.1 begins by comparing various chemical and physical permeabilization techniques, for cell suspensions with enhanced cofactor regeneration capacity. Section 5.2 considers the effects of glycerol dehydrogenase co-expression in intact cells, which includes both high-density and low-density suspensions. Section 5.3 deals with the effects of glycerol dehydrogenase in high-density suspensions of mechanically broken cells and cell free extract. Section 5.4 deals with the effects of glycerol dehydrogenase in chemically permeabilised cells, at both high and low culture densities. Section 5.5 discusses points of interest that can be drawn from a comparison of all the chemically and mechanically permeabilised cultures tested. Finally, Section 5.7 considers the application of the membrane channel protein AlkL in the resting *E. coli* system.

This chapter has been organised thematically, which sometimes involves the comparison and discussion of results from multiple separate experiments. The chronological progression of experiments is summarised in Table 5.1 (see page 85) and described in detail below.

Initially, a variety of chemical and physical membrane permeabilization treatments were compared to one another in resting cells co-expressing the CYP operon (CYP153A6 and its redox partners, FdR and Fdx) together with GLD, as well as in co-cultures of cells expressing A6/FdR/Fdx and cells expressing GLD. For disintegrated cells, co-cultures with or without GLD were also compared. Results from these

experiments are presented and discussed in Section 5.1. The results suggested that limitations in substrate transport and cofactor regeneration were interacting, not only with one another but also with the availability of CYP153A6.

A better understanding was needed regarding the efficacy of dehydrogenase co-expression. Suspensions of resting cells expressing A6/FdR/Fdx from the pET28b+ vector and co-expressing GLD from the pACYCDuet vector were compared to cells expressing A6/FdR/Fdx but carrying the empty pACYCDuet vector. This was done for intact cells, cells washed with acetone, ruptured cells, and cell free extract (CFE). Further biotransformations, with resting cells co-expressing A6/FdR/Fdx and GLD, were also carried out with cells in the presence of Triton X-100, and with disintegrated cells. These results are discussed in Sections 5.2.1, 5.3, and 5.4.1. The combination of glycerol dehydrogenase co-expression and mechanical cell breakage was found to be particularly effective, despite the fact that mechanical breakage and dehydrogenase co-expression were each found to be detrimental when applied alone. In contrast, the application of chemical permeabilizers was somewhat effective when applied alone, but this effectiveness was reduced when combined with glycerol dehydrogenase co-expression.

Octyl acetate by-product had been detected across many early experiments. As will be discussed in Section 5.5.3, this acetylation of product was believed to be an artefact of the pACYCDuet plasmid. Thus, new biocatalytic strains were created by transforming cells with the A6/FdR/Fdx operon on pET28b+, alongside either the empty pCDFDuet vector, or pCDFDuet containing genes for glycerol dehydrogenase. The new strains were applied, at a five-fold lower cell density than the experiments described above, either as intact cells, or in the presence of Polymyxin B or Triton X-100.

Section 5.2 compares intact cells at low density to intact cells from earlier high-density experiments. It was found that individual cells displayed similar rates of product formation during the first 12 – 24 h of the biotransformation, regardless of culture density and plasmid type. However, the low-density preparations were stable up to 72 h, while cells in high-density preparations had lost most or all of their activity after 24 h, although they had already reached higher volumetric titres by that point than low-density preparations would reach after 72 h.

Since use of the pCDFDuet plasmid did not appear to either boost or impair the performance of CYP153A6 in individual cells, and since no octyl acetate formation was detected when pCDFDuet was used instead of pACYCDuet, further experiments were carried out with the pCDFDuet strains. Low-density preparations were also deemed better candidates for further investigations into cofactor and transport, not only because of their enhanced stability, but also because the possibility of oxygen limitations was investigated and discounted at these densities (see Section 4.4).

Using low-density cultures, intact cells were again compared to cells exposed to Polymyxin B or Triton X-100. These comparisons were done for strains expressing A6/FdR/Fdx on pET28b+ and carrying empty pCDFDuet, as well as strains expressing A6/FdR/Fdx on pET28b+ and co-expressing GLD on pCDFDuet. Results are presented and discussed in Section 5.4.2.

Sections 5.5 and 5.6 discuss and summarise overall trends from across all the chemically and mechanically permeabilised systems tested.

Finally, another means of transport enhancement was explored: co-expression of the membrane channel protein AlkL from *Pseudomonas putida*, a passive transporter that evolved to target the import of alkanes and other hydrocarbons. Biotransformations were carried out with intact cells co-expressing A6/FdR/Fdx on pET28b+, with pCDFDuet carrying AlkL, glycerol dehydrogenase, both, or neither. These results are presented and discussed in Section 5.7.

Table 5.1: Experiments investigating the interactions between cofactor regeneration and cross-membrane transport, and their impact on biocatalyst performance. Experiments are listed in chronological order (as indicated by Exp. No.) and linked to sections of Chapter 5 which draw on the data from these experiments. A star (*) indicates experiments performed at a low cell density.

Exp. No.	Strain(s) being compared	Permeabilization technique(s) being compared	Relevant section(s)
1	Cells co-expressing A6/FdR/Fdx on pET28b+ and GLD on pACYCDuet	Intact cells Acetone-washed cells Cells exposed to Polymyxin B Ruptured cells Disintegrated cells Cell free extract	5.1.1
2	Cells expressing A6/FdR/Fdx on pET28b+ co-cultured with cells expressing GLD on pACYCDuet	Intact cells Acetone-washed cells Cells exposed to Polymyxin B Ruptured cells Disintegrated cells Cell free extract	5.1.1
	Cells expressing A6/FdR/Fdx on pET28b+ co-cultured with cells expressing GLD on pACYCDuet Cells expressing A6/FdR/Fdx from pET28b+ co-cultured with cells expressing empty pACYCDuet	Disintegrated cells	5.1.2
3	Cells co-expressing A6/FdR/Fdx on pET28b+ and GLD on pACYCDuet Cells co-expressing A6/FdR/Fdx on pET28b+ and empty pACYCDuet	Intact cells Acetone-washed cells	5.2.1 5.4.1
	Cells co-expressing A6/FdR/Fdx on pET28b+ and GLD on pACYCDuet	Cells exposed to Triton X-100	5.4.1
4	Cells co-expressing A6/FdR/Fdx on pET28b+ and GLD on pACYCDuet Cells co-expressing A6/FdR/Fdx on pET28b+ and empty pACYCDuet	Ruptured cells CFE	5.3
	Cells co-expressing A6/FdR/Fdx on pET28b+ and GLD on pACYCDuet	Disintegrated cells	5.3
5*	Cells co-expressing A6/FdR/Fdx on pET28b+ and GLD on pCDFDuet Cells co-expressing A6/FdR/Fdx on pET28b+ and empty pCDFDuet	Intact cells Cells exposed to Polymyxin B Cells exposed to Triton X-100	5.2.2 5.4.2
6*	Cells co-expressing A6/FdR/Fdx on pET28b+ and empty pCDFDuet Cells co-expressing A6/FdR/Fdx on pET28b+ and GLD on pCDFDuet Cells co-expressing A6/FdR/Fdx on pET28b+ and AlkL on pCDFDuet Cells co-expressing A6/FdR/Fdx on pET28b+ and AlkL and GLD on pCDFDuet	Intact cells	5.7

5.1 Effects of membrane permeabilization in cell suspensions with additional glycerol dehydrogenase

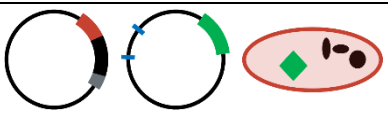
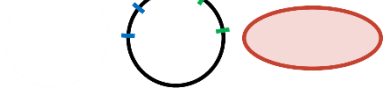
As discussed in Section 2.5.2, the cell envelope of *Escherichia coli* represents a substantial barrier to the import and export of hydrocarbons. In an attempt to overcome this barrier, this study exposed cells to a number of permeabilization treatments.

Chemical treatments included washing cells with 5% (v/v) acetone prior to resuspension in biotransformation buffer; such treatment disrupts the structure and dynamics of the lipid bilayer (Posokhov and Kyrychenko, 2013). Alternatively, Polymyxin B was added to the biotransformation buffer at a concentration of 30 μ M. Polymyxin B is an antibiotic which interferes with the structure of the outer membrane and promotes cell lysis (Lounatmaa and Nanninga, 1976; Ruff et al., 2016).

Physical breakage of the cell envelope was achieved via passage through a one-shot cell disruptor. Cells were treated at either 15 kpsi, in order to rupture the cell envelope; or at 30 kpsi, in order to disintegrate the cell. The soluble fraction of disintegrated cells was also applied separately, as cell free extract.

Initial experiments were carried out with high-density suspensions of resting cells. The amount of glycerol dehydrogenase (GLD) in the cultures had been enhanced via heterologous expression. Glycerol dehydrogenase boosts the cells' capacity to regenerate the NADH cofactor necessary for biotransformation. The dehydrogenase was either co-expressed alongside the CYP153A6, FdR and Fdx in a single strain, or the A6/FdR/Fdx and GLD were expressed in separate strains, which were then co-cultured for biotransformation.

Strains used in this section of work:

<i>E. coli</i> BL21(DE3) containing pET(CYP) and pACYC(GLD)	
<i>E. coli</i> BL21(DE3) containing pET(CYP)	
<i>E. coli</i> BL21(DE3) containing pACYC(GLD)	
<i>E. coli</i> BL21(DE3) containing pACYC	

5.1.1 Combining A6/FdR/Fdx and GLD: co-expression versus co-culturing

Initially, biotransformations were carried out using *Escherichia coli* co-expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid (A6/FdR/Fdx) alongside glycerol dehydrogenase from the pACYCDuet plasmid (GLD). Some of these cells were left intact; others were permeabilised by acetone washing or the addition of Polymyxin B, or by physical breakage, as described above. Cells converted n-octane into 1-octanol as well as some octyl acetate by-product. The performance of intact and permeabilised cells is compared in Figure 5.1.

A modified version of the above experiment was performed, in which *E. coli* expressing only A6/FdR/Fdx were co-cultured with *E. coli* expressing only GLD. Cells were treated using the same permeabilization techniques as before. The results are shown in Figure 5.2, and the two experiments are compared in Table 5.2.

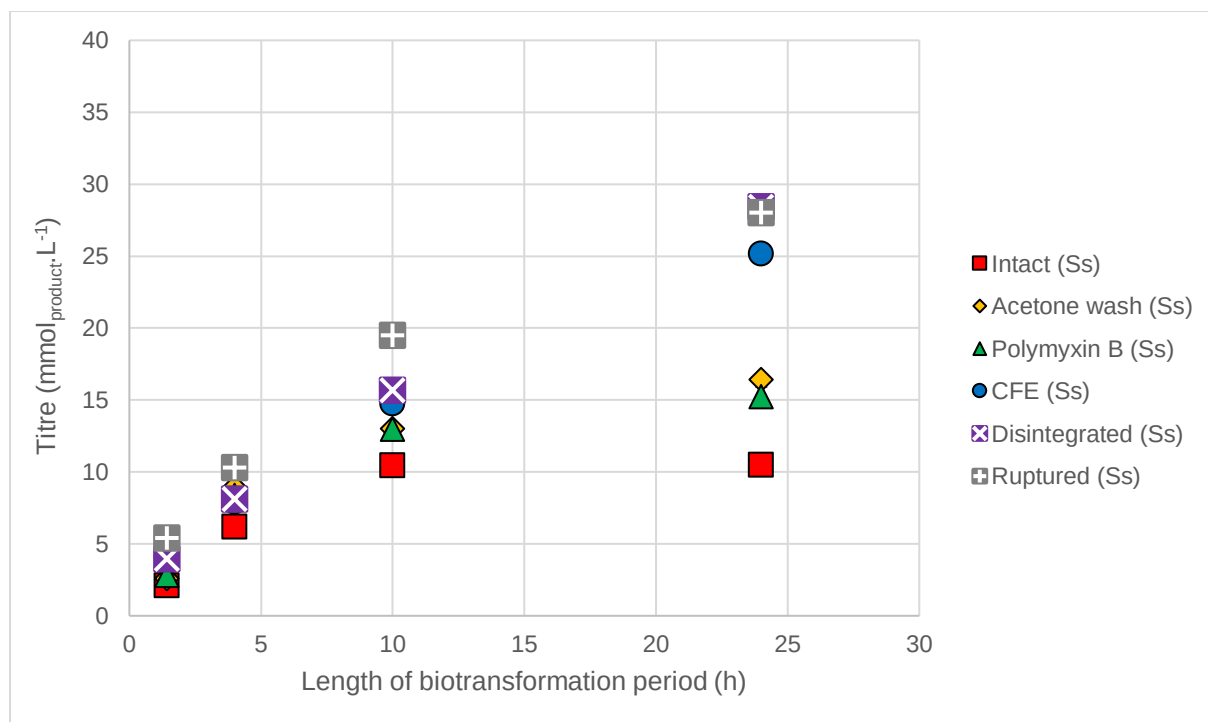


Figure 5.1: Effect of different membrane permeabilization techniques on the biotransformation of n-octane by *E. coli* expressing A6/FdR/Fdx on pET28b+ and co-expressing glycerol dehydrogenase on pACYCDuet. The biotransformation mixture consisted of 1.2 mL resting cells suspended in potassium phosphate buffer (average cell density 22.6 ± 4.5 g_{DCW}.L⁻¹) and 200 μ L n-octane. The various permeabilization techniques are described in detail in Section 3.3. Biotransformations were carried out in 40 mL amber vials. Two vials were sacrificed for sampling at each time point; error bars represent the standard deviation between the two vials.

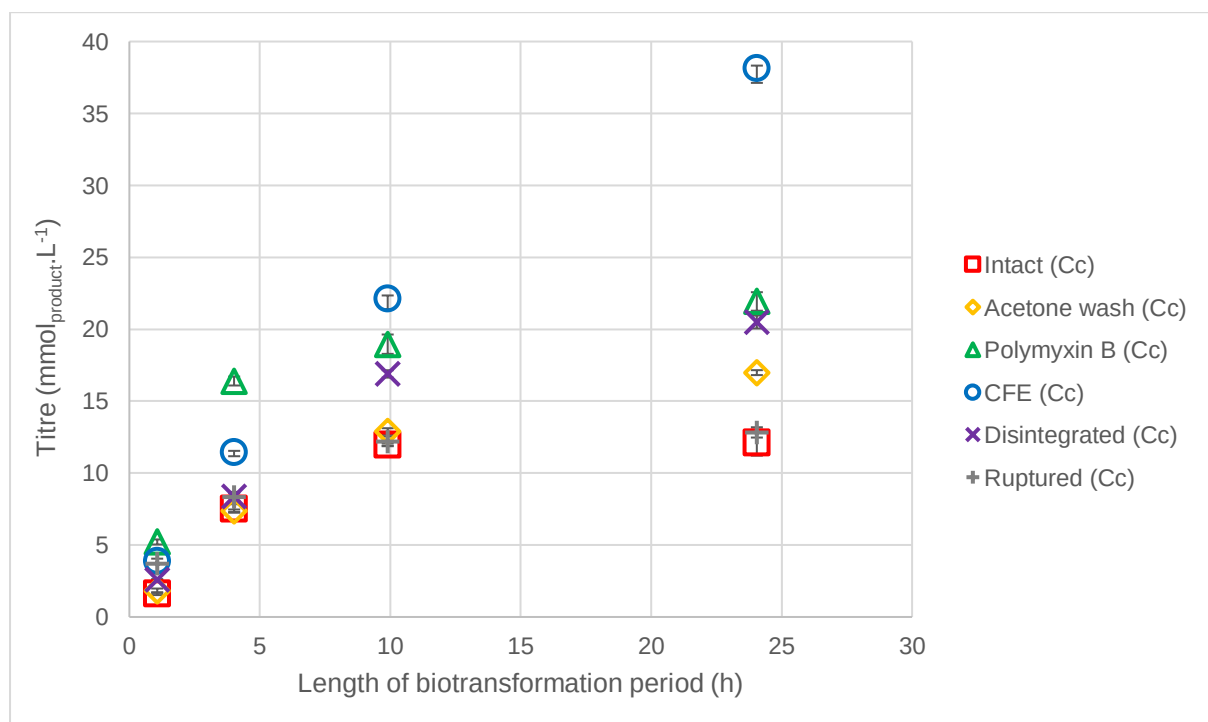


Figure 5.2: The experiment described in Figure 5.1 was repeated, but this time the cell preparation consisted of a 40:20 mix of *E. coli* expressing CYP153A6, Fdx, and FdR on pET28b+ and *E. coli* expressing glycerol dehydrogenase on pACYCDuet.

Table 5.2: Whole-cell biotransformation of n-octane by CYP153A6 and its redox partners, with the availability of glycerol dehydrogenase increased via either co-expression or co-culturing.

Permeabilization treatment	Maximum titre ^a (mmol _{product} .L ⁻¹)	Maximum yield on biomass (mmol _{product} .g _{DCW} ⁻¹)	Maximum volumetric P450 concentration ^b (μmol.L ⁻¹)	Estimated intracellular P450 concentration ^c (μmol.g _{DCW} ⁻¹)
Intact	10.49 ± 0.14	0.43 ± 0.02	7.42 ± 0.20	0.30 ± 0.02
Intact (co-culture)	12.12 ± 0.90	0.55 ± 0.04	5.59 ± 0.53	0.63 ± 0.06
Acetone wash	16.42 ± 0.30	0.63 ± 0.01	5.75 ± 0.30	0.22 ± 0.01
Acetone wash (co-culture)	16.99 ± 0.17	0.83 ± 0.05	4.85 ± 0.11	0.59 ± 0.04
Polymyxin B	15.24 ± 0.66	0.58 ± 0.03	7.29 ± 0.96	0.27 ± 0.04
Polymyxin B (co-culture)	21.93 ± 0.64	0.82 ± 0.06	5.37 ± 0.43	0.50 ± 0.05
CFE	25.16 ± 0.13	1.11 ± 0.21	2.67 ± 0.27	-
CFE (co-culture)	38.16 ± 1.03	1.84 ± 0.36	3.56 ± 0.18	-
Disintegrated	28.41 ± 0.24	1.68 ± 0.06	3.26 ± 0.00	0.20 ± 0.01
Disintegrated (co-culture)	20.51 ± 0.45	1.24 ± 0.10	4.06 ± 0.17	0.61 ± 0.05
Ruptured	28.03 ± 0.78	1.46 ± 0.06	4.86 ± 0.34	0.27 ± 0.03
Ruptured (co-culture)	12.83 ± 0.36	0.71 ± 0.06	4.73 ± 0.23	0.66 ± 0.06

- (a) Maximum titres and yields on biomass were calculated based on the 24 h sample point.
- (b) 'P450' refers to the active form of CYP153A6. Maximum P450 concentrations were most frequently observed at the 4 h sample point. Variation between maximum and minimum concentrations was usually less than 1.5 μM.
- (c) Since co-cultures were a combination of catalytically active and inactive cells, dividing volumetric concentration by culture density would not give the true intracellular P450 concentration in the active cells. Intracellular P450 concentrations in co-cultures were estimated based on the ratio of catalytically active and inactive cells in the biotransformation preparation.

In the case of co-expression, intact cells maintained their production of 1-octanol for only 10 h, producing a final titre of 10.5 ± 0.4 mmol_{product}.L⁻¹ and a final yield on biomass of 0.420 ± 0.026 mmol_{product}.g_{DCW}⁻¹. Chemical permeabilization did not substantially alter the course of reaction, but initial product formation rates did increase slightly. Also, biotransformation did not cease between 10 h and 24 h, although it slowed substantially. These minor improvements in performance resulted in final titres of 16.4 ± 0.3 mmol.L⁻¹ (0.629 ± 0.011 mmol.g_{DCW}⁻¹) for acetone washing, and 15.2 ± 0.7 mmol.L⁻¹ (0.577 ± 0.031 mmol.g_{DCW}⁻¹) for Polymyxin B addition. Physical breakage of the membrane substantially improved biocatalytic performance relative to intact cells. After 24 h, ruptured cells produced 28.0 ± 0.8 mmol.L⁻¹ (1.46 ± 0.06 mmol.g_{DCW}⁻¹), and disintegrated cells produced 28.4 ± 0.2 mmol.L⁻¹ (1.68 ± 0.06 mmol.g_{DCW}⁻¹). Cell free extract underperformed relative to suspensions of broken cells, but still outperformed intact cells, producing 25.2 ± 0.1 mmol.L⁻¹ after 24 h. These results confirm that cross-membrane transport is a substantial limitation in the whole-cell CYP153A6 system.

In the case of intact or chemically permeabilised cells, co-culturing produced similar results to co-expression. Intact cells were again the worst performers overall, with biotransformation ceasing entirely after 10 h. Washing cells with acetone or exposing them to Polymyxin B improved their performance relative to intact cells, with Polymyxin B having a particularly substantial effect on the initial rate. However, rates of product formation by chemically permeabilised cells began to decrease from around the 4 h time point, just as they had done in the co-expressing cells. Cell free extract was even more effective than it had been in the co-expression experiment, outperforming disintegrated or ruptured cells, which underperformed substantially relative to their co-expressing counterparts. Disintegrated cells still outperformed intact and acetone-washed cells when co-cultured, but where co-expressing cells had been stable for up to 24 h when disintegrated, co-cultured cells showed a substantial decrease in product formation rates between 10 h and 24 h. Ruptured cells performed no better than intact cells when applied as co-cultures.

These results can best be understood by considering intracellular P450 concentrations. Less than half of the cells in co-cultures were expressing A6/FR/Fdx. Thus, similar titres per unit volume or per unit *total* cells implies that product formation per unit of *catalytically active* cells was more than twice as high in co-cultures as it was in co-expressing cells. Volumetric concentrations of active CYP153A6 were similar between the two experiments, suggesting that intracellular enzyme concentrations were much lower in cells co-expressing GLD, which would fit with the results described in Section 4.2. This would suggest that, in the case of intact cells, the poor performance of co-expressing cells relative to co-cultures was linked to limitations in intracellular P450 concentration. Intracellular P450 concentrations in co-expressing cells were between 0.2 and 0.3 $\mu\text{mol.g}_{\text{DCW}}^{-1}$, which is at the lower end of the concentration range discussed in Section 4.3. However, all intracellular P450 concentrations were above 0.1 $\mu\text{mol.g}_{\text{DCW}}^{-1}$ (the expected limiting point for intact cells, based on results presented by Olaofe, 2012). Nevertheless, it is possible that the enhanced availability of substrate or cofactor led to enzyme availability becoming the limiting factor once cells were permeabilised.

Results obtained with cell free extracts also fit this pattern of enzyme limitation, since a 1.2-fold increase in volumetric P450 concentration was linked to a 1.5-fold increase in product formation. The trend was somewhat repressed in ruptured and disintegrated cells: co-cultures underperformed *overall* relative to their co-expressing counterparts, but each unit of catalytically active cells would still have been 10 – 80 % more productive than its co-expressing counterpart. This could be because additional glycerol dehydrogenase is less useful when it is not in close proximity to the A6/FdR/Fdx.

Overall, the comparison of co-expression and co-culturing suggests that the availability of cofactor and substrate affects the efficiency of CYP153A6, which in turn affects the concentration at which enzyme availability becomes a limiting factor.

5.1.2 Enhanced cofactor regeneration in preparations of disintegrated cells

Disintegrated cells expressing A6/FdR/Fdx were co-cultured with disintegrated cells carrying the empty pACYCDuet vector instead of GLD. As shown in Figure 5.3 (left axis, purple points), product titres from this co-culture were substantially reduced compared to co-cultures containing both A6/FdR/Fdx and GLD. Yields on biomass were also noticeably higher in the GLD-containing co-culture (Figure 5.3, right axis, green points).

The underperformance of co-cultures expressing empty vector was not due to the concentration of active CYP153A6, which was actually slightly higher than it was in the GLD-containing co-cultures.

These results suggest that enhanced expression of glycerol dehydrogenase can benefit the CYP153A6 system, even under conditions of co-culturing which reduce the proximity of GLD to the biocatalytic enzymes.

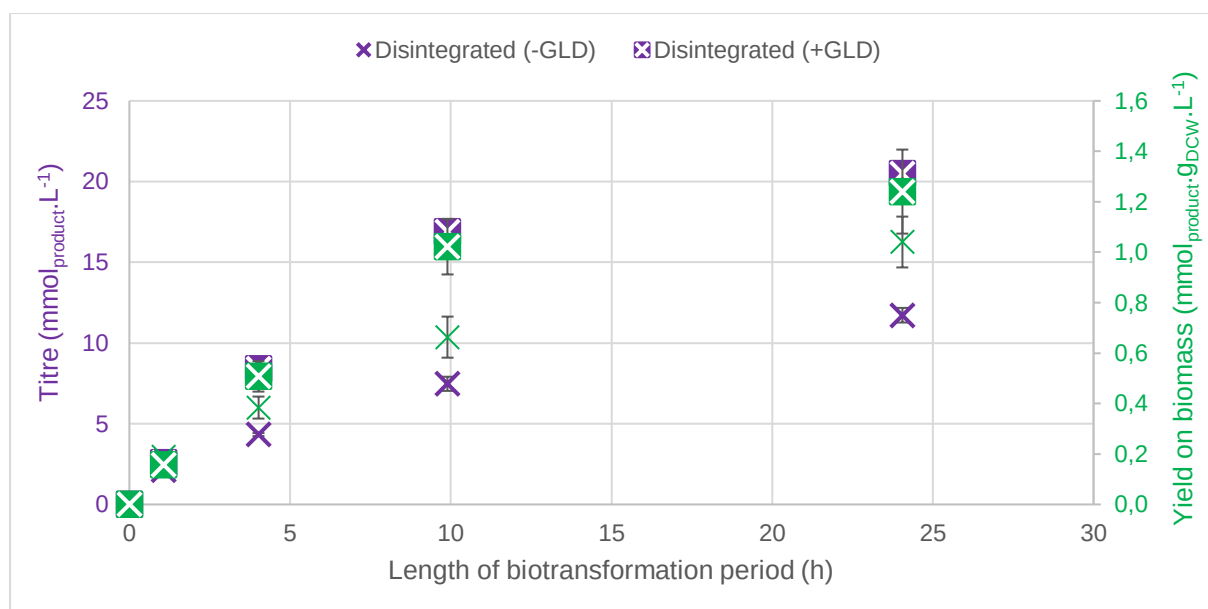


Figure 5.3: Effect of glycerol dehydrogenase on the titre and yield on biomass obtained by suspensions of disintegrated *E. coli* cells. Roughly 40% of cells in both suspensions were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. In '-GLD' suspensions (crosses), the remaining 60% of cells were 'blanks' carrying the empty pACYC vector. In '+GLD' suspensions (boxes), a further 40% of cells were expressing glycerol dehydrogenase from the pACYCDuet vector, with cells carrying empty pACYCDuet making up the remainder. Biotransformations were carried out in 40 mL amber vials, with 1 mL of cell suspension and 200 μ L of n-octane. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation between two vials. Purple data points represent volumetric titres at each time point; green data points represent yield on biomass at each timepoint.

Taken together with the results of Section 5.1.1, the experiments suggest a possible pattern of interacting limitations in enzyme, substrate, and cofactor:

Intact cells were limited by low intracellular concentrations of active CYP153A6. Individual cells therefore performed better in co-cultures, because intracellular P450 concentrations were higher. However, intact cells were also limited by the cross-membrane transport of n-octane, which is why they were the worst performers in both experiments.

Chemical permeabilization improved performance relative to intact cells, by improving the transport of n-octane to the biocatalytic enzyme. Under these circumstances, limitations in active CYP153A6 became more prominent, which is why chemically permeabilised cells performed better in co-cultures.

Cell free extracts no longer experienced significant limitations in accessing n-octane or NADH. Their performance therefore became limited only by the concentration of biocatalytic enzyme, which was higher when the A6/FdR/Fdx and GLD came from separate growth and expression cultures.

Rupturing or disintegrating the cell envelope overcame transport limitations, which led mechanically broken cells to be the best performers in the co-expression experiment. However, the availability of GLD played a key role in the performance co-cultures of disintegrated cells, so it may have been key to the performance of ruptured cells as well. Co-expressing cells may have derived greater benefits from GLD, since the biocatalytic enzymes and the cofactor regeneration enzymes would have been in closer proximity.

Mechanical breakage was somewhat less effective than chemical permeabilization in co-cultures, but considerably more effective when A6/FdR/Fdx and GLD were co-expressed in the same strain. This suggests that chemically permeabilised cells were not benefiting from additional cofactor regeneration capacity to the same extent as mechanically permeabilised cells. This may be because chemical permeabilizers partition into the cell membrane, altering its structure, while the mechanical pressure breaks the cell envelope apart. The latter may cause greater disruption to cell metabolic processes, thus creating a greater need for the cofactor regeneration capacity of GLD.

5.2 Effects of glycerol dehydrogenase co-expression in intact cells

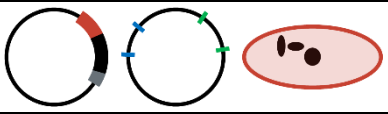
Initial experiments, as described in the previous section, showed that the interactions between substrate supply and cofactor regeneration needed to be better understood. The experiments presented in this section compared two strains of *Escherichia coli*, both of which were expressing CYP153A6, FdR and Fdx from pET28b+ (A6/FdR/Fdx); one strain carried an empty Duet vector, while the other strain was co-expressing glycerol dehydrogenase from the Duet vector (GLD).

The effects of glycerol dehydrogenase co-expression were initially investigated using high-density suspensions of intact cells. Cells were resuspended in biotransformation buffer to a wet cell weight of approximately 200 g_{WCW}.L⁻¹, resulting in biomass concentrations of 26.3 ± 1.7 g_{DCW}.L⁻¹ for cells without additional dehydrogenase, and 37.9 ± 2.8 g_{DCW}.L⁻¹ for cells with additional dehydrogenase. As will be discussed in this section, cells co-expressing GLD experienced sharp productivity losses between the 10 h and 24 h sampling points. This rapid decrease in product formation was not preceded by a substantial loss of active CYP153A6; nor did it indicate an absolute toxicity threshold, since cells without additional dehydrogenase continued to accumulate product to higher concentrations. This suggested that stability of the overall whole-cell system was negatively affected either by increased glycerol dehydrogenase or by increased cell density.

Additional biotransformations were carried out in low-density cells suspensions: 4.31 ± 0.73 g_{DCW}.L⁻¹ for cells without additional dehydrogenase, and 4.36 ± 1.02 g_{DCW}.L⁻¹ for cells with additional dehydrogenase. Oxygen limitations had been discounted at these biomass loadings (see Section 4.4). The plasmid carrying genes for glycerol dehydrogenase was also changed, from pACYCDuet in the high-density experiments, to pCDFDuet in the low-density experiments. The two plasmids have copy numbers within the same order of magnitude, but pCDFDuet does not present the same degree of by-product formation (for reasons that are discussed in detail in Section 5.5.3). At low cell densities, the co-expression of glycerol dehydrogenase did not have a substantial effect on either product titres or biocatalyst stability.

5.2.1 Effects of glycerol dehydrogenase at high cell density

Strains used in this section of work:

<i>E. coli</i> BL21(DE3) containing pET(CYP) and pACYC	
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pACYC(GLD)	

Biotransformations were carried out for up to 47 h with intact cells. The time course of these reactions is shown in Figure 5.4, as volumetric titres and as yields on biomass. Intact cells *without* GLD produced a maximum titre of 29.8 ± 0.4 mmol_{product}.L⁻¹ after 47 h, and a yield on biomass of 1.08 ± 0.03 mmol_{product}.g_{DCW}⁻¹. Cells demonstrated an initial product formation rate of 1.64 ± 0.17 mmol.L⁻¹.h⁻¹ over the first two hours of biotransformation. Production was slower between 4 h and 10 h (1.30 mmol.L⁻¹.h⁻¹ on average). The decreased rate became more noticeable between 10 h and 23 h (0.72 mmol.L⁻¹.h⁻¹) and slowed even further between 23 h and 47 h (0.23 mmol.L⁻¹.h⁻¹).

The co-expression of GLD was detrimental to the performance of intact cells. Overall product formation was roughly halved, with maximum titre decreasing to 17.7 ± 0.4 mmol.L⁻¹, and yield on biomass decreasing to 0.496 ± 0.030 mmol.g_{DCW}⁻¹. The co-expression of GLD did not affect the activity of the whole-cell biocatalyst during the early stages of the biotransformation: average product formation rates were 1.71 ± 0.47 mmol.L⁻¹.h⁻¹ over the first two hours and 1.45 mmol.L⁻¹.h⁻¹ between 4 h and 10 h. However, the presence of GLD reduced biocatalyst stability: the reaction levelled off more sharply between 10 h and 23 h (0.18 mmol.L⁻¹.h⁻¹), and no additional product was formed after 23 h.

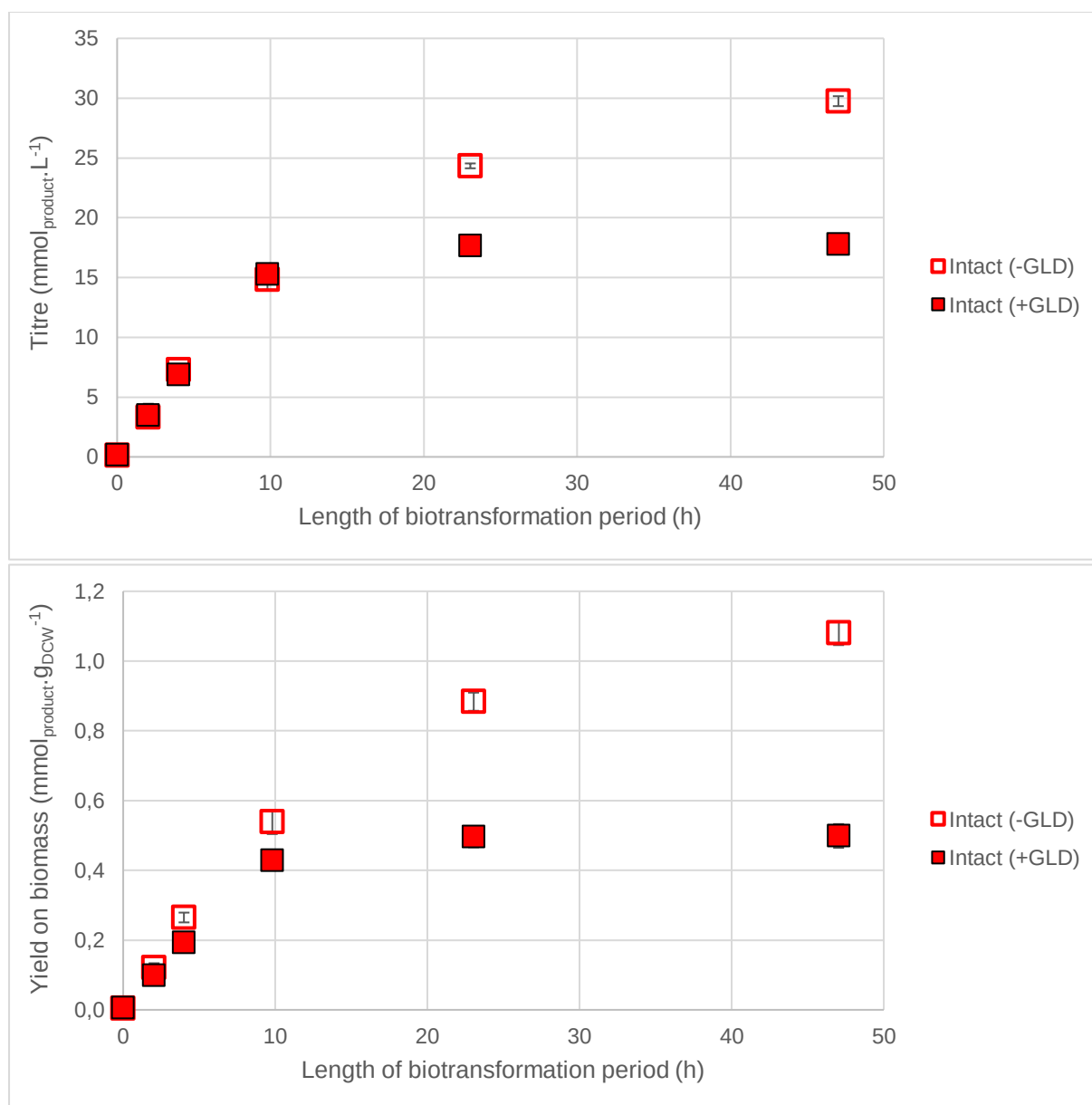


Figure 5.4: Effect of glycerol dehydrogenase co-expression on biotransformation of n-octane by high-density suspensions of intact cells. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. In '-GLD' suspensions, cells were carrying the empty pACYCDuet plasmid. In '+GLD' suspensions, cells were co-expressing glycerol dehydrogenase from the pACYCDuet plasmid. Biotransformations were carried out in 40 mL amber vials, with 1.2 mL of cell suspension and 200 μ L of n-octane. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation between two vials.

Figure 5.5 summarises key performance indicators for intact cells. The co-expression of GLD had little impact on the initial reaction rate, maximum space time yield, or maximum turnover frequency, but substantially reduced the maximum titre and yield on biomass, and reduced the time for which biotransformation could be maintained. In other words, biocatalyst activity did not improve when cofactor regeneration was enhanced, and biocatalyst stability was negatively affected.

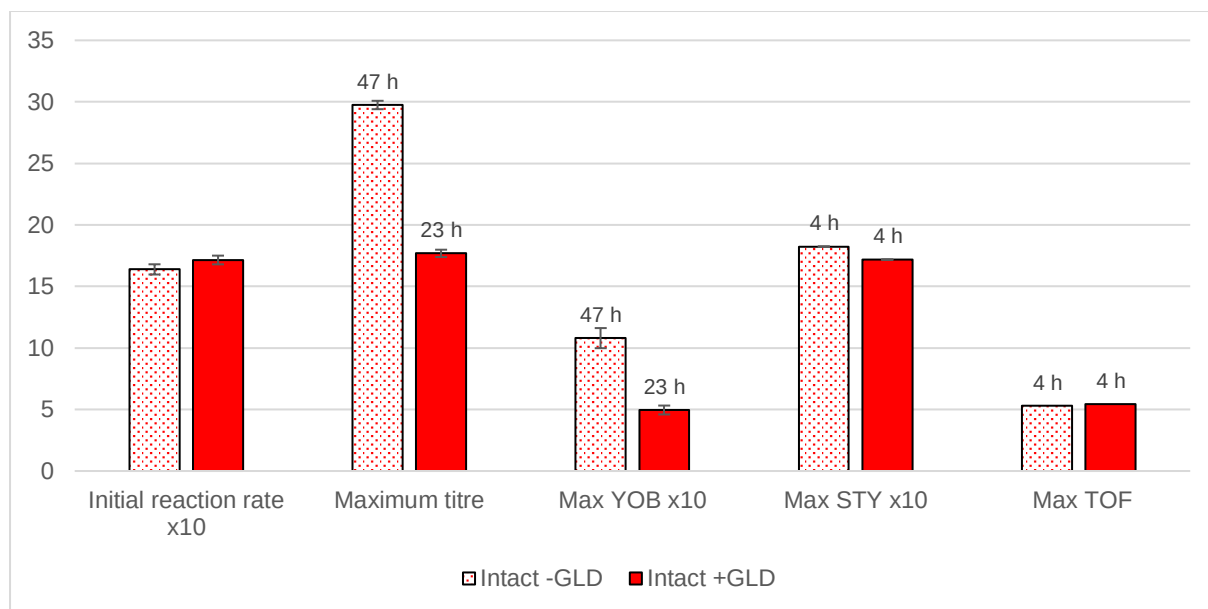


Figure 5.5: Key performance indicators for biotransformation systems consisting of high-density suspensions of intact cells. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. In '-GLD' suspensions, cells were carrying the empty pACYC vector. In '+GLD' suspensions, cells were co-expressing glycerol dehydrogenase from the pACYCDuet plasmid. Biotransformations were carried out in 40 mL amber vials, with 1 mL of cell suspension and 200 μ L of n-octane. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation between two vials. Units are as follows: Initial reaction rate: $\text{mmol.L}^{-1}.\text{h}^{-1}$, Maximum titre: mmol.L^{-1} , Maximum YOB (yield on biomass): $\text{mmol.g}_{\text{DCW}}^{-1}$, Maximum STY (space time yield): $\text{mmol.L}^{-1}.\text{h}^{-1}$, Maximum TOF (turnover frequency): $\text{mol}_{\text{product}}.\text{mol}_{\text{P450}}^{-1}.\text{min}^{-1}$. Labels above data bars indicate the time point at which the various maxima were observed.

Results with intact cells are in line with several other studies on P450 systems. Gudimanchi et al. (2011) found that productivity per cell over 4 h increased by approximately a factor of three when additional glycerol dehydrogenase was expressed in cells induced by IPTG, but found no significant increase for cells induced by lactose; the different induction methods are known to have a profound effect on the starting cofactor pool, which could mean that additional regenerative capacity is helpful in one case but not the other. For the cultures in this study, lactose was used for induction, meaning that the initial cofactor pool may not have required the assistance that additional dehydrogenase could provide. However, dehydrogenase was also seen to have substantial negative effects on longer-term performance of high-density cultures of whole cells. Parallels can be drawn to Tieves et al. (2016), where the co-expression of glucose dehydrogenase improved the initial rate of n-octane conversion to 2-octanol by CYP154A8, but overall, the productivity did not increase. Pennec et al. (2015) also found dehydrogenase co-expression to be an ineffective strategy once biotransformation times were extended.

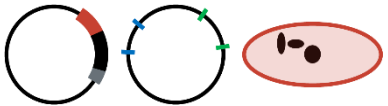
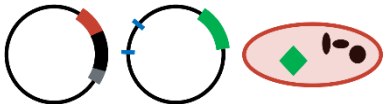
Metabolic burden is a potential explanation for the inability of the cells to capitalise on enhanced cofactor regeneration for biotransformation. The detrimental effects of diverting cellular energy carriers and raw materials towards heterologous protein production are well-established (Glick, 1995). Metabolic burden increases with increasing plasmid size and copy number (Cserjan-Puschmann et al., 1999; Rozkov et al., 2004). Both pET28b+ and pACYCDuet are fairly large vectors, and pET28b+ has a high copy number. Expression of the additional proteins imposes a more severe burden than merely maintaining the plasmids (Rozkov et al., 2004), which could explain why cells producing the glycerol dehydrogenase performed more poorly relative to control cells carrying the empty pACYCDuet vector.

However, metabolic burden alone cannot explain the decreased productivities, which are at odds with several studies that successfully used the co-expression of dehydrogenases to improve production. For example, Kataoka et al. (1999) doubled the rate of reduction of ethyl 4-chloro-3-oxobutanoate by aldehyde reductase through co-expression of glucose dehydrogenase. Mouri et al. (2006) reported ten-fold increases in camphor conversion when a cytochrome P450cam system was made to co-express glycerol dehydrogenase. Schewe et al. (2008) improved cell-specific production of α -pinene oxidase by CYP102A1-QM through co-expression of glucose dehydrogenase and a glucose

transport facilitator protein. Clearly, the benefits of enhanced cofactor regeneration can outweigh the metabolic costs, under the right circumstances. However, in the CYP153A6 system, the co-expression of dehydrogenase was detrimental to intact cells. It made little difference to initial rates of product formation, while decreasing the stability of the biocatalytic system, leading to lower titres overall. This would fit the hypothesis that cells with cross-membrane transport limitations cannot benefit from enhanced cofactor regeneration.

5.2.2 Effects of glycerol dehydrogenase at low cell density

Strains used in this section of the work:

<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF	
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(GLD)	

The volumetric titres and yields on biomass from a low-density biotransformation are presented in Figure 5.6, and compared to results obtained for intact cells at high density. After two days of biotransformation, low-density cultures produced lower volumetric titres than high-density cultures. Cells co-expressing GLD produced $11.5 \pm 1.8 \text{ mmol.L}^{-1}$ after 49 h, which is roughly 65% of the titre from high-density suspensions of the same strain. Cells carrying the empty vector produced a titre of $10.5 \pm 0.7 \text{ mmol.L}^{-1}$ over the same period, which is roughly 35% of the titre from high-density suspensions without GLD. When biotransformation was extended to 71 h, low-density cells with GLD produced a final titre of $16.4 \pm 1.4 \text{ mmol.L}^{-1}$; cells without GLD produced a slightly lower titre of $12.8 \pm 1.8 \text{ mmol.L}^{-1}$.

High-density suspensions were at a biomass loading of $27.5 \pm 0.8 \text{ g}_{\text{DCW}}.\text{L}^{-1}$ for cells without GLD, or $35.7 \pm 2.0 \text{ g}_{\text{DCW}}.\text{L}^{-1}$ for cells co-expressing GLD. Biomass loadings were an order of magnitude lower for low-density suspensions: $4.55 \pm 0.13 \text{ g}_{\text{DCW}}.\text{L}^{-1}$. Reducing the culture density improved biocatalytic stability, as can be seen from a comparison of yields on biomass for low-density and high-density suspensions. For cells co-expressing GLD, the yield on biomass after 47 – 49 h was $2.48 \pm 0.42 \text{ mmol.g}_{\text{DCW}}^{-1}$ at low density, which is an increase of almost five-fold compared to the yield of $0.50 \pm 0.03 \text{ mmol.g}_{\text{DCW}}^{-1}$ which was achieved at high density. For cells carrying the empty vector, yields on biomass were $2.25 \pm 0.16 \text{ mmol.g}_{\text{DCW}}^{-1}$ at low density, which is roughly double the yield of $1.08 \pm 0.03 \text{ mmol.g}_{\text{DCW}}^{-1}$ that was achieved at high density.

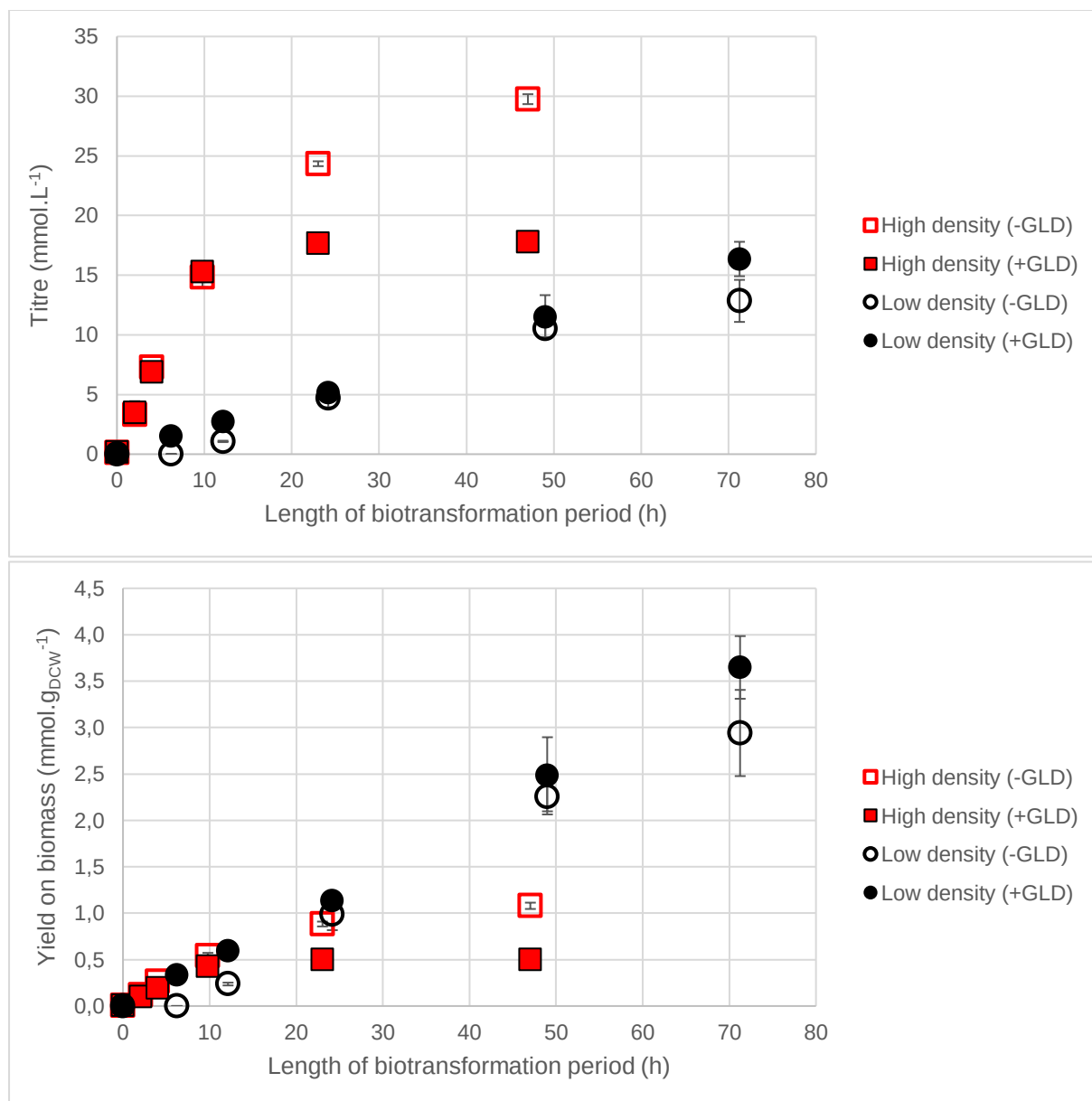


Figure 5.6: Effects of glycerol dehydrogenase co-expression in suspensions of intact cells. Biotransformations were carried out at low cell density ($4.55 \pm 0.13 \text{ g}_{\text{DCW}}\cdot\text{L}^{-1}$) and high cell density ($31.6 \pm 4.2 \text{ g}_{\text{DCW}}\cdot\text{L}^{-1}$) using resting whole cells suspended in phosphate buffer. All cells were expressing CYP153A6, Fdx, and FdR from pET28b+. Squares represent high-density suspensions; the Duet vector carried by these cells was pACYCDuet. Circles represent low-density suspensions; the Duet vector carried by these cells was pCDFDuet. Cells carrying an empty vector (-GLD) are represented by empty shapes; cells co-expressing heterologous glycerol dehydrogenase (+GLD) are represented by filled shapes. Biotransformations were carried out in 40 mL vials, some of which were sacrificed for sampling at each time point; error bars represent the standard deviation over at least two vials.

Figure 5.7 compares the average rate of product formation, per unit of biomass, across various periods of the biotransformation. For cells co-expressing GLD, low-density cultures showed a fair amount of variance between samples, but on average were very stable over the course of 71 h. They demonstrated an initial product formation rate of $0.0535 \text{ mmol}\cdot\text{g}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$ during the first 6 h of biotransformation, which is comparable to both the $0.0650 \text{ mmol}\cdot\text{g}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$ generated by high-density cultures without GLD, and the $0.0469 \text{ mmol}\cdot\text{g}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$ generated by high-density cultures co-expressing GLD. Low-density cultures did not experience “sudden death” after 24 h when GLD was present, as high-density cultures had done. Between 24 h and 49 h, they produced an average of $0.0543 \text{ mmol}\cdot\text{g}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$ (high-density cultures had produced less than $0.01 \text{ mmol}\cdot\text{g}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$ over the same period). Low-density cells with GLD were still producing $0.0525 \text{ mmol}\cdot\text{g}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$ between 49 h and 71 h.

Low-density cultures carrying the empty vector demonstrated an unusual lag, with no product formation detected between 0 h and 6 h. However, for the rest of the biotransformation, product formation rates in low-density cultures were similar for cells with and without GLD. For cells without GLD, product formation rates appeared to peak between 12 h and 24 h, and thereafter to decrease slightly, whereas they had been relatively stable for cells co-expressing GLD. This is a very different pattern from high-density cultures, in which product formation rates decreased steadily over the course of biotransformation for cells without GLD, and decreased sharply after 12 h for cells with GLD.

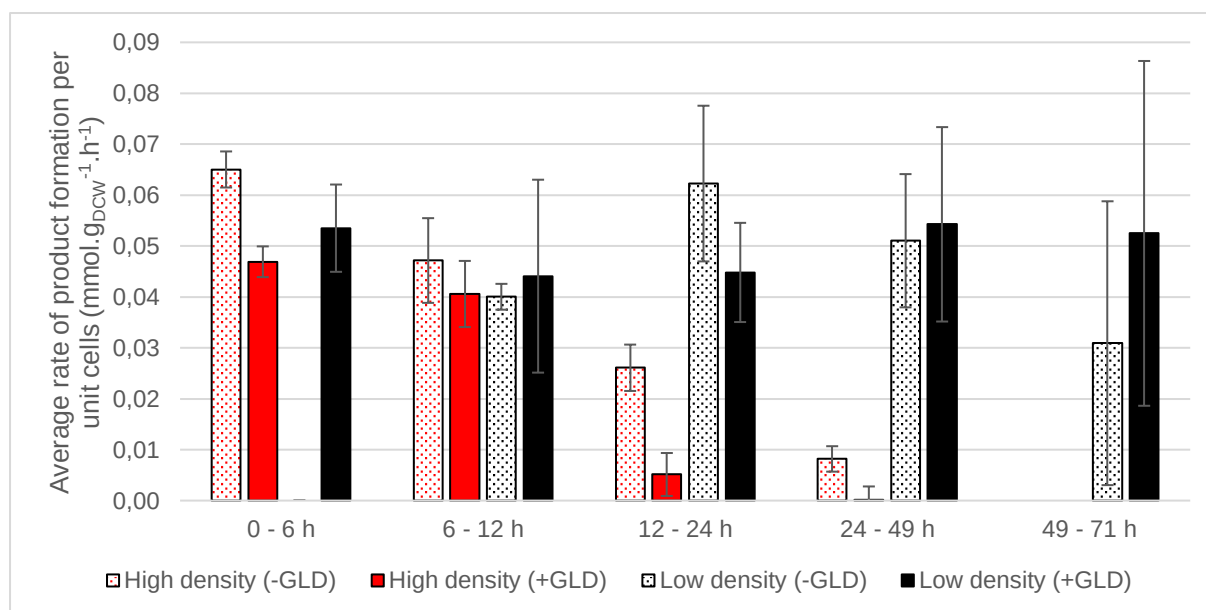


Figure 5.7: Effect of culture density on the average rates of product formation over various periods of the biotransformation, for intact cells with and without additional glycerol dehydrogenase. Biotransformations were carried out at low cell density ($4.55 \pm 0.13 \text{ g}_{\text{DCW}}\cdot\text{L}^{-1}$) and high cell density ($31.6 \pm 4.2 \text{ g}_{\text{DCW}}\cdot\text{L}^{-1}$) using resting whole cells suspended in phosphate buffer. All cells were expressing CYP153A6, Fdx, and FdR from pET28b+. Red bars represent high-density suspensions; the Duet vector carried by these cells was pACYCDuet. Black bars represent low-density suspensions; the Duet vector carried by these cells was pCDFDuet. Cells carrying an empty vector (-GLD) are represented by dotted bars; cells co-expressing glycerol dehydrogenase (+GLD) are represented by solid bars. Biotransformations were carried out in 40 mL vials, some of which were sacrificed for sampling at each time point; error bars represent the standard deviation over at least two vials.

As shown in Figure 5.8, the intracellular concentration of active CYP153A6 was less stable in the low-density cultures, especially in cells without GLD, which were able to express CYP153A6 to a high level during the growth and expression stage, but lost enzyme rapidly as the biotransformation progressed. The expression of CYP153A6 appears to be more strongly impacted by co-expression from pCDFDuet than from pACYCDuet: intracellular P450 dropped by almost three-fold when glycerol dehydrogenase was co-expressed from pCDFDuet, whereas it had dropped by a factor of only 1.56 when the dehydrogenase was co-expressed from pACYCDuet. However, low intracellular P450 concentrations do not appear to have limited biotransformation in low-density cultures, as is evident from the poor performance of cells without GLD, in spite of their much higher P450 concentrations. Limitations in substrate availability may have overshadowed limitations in enzyme concentration in these cultures.

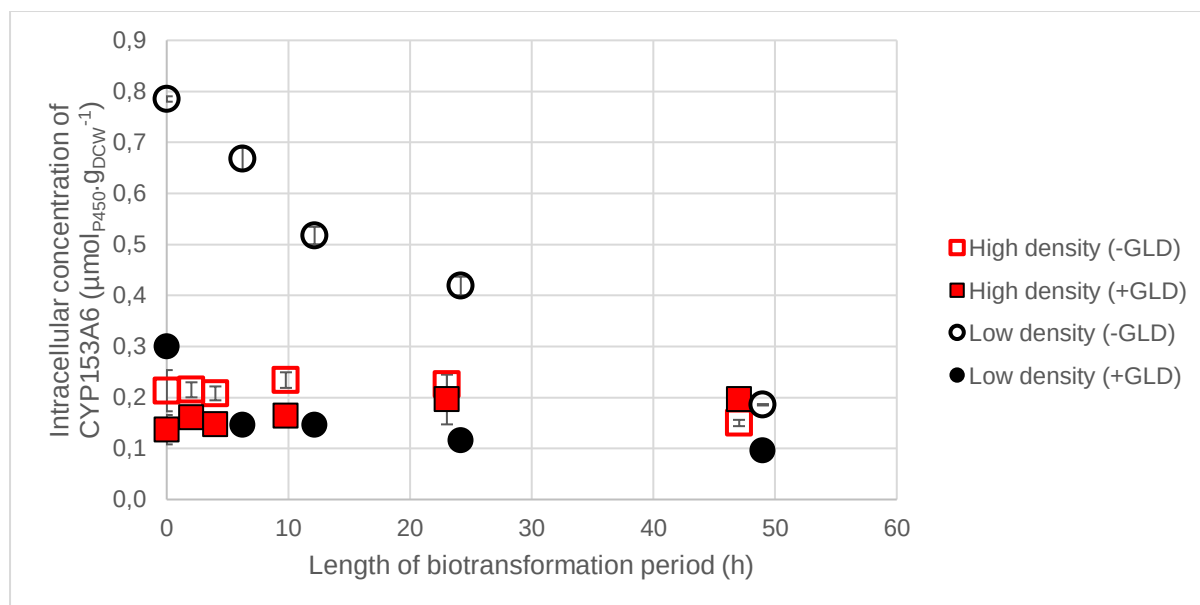


Figure 5.8: Effect of culture density on the stability of CYP153A6. Biotransformations were carried out at low cell density ($4.55 \pm 0.13 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$) and high cell density ($31.6 \pm 4.2 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$) using resting whole cells suspended in phosphate buffer. All cells were expressing CYP153A6, Fdx, and FdR from pET28b+. Red squares represent high-density suspensions; the Duet vector carried by these cells was pACYCDuet. Black circles represent low-density suspensions; the Duet vector carried by these cells was pCDFDuet. Cells carrying an empty vector (-GLD) are represented by empty shapes; cells co-expressing glycerol dehydrogenase (+GLD) are represented by filled shapes. Biotransformations were carried out in 40 mL vials, some of which were sacrificed for sampling at each time point; error bars represent the standard deviation over at least two vials.

Comparing the results of high-density and low-density experiments reveals that cultures behaved similarly in terms of initial productivity per cell, but showed significant differences in stability (which was better in low-density cultures) and in volumetric performance (which was better in high-density cultures).

In high-density cultures, excess glycerol dehydrogenase exhibited a destabilising effect, leading to the eventual cessation of biotransformation. However, this productivity loss did not emerge at low cell densities. The performance of the CYP153A6 system in cell free extract has been shown to be impeded by the presence of additional glucose dehydrogenase, which merely increased the rate of medium acidification rather than contributing to biotransformation (Pennec et al., 2015). Similarly, the negative effects of high cell density have also been observed in whole-cell systems. Willrodt et al. (2016) found that heterologous limonene production by resting cells showed decreased biocatalytic stability at high biomass concentrations. In work with CYP153A6, Gudimanchi et al. (2012) found that product formation per gram of resting cells decreased as biomass concentration was increased from 7 to $28 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$; at the highest cell density, initial volumetric rates were highest but fell rapidly after 5 h.

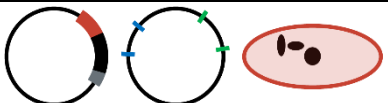
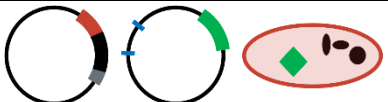
Oxygen limitation in high-density cultures is a plausible explanation for stability loss; it would account for the fact that the high-density and low-density cultures in this study exhibited similar product formation rates per unit biomass over the first 10 – 12 h of biotransformation, but differed in their ability to maintain these rates over longer periods. High-density cultures with additional dehydrogenase might also have become oxygen-limited more quickly if uncoupling was leading to wasteful increased consumption of oxygen and the associated formation of reactive oxygen species. The system studied by Gudimanchi et al. (2012) was more stable when the initial intracellular P450 concentration was $0.53 \text{ μmol} \cdot \text{g}_{\text{DCW}}^{-1}$, as opposed to $0.76 \text{ μmol} \cdot \text{g}_{\text{DCW}}^{-1}$, suggesting that reactive oxygen species were being formed, destabilising the biocatalyst. In this study, the more stable low-density cultures also had lower concentrations of CYP153A6 per cell, which could have contributed to their enhanced stability. Alternatively, an abnormal amount of NADH in the cofactor pool may simply have had wide-ranging implications for cell physiology, causing cells to divert oxygen away from biotransformation.

Despite their loss of performance as biotransformation progressed, the high-density cell suspensions achieved much higher volumetric titres, when considered over either 24 h or 48 h periods. This suggests that biomass loading is a key focus area for optimisation of the CYP153A6 system: lower cell densities improve stability, leading to higher yields per unit of biomass, but reduce the amount of product formed per unit volume; and vice versa.

Regardless of cell density, co-expression of glycerol dehydrogenase did not lead to meaningful improvements in product formation rates for intact cells. At low cell density, the benefits that additional dehydrogenase provided were minor; at high cell density, dehydrogenase co-expression was detrimental to biocatalyst performance. This supports the hypothesis that the cell's capacity for cofactor regeneration is interacting with other factors to determine whether the additional cofactor is utilised effectively, or self-destructively.

5.3 Effects of glycerol dehydrogenase co-expression in mechanically broken cells

Strains used in this section of work:

<i>E. coli</i> BL21(DE3) containing pET(CYP) and pACYC	
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pACYC(GLD)	

As discussed in Section 5.1, strains that were co-expressing glycerol dehydrogenase had performed best when applied as cell free extract (CFE) or mechanically broken cells, outperforming intact or chemically permeabilised cells of the same strain. Co-cultures of disintegrated cells had also performed more poorly when cells expressing glycerol dehydrogenase from pACYCDuet were replaced by cells carrying the empty pACYCDuet plasmid. In order to better understand the role GLD was playing in the enhanced performance of mechanically broken cells, strains co-expressing A6/FdR/Fdx and GLD were compared to strains expressing only A6/FdR/Fdx (but still carrying empty pACYCDuet). Cells were applied as cell free extract (the soluble fraction of a cell suspension passed through a one-shot cell disruptor at 30 kpsi) or as ruptured cells (cell suspension passed through a disruptor at 15 kpsi). The strain co-expressing GLD was also applied as disintegrated cells (cell suspension passed through a disruptor at 30 kpsi).

The results are presented in Figure 5.9. Ruptured cells co-expressing GLD produced a titre of $42.9 \pm 3.0 \text{ mmol}_{\text{product}} \cdot \text{L}^{-1}$ after 48 h. This represents a 44% increase relative to intact cells without GLD. The co-expression of GLD had a slight, but not significant, positive effect on initial rates, which rose to $1.76 \pm 0.02 \text{ mmol}_{\text{product}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ (from $1.64 \pm 0.17 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ for intact cells). However, ruptured cells co-expressing GLD were considerably more stable than intact cells; as shown in Figure 5.10, average rates of product formation for ruptured cells were higher throughout the biotransformation, with the improvement becoming more significant as the biotransformation progressed.

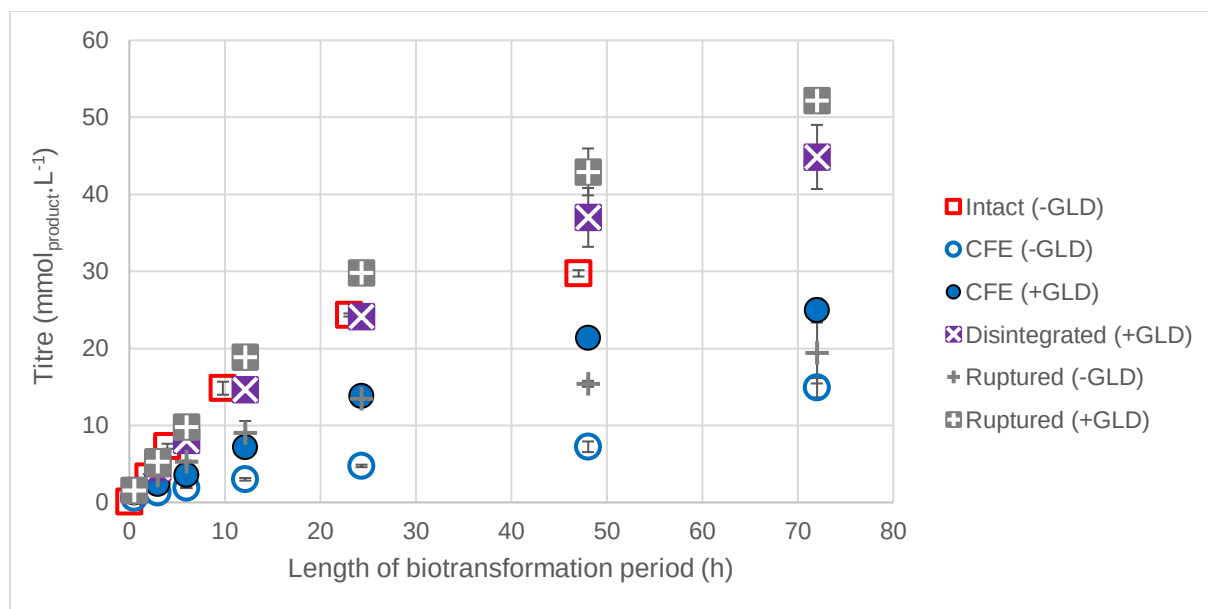


Figure 5.9: Effects of glycerol dehydrogenase co-expression on cell free extract and mechanically broken cells. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. In '-GLD' suspensions, cells were carrying the empty pACYC vector. In '+GLD' suspensions, cells were co-expressing glycerol dehydrogenase from the pACYCDuet plasmid. Biotransformations were carried out in 40 mL amber vials, with 1.2 mL of cell suspension and 200 μ L of n-octane. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation between two vials. Results obtained from intact cells without GLD are included for comparison.

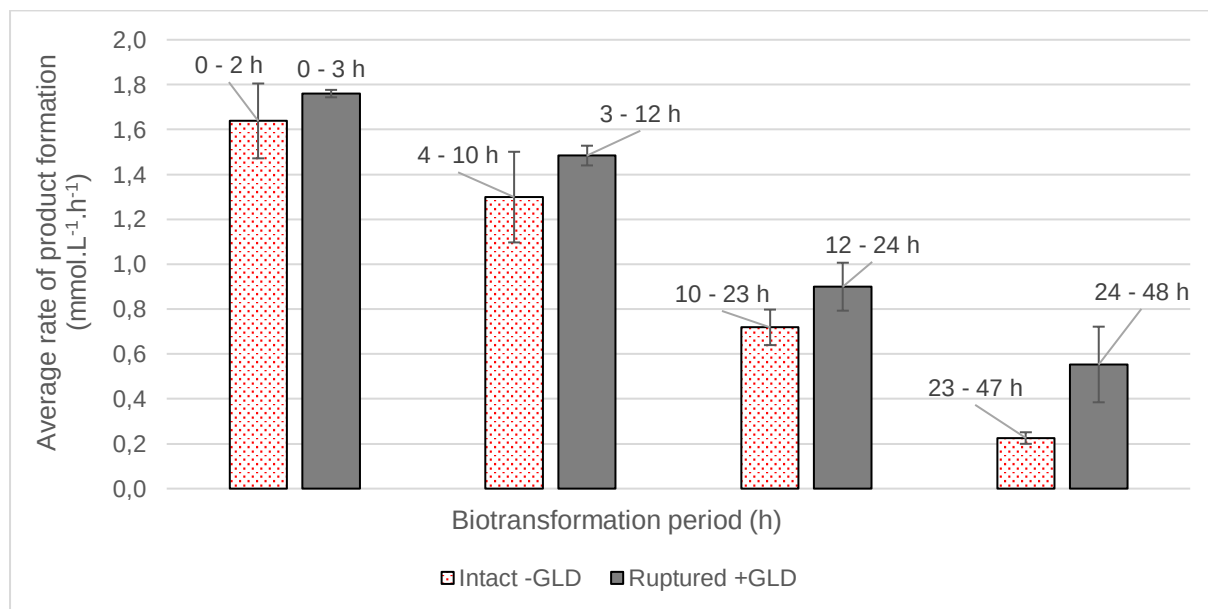


Figure 5.10: Average rates of product formation for ruptured cells with enhanced cofactor regeneration, relative to intact cells. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. Intact cells carried the empty pACYC vector. Ruptured cells were co-expressing glycerol dehydrogenase from the pACYCDuet plasmid. Biotransformations were carried out in 40 mL amber vials, with 1.2 mL of cell suspension and 200 μ L of n-octane. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation between two vials.

Unlike intact cells, ruptured cells performed considerably worse when GLD was not present. Without GLD co-expression, ruptured cells produced only 15.4 mmol.L^{-1} product after 48 h (roughly half the final titre of intact cells without GLD, and almost three-fold lower than ruptured cells with GLD). The maximum titre from ruptured cells without GLD was only $19.4 \pm 4.0 \text{ mmol.L}^{-1}$ after 72 h, compared to $52.2 \pm 0.8 \text{ mmol.L}^{-1}$ from ruptured cells with GLD. Breakage of the membrane also had a negative effect on initial rates of product formation when GLD was not present, reducing them from $1.64 \pm 0.17 \text{ mmol.L}^{-1}.\text{h}^{-1}$ (intact cells) to $1.16 \pm 0.004 \text{ mmol.L}^{-1}.\text{h}^{-1}$ (ruptured cells).

Disintegrated cells co-expressing GLD performed similarly to ruptured cells of the same strain, although rates of product formation were slightly lower from around 6 h onwards, leading to a titre of $37.0 \pm 3.8 \text{ mmol.L}^{-1}$ after 48 h. This represents a 14% decrease relative to ruptured cells with GLD, but a 24% increase relative to intact cells without GLD. This is in line with previous results with co-expressing cells, as presented in Section 5.1.1, in which suspensions of ruptured or disintegrated cells performed similarly, and both performed substantially better than intact cells.

As can be seen from Figure 5.11, the enhanced performance of ruptured and disintegrated cells cannot be attributed to an increase in the concentration of active CYP153A6, which was within a similar range across the various cell suspensions, and was less stable during the first 24 h of biotransformation for mechanically broken cells than for intact cells.

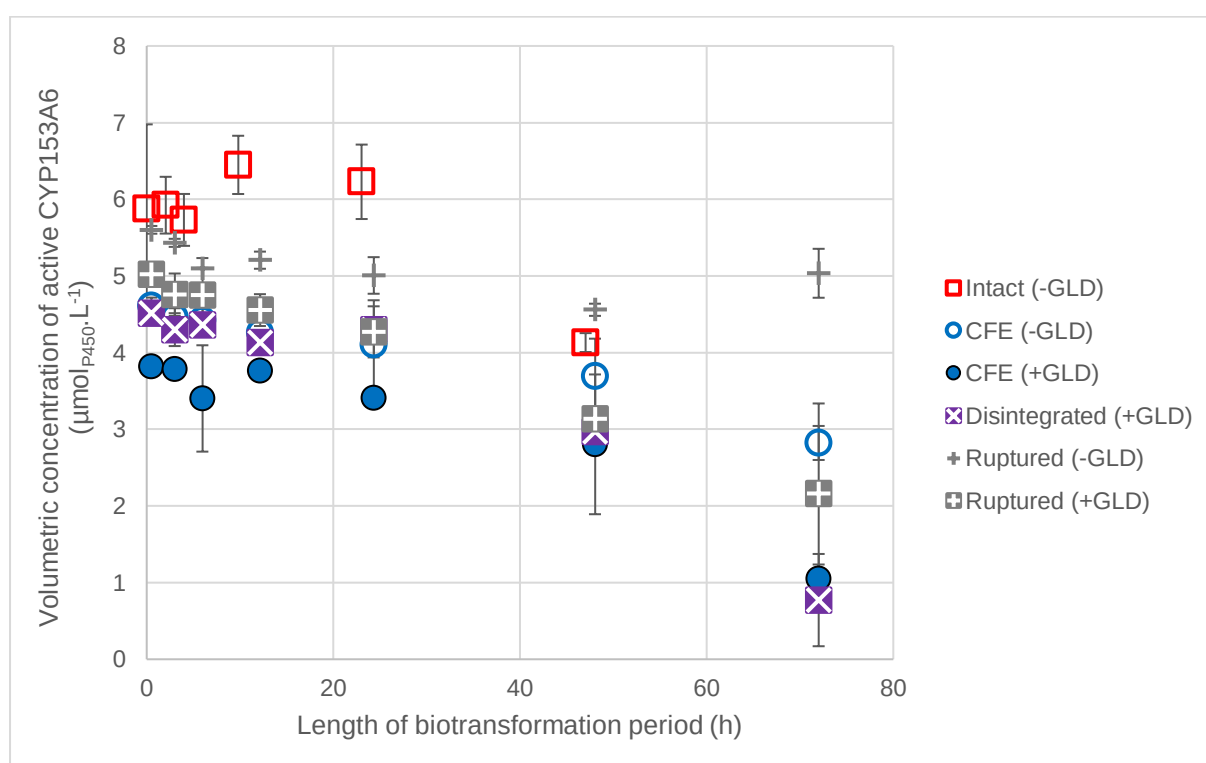


Figure 5.11: Effects of glycerol dehydrogenase co-expression on the concentration of active CYP153A6 in cell free extract and mechanically broken cells. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. In '-GLD' suspensions, cells were carrying the empty pACYC vector. In '+GLD' suspensions, cells were co-expressing glycerol dehydrogenase from the pACYCDuet plasmid. Biotransformations were carried out in 40 mL amber vials, with 1 mL of cell suspension and 200 μL of n-octane. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation between two vials. Results obtained from intact cells without GLD are included for comparison.

Cell free extracts performed poorly relative to intact cells. CFE from cells without GLD achieved an average product formation rate of $0.422 \pm 0.013 \text{ mmol.L}^{-1}.\text{h}^{-1}$ over the first three hours of biotransformation, and produced a final titre of only $14.9 \pm 1.2 \text{ mmol.L}^{-1}$ after 72 h. As with disintegrated and ruptured cells, the co-expression of GLD improved performance. Average initial rate doubled to $0.798 \pm 0.057 \text{ mmol.L}^{-1}.\text{h}^{-1}$ and final titre increased to $25.0 \pm 1.5 \text{ mmol.L}^{-1}$ after 72 h. However, titres were still significantly lower than titres achieved by intact cells without GLD, let alone by ruptured or

disintegrated cells co-expressing GLD. CFE from cells co-expressing GLD did outperform suspensions of ruptured cells *without* GLD, which fits with the fact that cell free extracts outperformed ruptured cells in previous co-culture experiments (see Figure 5.1). The results suggest that there is a substantial benefit to the containment effect provided by whole cells, which naturally keep biotransformation enzymes and cofactor regeneration enzymes in close proximity to one another. Volumetric concentrations of active CYP153A6 were lower in CFE than in cell suspensions; this explains some of the performance loss. However, CFE with GLD performed substantially better than CFE without GLD, despite lower P450 concentrations throughout the biotransformation. This confirms that enhanced cofactor regeneration can be beneficial when transport limitations are overcome.

Maximum turnover frequencies for the various systems are given in Table 5.3. It is important to note that performance per unit enzyme is difficult to interpret in systems where activity is not directly correlated to enzyme concentration. Previous studies on this system showed that cultures with higher cell densities and higher concentrations of active CYP153A6 had lower turnover frequencies than systems with lower P450 concentrations, but these lower turnover frequencies did not correlate with reduced titres or reduced volumetric rates (Gudimich et al., 2012; Olaofe et al., 2013). The same is true for this study.

Table 5.3: Maximum turnover frequencies for the various mechanically permeabilised systems

Type of permeabilization treatment	Maximum turnover frequency ($\text{mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$)	Time point at which Max. TOF was measured
Intact cells (-GLD)	5.3 ± 0.002	4 h
Intact cells (+GLD)	5.4 ± 0.001	4 h
Ruptured cells (-GLD)	10.3 ± 0.005	1 h
Ruptured cells (+GLD)	6.6 ± 0.016	1 h
Disintegrated cells (+GLD)	13.4 ± 0.002	72 h
CFE (-GLD)	4.2 ± 0.003	1 h
CFE (+GLD)	10.9 ± 0.031	1 h

The results with mechanically broken cells and cell free extracts suggest a trade-off between the costs of reduced cell integrity and the benefits of improved substrate import and product export. Breaking the cell envelope facilitated substrate entry. However, it also allowed for leakage of intracellular components. Consequently, the multiple system components (CYP153A6, Fdx, FdR, NADH, and dehydrogenases) may have started out relatively contained in ruptured and disintegrated cells, but over time these components would have diffused throughout the aqueous phase (especially cofactor, the smallest component). This distribution would have reduced the frequency of interaction between components. This would explain why cell free extract, with its total absence of cellular containment, demonstrated significantly lower rates than cell-based systems. In the case of mechanically broken cells without additional dehydrogenase, the membrane damage and associated improvement in substrate transport may not have been sufficient to compensate for the cofactor lost during the preparation steps, so rates of product formation decreased. A similar phenomenon has been observed in commercial processes for the conversion of fumaric acid to L-malic acid by the enzyme fumarase: whole cells permeabilised by detergents showed reduced substrate uptake limitations, but were also shown to lose cofactors (Schrewe et al., 2013a). Since cofactor availability is critical in the case of P450-based biotransformations, cofactor leakage was highly detrimental to system performance. However, if glycerol dehydrogenase was co-expressed, the additional dehydrogenase compensated for the reduced cofactor pool that resulted from membrane breakage, thus allowing the cells to take advantage of improvements in substrate transport. Initial rates of product formation increased relative to intact cells, and final titres were higher. Similar beneficial effects were observed by Kaluzna et al. (2016), who successfully employed the heterologous expression of glucose dehydrogenase to compensate for cofactor loss during cell breakage via freeze-thaw cycles.

Biotransformation with mechanically treated cells did not appear to be limited by enzyme expression or availability. The mechanical permeabilization process resulted in some loss of biomass and a decrease in the *volumetric* concentration of active CYP153A6. The co-expression of glycerol dehydrogenase has also been shown to depress the *intracellular* concentration of active CYP153A6. Yet ruptured or disintegrated cells produced higher titres than intact cells, provided glycerol dehydrogenase expression had been enhanced. These significant increases in product formation came about because the enzymes were provided with the substrate and cofactor needed for bioconversion at an increased rate, overcoming key limitations. Balancing substrate supply and cofactor regeneration allowed the cell to utilise its biocatalytic enzymes more efficiently and consistently over an extended biotransformation period. Even so, all of the systems demonstrated maximum turnover frequencies that were substantially lower than the activities reported for CYP153A6 as free enzyme (Funhoff et al., 2006), indicating that system limitations were not fully overcome.

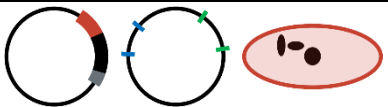
5.4 Effects of glycerol dehydrogenase co-expression in chemically permeabilised cells

As discussed in Section 5.1, initial experiments had suggested that chemical permeabilization was less effective than mechanical permeabilization when cells were co-expressing glycerol dehydrogenase, but more effective when glycerol dehydrogenase was expressed in separate cells. However, as discussed in Section 5.2, experiments with low-density intact cells suggested that the efficacy of glycerol dehydrogenase depended to some extent on cell density.

To further investigate the interaction between cofactor regeneration and cross-membrane transport, cells with and without GLD were permeabilised with acetone, Polymyxin B, or Triton X-100. Experiments were carried out at both high and low cell densities.

5.4.1 Combining glycerol dehydrogenase and chemical permeabilization at high cell density

Strains used in this section of work:

<i>E. coli</i> BL21(DE3) containing pET(CYP) and pACYC	
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pACYC(GLD)	

Strains with and without GLD were washed with 5% (v/v) acetone and compared over 47 h of biotransformation. Since results from previous experiments had suggested that acetone and Polymyxin B might not be providing sufficient enhancement of n-octane transport, cells co-expressing GLD were also applied in biotransformation buffer containing 0.25% (v/v) of the detergent Triton X-100, which is known to solubilise hydrophobic substrates, and to disrupt the outer membrane without affecting the cytoplasmic membrane (Canovas et al., 2005). Cells mixed with Triton X-100 were then compared to cells from the same strain that had been washed with acetone. Results are shown in Figure 5.12.

As with the experiments described in Section 5.1, washing cells with acetone improved their performance relative to intact cells, but not substantially. Acetone-washed cells responded to the presence or absence of GLD in much the same way as intact cells. Productivity was highest during the early stages of the biotransformation, and decreased after the 10 h mark, with the decrease being more marked if GLD was present. Cells co-expressing GLD showed almost no biocatalytic activity between 23 h and 47 h.

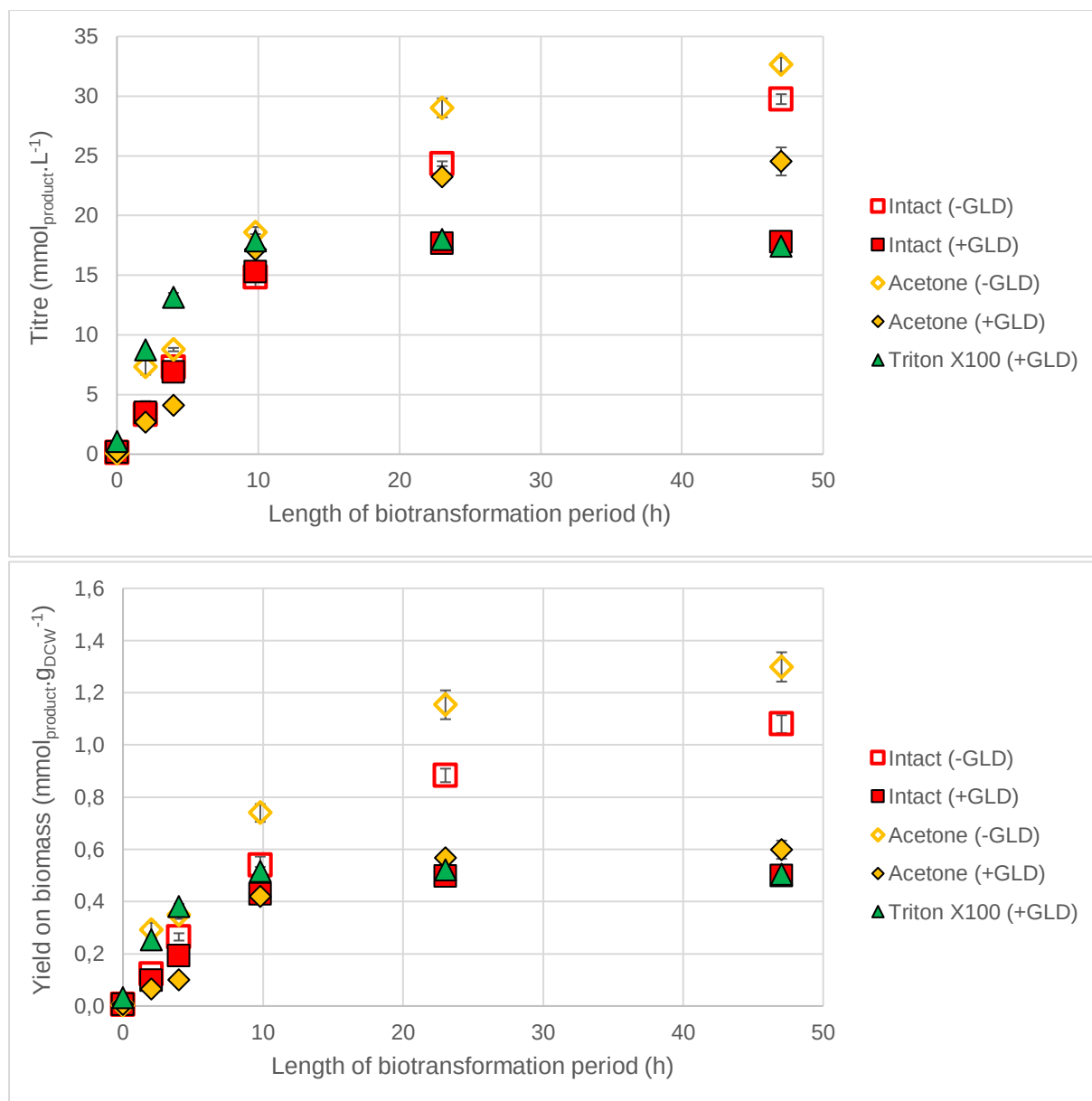


Figure 5.12: Effects of acetone and Triton X-100 on the biotransformation of n-octane at high cell density, by *E. coli* expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. Some cells (+GLD, filled shapes) were co-expressing glycerol dehydrogenase from the pACYCDuet plasmid. Other cells (-GLD, empty shapes) carried the empty pACYCDuet plasmid. Biotransformations were carried out in 40 mL amber vials, with 1.2 mL of cell suspension and 200 μ L of n-octane. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation between two vials. Results obtained from intact cells are included for comparison.

Acetone-washed cells co-expressing GLD also demonstrated a reduced product formation rate during the early hours of the biotransformation, relative to cells carrying empty pACYCDuet. This difference may be partly attributed to lower intracellular concentrations of active CYP153A6, as shown in Figure 5.13. However, the loss of activity from 10 h onwards cannot be attributed to loss of active enzyme, since P450 concentrations remained stable throughout the biotransformation when GLD was present. Expression of additional dehydrogenase was therefore directly or indirectly responsible for reduced biocatalyst stability in acetone-washed cells. This suggests that acetone permeabilization provides only a small increase in substrate access, with an associated small increase in product formation rate – enough to gain a slight advantage over intact cells, but not enough to outpace the cell's natural NADH regeneration rates; hence no benefit can be derived from dehydrogenase co-expression.

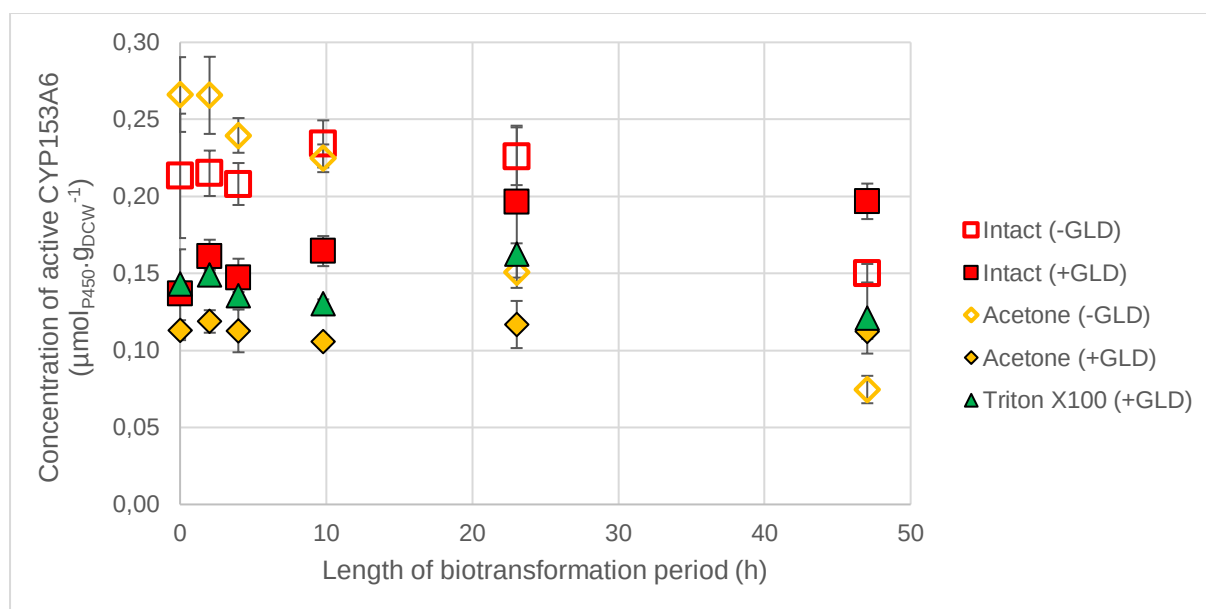


Figure 5.13: Intracellular concentrations of active CYP153A6 across two days of biotransformation, for intact cells, cells washed with 5% (v/v) acetone, and cells mixed with 0.25% (v/v) Triton X-100. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. In '-GLD' suspensions (empty shapes), cells were carrying the empty pACYCDuet plasmid. In '+GLD' suspensions (filled shapes), cells were co-expressing glycerol dehydrogenase from the pACYCDuet plasmid. Biotransformations were carried out in 40 mL amber vials, with 1.2 mL of cell suspension and 200 μ L of n-octane. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation between two vials.

When Triton X-100 was added to the biotransformation buffer, the average rate of product formation over the first two hours of biotransformation was $4.30 \text{ mmol}_{\text{product}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$. This is an increase of over three-fold relative to acetone-washed cells of the same strain, which produced only $1.31 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ over the same period, and an increase of over two-fold relative to intact cells, in which both strains produced between 1.6 and $1.7 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$. This performance enhancement was not due to increased intracellular concentrations of active CYP153A6, which the co-expression of GLD had suppressed in the typical fashion. Per unit of biomass, cells in the presence of Triton X-100 matched the initial performance of acetone-washed cells not burdened by GLD, despite having much lower intracellular P450 concentrations. It is possible that Triton X-100 enhances substrate transport to a greater degree than acetone washing, and that additional cofactor regeneration capacity becomes important for more permeabilised cells (as it was for mechanically broken cells in Section 5.3).

After its initial spurt, the rate of product formation dropped off rapidly in the presence of Triton X-100. On average, it was only $0.81 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ between 4 h and 10 h, which is only 56% of the average rate displayed by intact cells over the same period. No further product formation was detected after 10 h. Thus, maximum titre was only $17.8 \pm 0.6 \text{ mmol} \cdot \text{L}^{-1}$, and maximum yield on biomass was only $0.52 \pm 0.02 \text{ mmol} \cdot \text{g}_{\text{DCW}}^{-1}$. In comparison, acetone-washed cells without GLD were able to produce a maximum titre of $32.7 \pm 0.6 \text{ mmol} \cdot \text{L}^{-1}$, and a maximum yield on biomass of $1.30 \pm 0.06 \text{ mmol} \cdot \text{g}_{\text{DCW}}^{-1}$.

Key performance indicators for the chemically permeabilised systems are shown in Figure 5.14. Acetone permeabilization had a clear positive effect on the initial rate of product formation for cells without GLD, while Triton X-100 had a substantial positive effect on the initial rate for cells with GLD. This is further evidence that transport of alkane substrate is a key limitation. However, the co-expression of GLD had a negative effect on both the activity and the stability of acetone-washed cells. The Triton X-100 system was also highly unstable. Thus, unlike mechanical permeabilization techniques, chemical permeabilization did not lead to large improvements in titre or yield on biomass, relative to untreated whole cells.

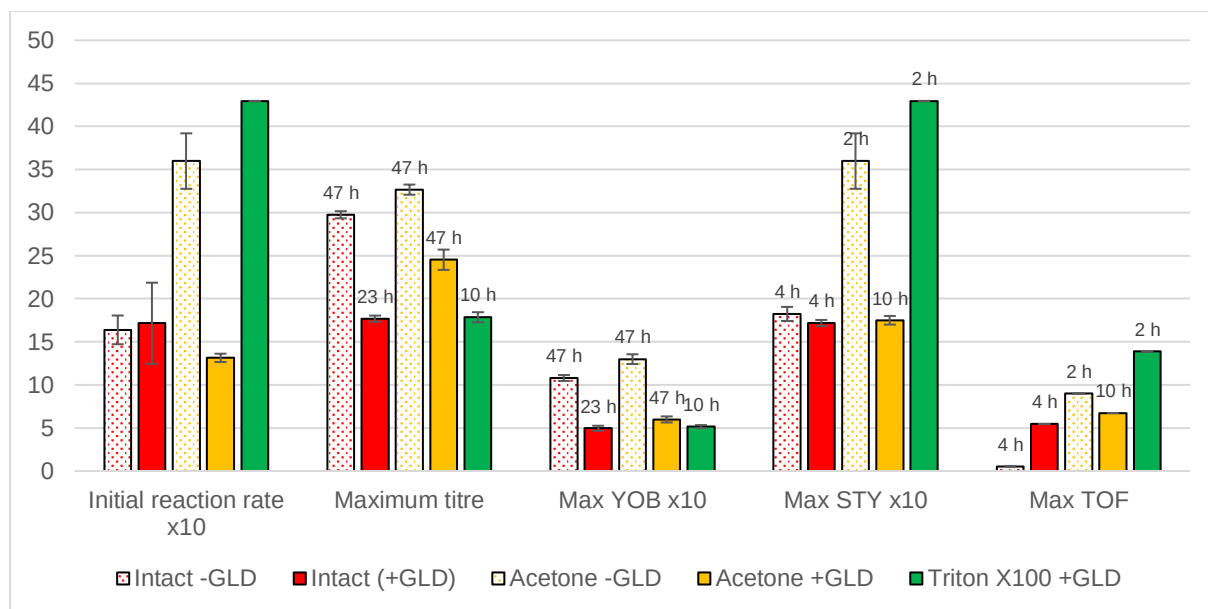


Figure 5.14: Key performance indicators for biotransformation systems consisting of whole cells washed with 5% (v/v) acetone prior to biotransformation, or mixed with 0.25% (v/v) Triton X-100 in the biotransformation buffer. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. In '-GLD' suspensions, cells were carrying the empty pACYC vector. In '+GLD' suspensions, cells were co-expressing glycerol dehydrogenase from the pACYCDuet plasmid. Biotransformations were carried out in 40 mL amber vials, with 1.2 mL of cell suspension and 200 μ L of n-octane. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation between two vials. Units are as follows: Initial reaction rate: $\text{mmol.L}^{-1}.\text{h}^{-1}$, Maximum titre: mmol.L^{-1} , Maximum YOB (yield on biomass): $\text{mmol.g}_{\text{DCW}}^{-1}$, Maximum STY (space time yield): $\text{mmol.L}^{-1}.\text{h}^{-1}$, Maximum TOF (turnover frequency): $\text{mol}_{\text{product}}.\text{mol}_{\text{P450}}^{-1}.\text{min}^{-1}$. Labels above data bars indicate the time point at which the various maxima were observed. Results obtained from intact cells are included for comparison.

5.4.2 Combining glycerol dehydrogenase and permeabilization at low cell density

Strains used in this section of the work:

<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF	
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(GLD)	

Treatment with Polymyxin B or Triton X-100 was shown to match or exceed the results obtained via treatment with acetone, in high-density preparations of strains co-expressing GLD (see Figure 5.1 and Figure 5.14). These chemicals were subsequently applied to low-density cell preparations, in which the efficacy of GLD was tested by comparing strains co-expressing glycerol dehydrogenase to strains carrying empty vector. Intact cells without GLD were applied as a control.

Biotransformations were carried out over two days. Figure 5.15 presents the product titres and yields on biomass obtained at each time point. Relative to intact cells, the presence of Polymyxin B or Triton X-100 in the biotransformation buffer led to improved performance throughout the biotransformation, whether or not cells were co-expressing GLD. After 50 h, the titre for intact cells was $10.6 \pm 0.3 \text{ mmol.L}^{-1}$, and the yield on biomass was $3.13 \pm 0.12 \text{ mmol.g}_{\text{DCW}}^{-1}$. The best-performing culture consisted of cells without GLD, treated with Polymyxin B, which produced a titre of $23.0 \pm 1.1 \text{ mmol.L}^{-1}$ and a yield on biomass of $6.80 \pm 0.35 \text{ mmol.g}_{\text{DCW}}^{-1}$. This represents a two-fold increase relative to unmodified cells. Even the worst performers from among the chemically permeabilised cultures were able to out-perform intact cells. Cells without GLD, treated with Triton X-100, produced a titre of $15.7 \pm 1.9 \text{ mmol.L}^{-1}$ and a yield on biomass of $4.64 \pm 0.56 \text{ mmol.g}_{\text{DCW}}^{-1}$ (an improvement of almost 50% over intact cells), and cells with GLD, treated with Polymyxin B, produced

a titre of $16.4 \pm 0.4 \text{ mmol.L}^{-1}$ and a yield on biomass of $4.18 \pm 0.19 \text{ mmol.g}_{\text{DCW}}^{-1}$ (an improvement of over 30% relative to intact cells).

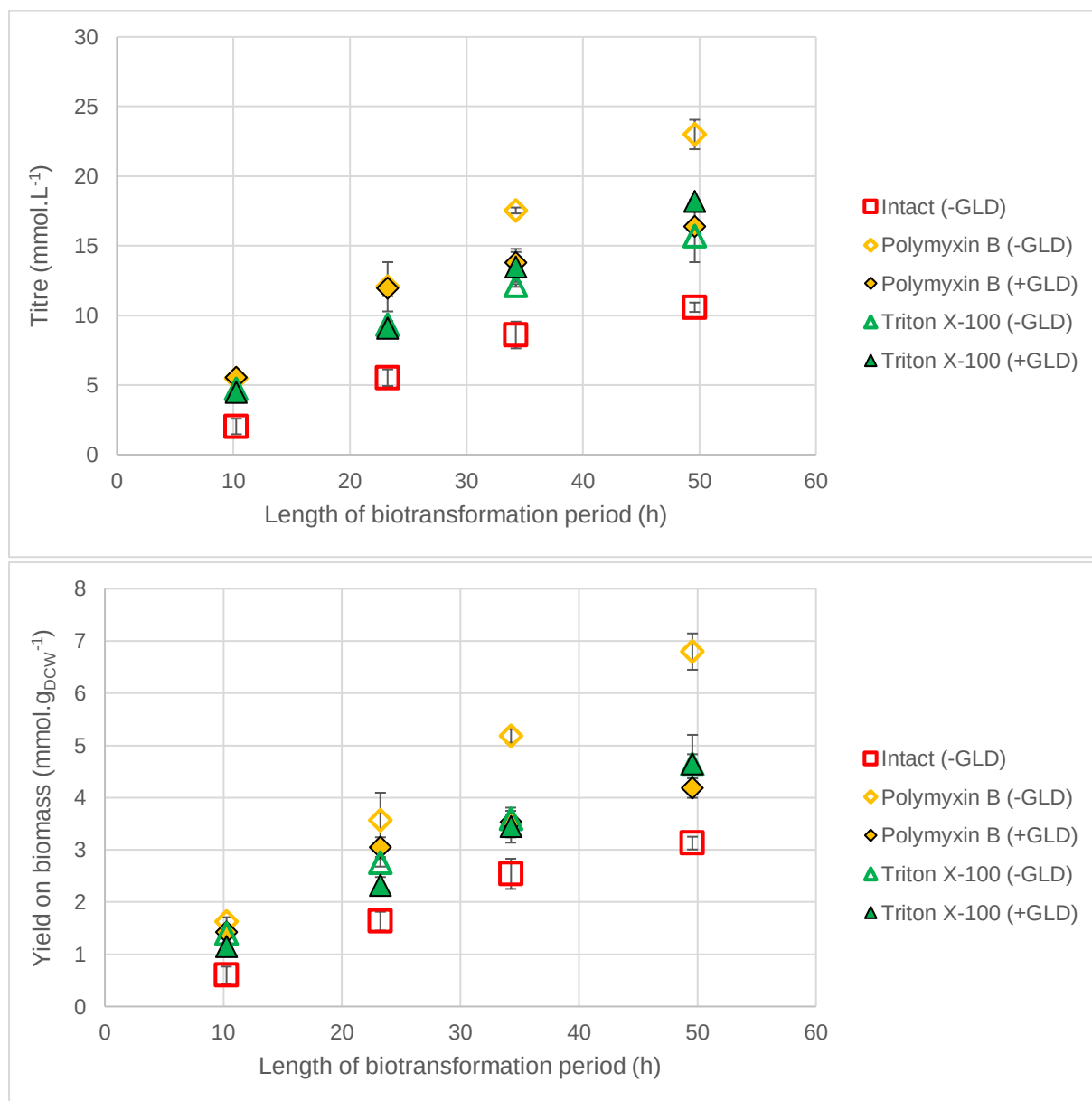


Figure 5.15: Combining chemical permeabilization and dehydrogenase co-expression in low-density suspensions of resting cells. All cells were carrying the pET28b+ vector containing CYP153A6, Fdx, and FdR, as well as the pCDFDuet vector, which was either empty (-GLD, empty shapes) or expressing glycerol dehydrogenase (+GLD, filled shapes). In selected cell suspensions, the buffer included either 30 μM Polymyxin B (yellow diamonds) or 0.25% (v/v) Triton X-100 (green triangles). Intact cells without GLD (empty red squares) were included as a control. Biotransformations were carried out in 40 mL amber vials, some of which were sacrificed for sampling at each time point; error bars represent the standard deviation over three vials.

For intact cells without GLD, the average rate of product formation over the first 10 h of biotransformation was $0.197 \pm 0.055 \text{ mmol.L}^{-1}.\text{h}^{-1}$. When Polymyxin B was present, this initial rate almost tripled, increasing to $0.537 \pm 0.025 \text{ mmol.L}^{-1}.\text{h}^{-1}$. When Triton X-100 was present, the initial rate more than doubled, reaching $0.460 \pm 0.015 \text{ mmol.L}^{-1}.\text{h}^{-1}$.

The co-expression of GLD depressed performance in cells permeabilised by Polymyxin B. Initial rates were not significantly affected, with cells producing $0.542 \pm 0.018 \text{ mmol.L}^{-1}.\text{h}^{-1}$ over the first two hours of biotransformation. However, cells lost activity more rapidly, with a particularly noticeable decrease after 24 h. When Triton X-100 was the permeabilizer, the initial rate was $0.438 \pm 0.019 \text{ mmol.L}^{-1}.\text{h}^{-1}$,

which again is statistically equivalent to the initial rate for cells without GLD. Dehydrogenase co-expression had very little impact on the stability of cells in the presence of Triton X-100.

The manipulation of δ -ALA concentrations during the growth and expression period ensured that all cultures had a similar intracellular concentration of active CYP153A6 at the beginning of the biotransformation, as shown in Table 5.4. However, CYP153A6 was more stable in cells that were co-expressing glycerol dehydrogenase. A similar trend was observed for intact and acetone-washed cells at high density (see Figure 5.13 in previous section).

Table 5.4: Intracellular concentrations of active CYP153A6 at the beginning and end of biotransformations with chemically permeabilised cells at low density

	P450 concentration at 0 h ($\mu\text{mol.g}_{\text{DCW}}^{-1}$)	P450 concentration at 50 h ($\mu\text{mol.g}_{\text{DCW}}^{-1}$)	P450 remaining after 50 h
Intact cells (-GLD)	0.184 ± 0.008	0.040 ± 0.003	22 %
Polymyxin B (-GLD)	0.184 ± 0.008	0.054 ± 0.035	29 %
Polymyxin B (+GLD)	0.165 ± 0.008	0.131 ± 0.018	80 %
Triton X-100 (-GLD)	0.184 ± 0.008	0.005 ± 0.000	3 %
Triton X-100 (+GLD)	0.165 ± 0.008	0.211 ± 0.030	128 %

However, the loss of active enzyme had little impact on the stability of the whole-cell biocatalyst, as shown in Figure 5.16. Intact cells achieved a space time yield of around $0.2 \text{ mmol.L}^{-1}.\text{h}^{-1}$, irrespective of the length of the biotransformation, despite losing almost 80% of their active CYP153A6. When Polymyxin B was applied, space time yields in cells with GLD fell from $0.54 \text{ mmol.L}^{-1}.\text{h}^{-1}$ after 10 h to $0.33 \text{ mmol.L}^{-1}.\text{h}^{-1}$ after 50 h, while space time yields in cells without GLD only fell to $0.46 \text{ mmol.L}^{-1}.\text{h}^{-1}$. This was in spite of the fact that cells with GLD maintained 80% of their initial P450 concentration, while cells without GLD had lost 71% of their active CYP153A6 after 50 h. When Triton X-100 was applied, space time yields for cells with and without GLD fell at similar rates, even though cells without GLD lost almost all of their active CYP153A6 during biotransformation. Space time yields were slightly less stable in chemically permeabilised cells than in intact cells, but permeabilised cells nevertheless achieved higher space time yields at all time points.

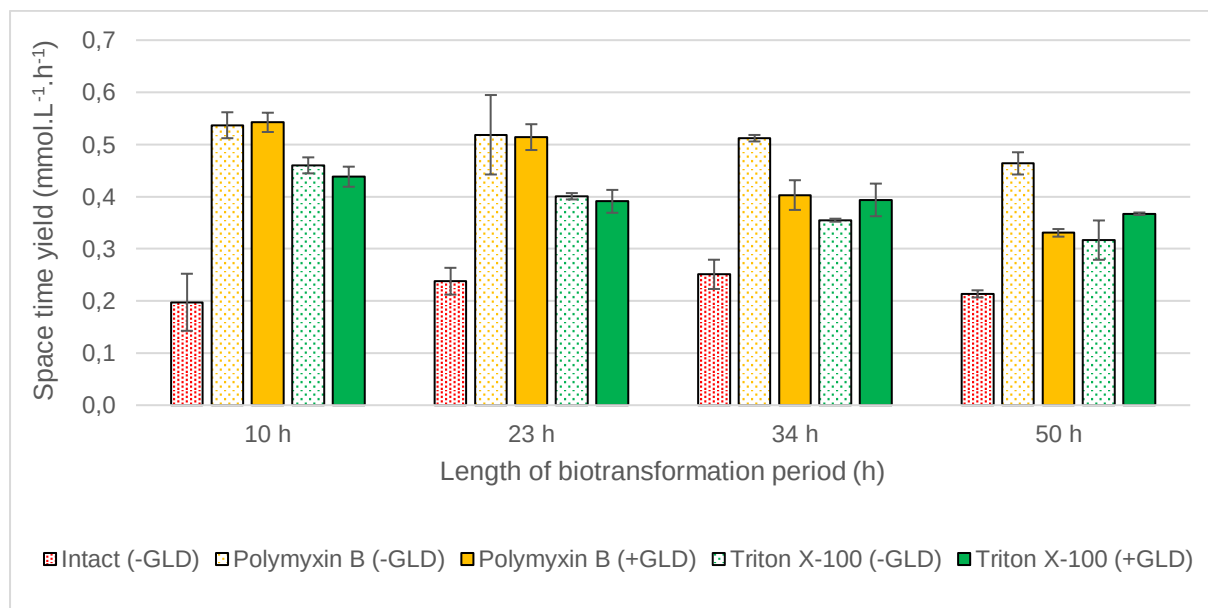


Figure 5.16: Effect of chemical permeabilization and glycerol dehydrogenase co-expression on biocatalyst stability, as indicated by changes in space time yield over the course of the biotransformation. All cells were carrying the pET28b+ vector containing CYP153A6, Fdx, and FdR, as well as the pCDFDuet vector, which was either empty (-GLD, dotted bars) or expressing glycerol dehydrogenase (+GLD, solid bars). In selected cell suspensions, the buffer included either 30 μM Polymyxin B (yellow bars) or 0.25% (v/v) Triton X-100 (green bars). Intact cells without GLD (dotted red bars) were included as a control. Biotransformations were carried out in 40 mL amber vials, some of which were sacrificed for sampling at each time point; error bars represent the standard deviation over three vials.

Comparing these results with previous experiments, it can be seen that Triton X-100 allowed cells with GLD to achieve an average rate of product formation of $0.053 \pm 0.002 \text{ mmol.g}_{\text{DCW}}^{-1}.\text{h}^{-1}$ in high-density cultures, and $0.112 \pm 0.007 \text{ mmol.g}_{\text{DCW}}^{-1}.\text{h}^{-1}$ in low-density cultures, over the first 10 h of biotransformation. Meanwhile, Polymyxin B allowed cells with GLD to achieve $0.048 \pm 0.002 \text{ mmol.g}_{\text{DCW}}^{-1}.\text{h}^{-1}$ in high-density cultures, and $0.138 \pm 0.007 \text{ mmol.g}_{\text{DCW}}^{-1}.\text{h}^{-1}$ in low-density cultures. In other words, Polymyxin B was more effective than Triton X-100 when culture density was low, but Triton X-100 was more effective when culture density was high. This suggests that the reduced effectiveness of Polymyxin B at high densities may have been due to an insufficient dosage. Triton X-100 improves the inter-phase transport of n-octane as well as permeabilising the outer membrane, while Polymyxin B has only permeabilising effects. As such, the same volume of Polymyxin B will become less efficacious as the cell density increases, while the same volume of Triton X-100 will retain its substrate solubilisation effects, although it will cause less membrane permeabilization. Indeed, Grant et al. (2012) found that the membrane permeabilising agent polyethyleneimine had no significant effect on biotransformation of n-dodecane by AlkB in growing *Escherichia coli*, while the detergents DMSO and Triton X-100 did. However, growing cells might be expected to derive fewer benefits from chemical permeabilization because they are better able to repair their membranes, reducing the degree of permeabilization.

Meanwhile, co-expression of additional dehydrogenase continued to be ineffective in chemically permeabilised systems, despite permeabilization allowing for a significant increase in the rate of product formation. This suggests that resting *E. coli* are able to regenerate cofactor rapidly enough to meet the demands of biotransformation. An implication of this finding is that additional dehydrogenase was only helpful in the case of mechanically broken cells because it compensated for cofactor leakage and loss of metabolic capacity. Cofactor supply does not appear to be a key limitation in the CYP153A6 resting whole-cell system, at the intracellular concentrations of CYP153A6 and substrate currently being achieved, although it clearly can limit the system if its concentration is sufficiently reduced.

5.5 Membrane integrity, NADH regeneration capacity, and biocatalyst performance

5.5.1 Stability of CYP153A6

The cell membrane plays an important role in maintaining homeostasis and protecting the cell from outside threats. Therefore, interfering with the membrane – through physical breakage or chemical permeabilization – might affect the ability of the resting cells to maintain their concentration of active CYP153A6, among other things. Volumetric P450 concentration was therefore tracked over the course of multiple biotransformations.

Apart from one run with low density cultures, starting concentrations of active CYP153A6 were within the range of $0.1 - 0.3 \mu\text{mol.P450.g}_{\text{DCW}}^{-1}$. Few cultures fell below $0.1 \mu\text{mol.g}^{-1}$, and then only around or after the two-day mark. These P450 concentrations are outside the limiting range for intact cells, but enzyme limitations cannot be ruled out in the case of permeabilised cells.

Enhanced stability of CYP153A6 was observed when intact or chemically permeabilised cells were co-expressing glycerol dehydrogenase. These cells also tended to achieve lower product titres, suggesting that CYP153A6 instability is linked to product formation. This is supported by the fact that ruptured cells demonstrated reduced P450 stability when glycerol dehydrogenase was co-expressed and product titres improved (see Section 5.3), while acetone-washed cells demonstrated reduced P450 stability when GLD was *not* expressed and product titres improved (see Section 5.4.1). Other researchers have linked cytochrome P450 instability to hydroxylation activity. In previous studies on this system, the CYP153A6 concentration was shown to be more stable in the absence of n-octane, and hence the absence of biotransformation (Gudimich et al., 2012). In the hydroxylation of dodecanoic acid by fusion construct CYP153A_{M.aq.}-CPR_{BM3}, the concentration of active enzyme halved during the first two hours of biotransformation, but this rapid attrition ceased after all the substrate was consumed, with enzyme concentration remaining fairly constant over the remainder of the 24 h period

(Lundemo et al., 2016). However, the theories of product toxicity or self-destruction during the reaction cycle are challenged by the results obtained with low-density cells: the co-expression of GLD had minimal impact on biocatalyst performance at low density, but had a substantial impact on CYP153A6 stability.

This would seem to suggest a beneficial effect conveyed by the additional dehydrogenase. However, GLD co-expression may instead be impacting biocatalyst stability indirectly, by reducing the expression of CYP153A6. As the scatter plot in Figure 5.17 shows, the strongest predictor of CYP153A6 stability was simply the starting concentration of P450. Cultures with a higher initial P450 concentration tended to lose active CYP153A6 more rapidly, while cultures with a very low initial P450 concentration demonstrated enhanced enzyme stability. This suggests that resting cells were tending towards their limiting P450 concentration as the biotransformation progressed. Similar results have been found in previous studies on this system: Gudimich et al. (2012) carried out n-octane biotransformations with *Escherichia coli* expressing CYP153A6, and found that when the low-expression BL21(DE3)pLysE strain was used as host, initial P450 content was only $0.15 \mu\text{mol.g}_{\text{DCW}}^{-1}$ but remained relatively stable over 48 h of biotransformation. The higher-expression BL21(DE3) strain was shown to provide high initial P450 concentrations, but these concentrations were unstable, quickly falling towards $0.1 \mu\text{mol.g}_{\text{DCW}}^{-1}$. In other studies, whole cells with an initial CYP153A6 concentration of $0.1 - 0.2 \mu\text{M}$ ($0.025 - 0.067 \mu\text{mol.g}_{\text{DCW}}^{-1}$) showed no significant decrease in P450 concentration after 1 h of bioconversion (Koch et al., 2009).

The loss of active P450 concentration may indicate a very high degree of uncoupling and associated formation of hydrogen peroxide, subsequently inactivating the enzyme through oxidation of the porphyrin ring in the haem group (Schrewe et al., 2013a). Given that CYP153A6 suffers minimal uncoupling *in vitro* (Arnold et al., 2012), one possible explanation for high uncoupling rates *in vivo* would be that excess enzymes were competing for limited substrate, and those unable to bind substrate would, when supplied with reducing equivalents, instead activate bound oxygen molecules to form hydrogen peroxide, and subsequently self-destruct. However, this is at odds with our current understanding of the reaction cycle, which is supposed to be initiated by substrate binding (Meunier et al., 2004).

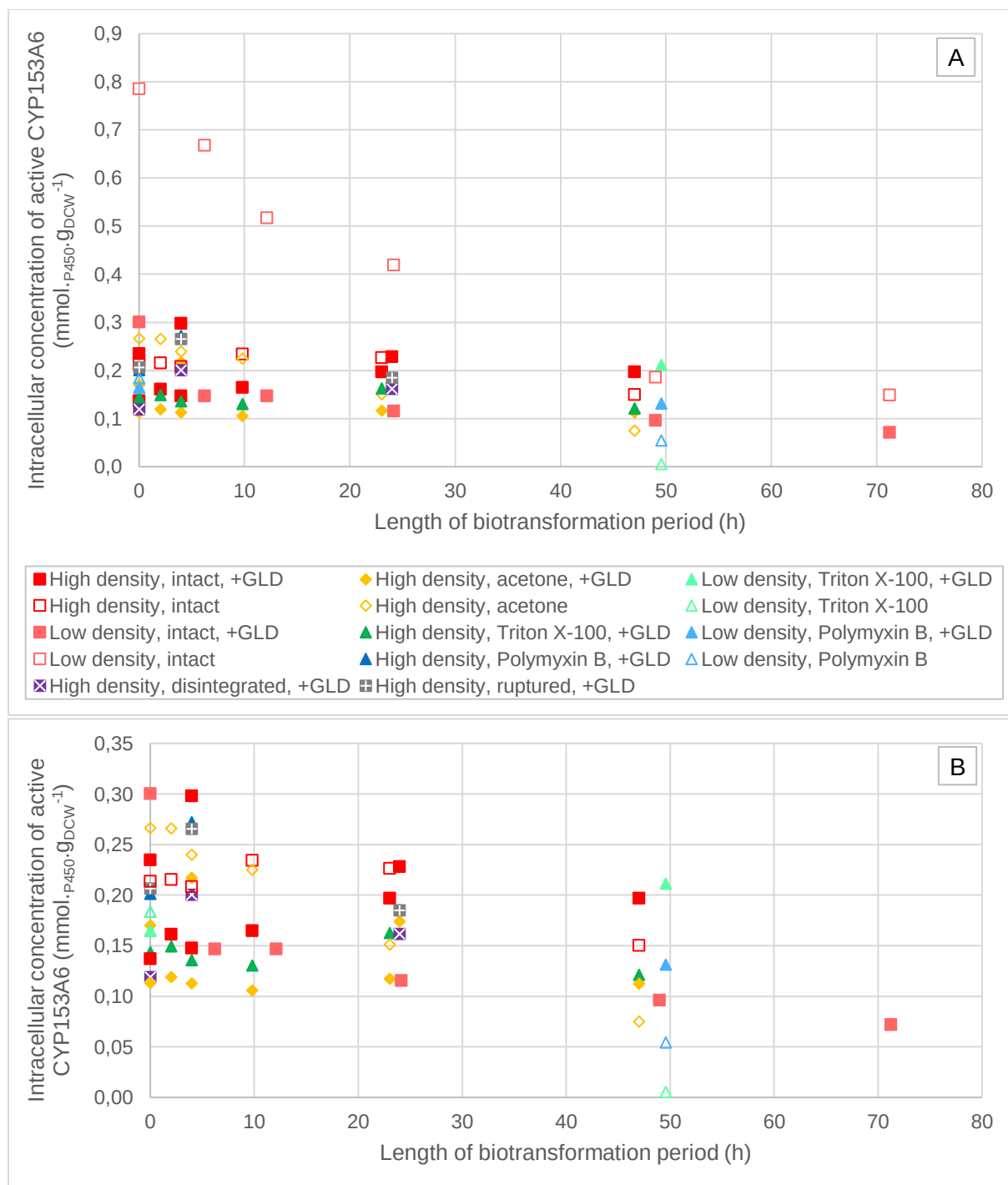


Figure 5.17: Intracellular concentrations of active CYP153A6 over the course of various resting-cell biotransformations. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ vector. Some cells were co-expressing glycerol dehydrogenase from a separate Duet vector (+GLD); other cells were carrying the empty Duet vector. For certain cultures, permeabilization was carried out according to the methods described in Section 3.3. Biotransformations were carried out in 40 mL amber vials. At least two vials were sacrificed for sampling per data point. Fig. 5.17-B is a magnified version of Fig. 5.17-A.

5.5.2 Stability of the whole-cell biocatalyst

As was shown in Figure 5.12, if GLD was co-expressed, high-density intact or chemically permeabilised cells ceased production after reaching titres of 15 – 25 mmol.L⁻¹, equivalent to yields on biomass of 0.5 – 0.6 mmol.g_{DCW}⁻¹. Meanwhile, low-density intact cells were only reaching 15 mmol.L⁻¹ after 72 h of biotransformation, but were producing yields on biomass in excess of 2.5 mmol.g_{DCW}⁻¹ and showing little or no signs of instability. Furthermore, as was shown in Figure 5.6, low-density and high-density cultures of intact cells attained similar initial rates of product formation per unit of biomass. Yet low-density cultures were able to maintain these initial rates for substantially longer than high-density cultures. This would suggest that biocatalyst instability was linked to volumetric product concentrations, rather than the amount of 1-octanol being produced per cell. However, as shown in Figure 5.9, ruptured or disintegrated cells (co-expressing GLD) reached titres of 40 – 50 mmol.L⁻¹, without losing much activity. This would suggest that it was not the absolute product concentration *per se* that caused the performance loss in intact or chemically permeabilised cells.

Taking data from all experiments of two days or longer, the space time yield after 10 – 12 h (mmol_{product}.L⁻¹.h⁻¹) was compared to the space time yield after 47 – 50 h. The difference was divided by the number of hours between the two sampling points, giving the average rate at which the biotransformation was levelling off (mmol_{product}.L⁻¹.h⁻²). For each system, this decrease in biocatalytic performance was plotted against the space time yield after 10 – 12 h (mmol_{product}.L⁻¹.h⁻¹), the average rate of product formation per unit biomass over the first 10 – 12 h (mmol_{product}.g_{DCW}⁻¹.h⁻¹), the turnover frequency after 10 – 12 h (mol_{product}.mol_{P450}⁻¹.min⁻¹), and the product titre (mmol.L⁻¹) and yield on biomass (mmol.g_{DCW}⁻¹) achieved after 47 – 50 h. Table 5.5 lists the R² values obtained from linear least squares regression of the plots of each of these various datasets.

Table 5.5: Rate of loss of biocatalytic performance (calculated by comparing space time yield after 10 – 12 h to space time yield after 47 – 50 h) was plotted against five key performance indicators, for 16 different biotransformation systems. Linear least squares regression was used to determine the likelihood of a correlation between variables.

Independent variable	R ² value from linear least squares regression
Space time yield after 10 – 12 h of biotransformation	0.92
Average rate of product formation per unit biomass over the first 10 – 12 h of biotransformation	0.18
Turnover frequency after 10 – 12 h of biotransformation	0.03
Volumetric titre after 47 – 50 h of biotransformation	0.18
Yield on biomass after 47 – 50 h of biotransformation	0.59

Space time yield after 10 – 12 h (which provides a good sense of the initial rate of product formation) was the only indicator to show a strong correlation with the rate of biocatalytic performance loss. A graphic representation of this data is given in Figure 5.18.

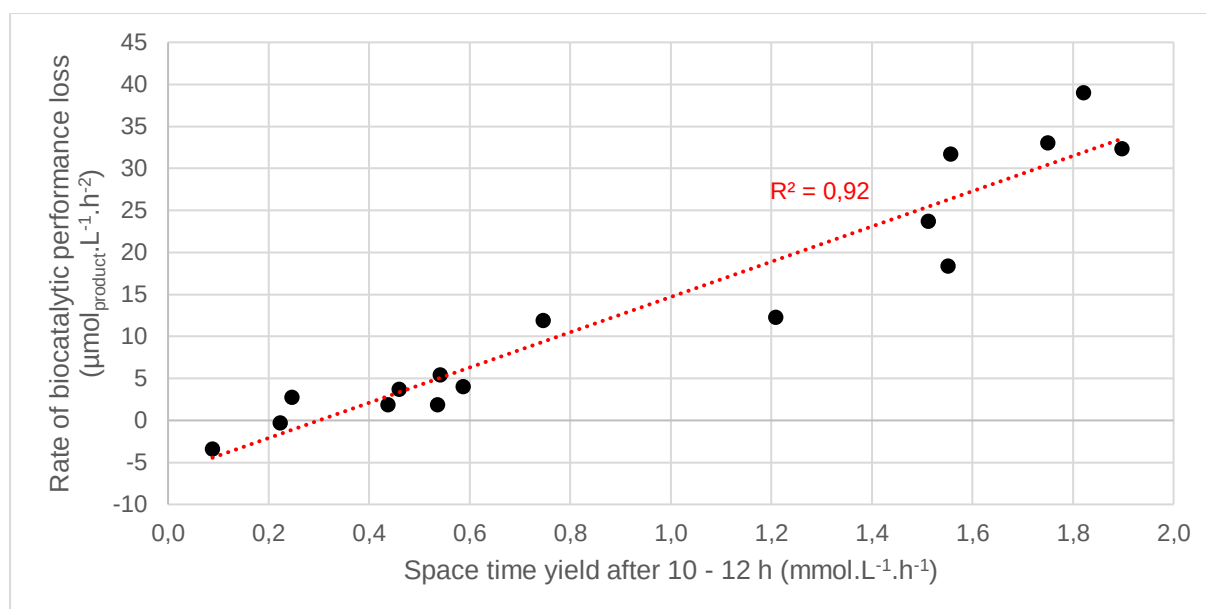


Figure 5.18: Rate of biocatalytic performance loss as a function of space time yield over the first 10 – 12 h of biotransformation. Data points were drawn from both low- and high-density systems, and include intact, mechanically broken, and chemically permeabilised cells.

Organic compounds with low $\log P_{OW}$ values are known to be highly toxic to biological systems; they disrupt the proton gradient by interfering with membrane structure, they divert cellular resources by prompting the upregulation of efflux protein expression, and they can interact with enzymes in detrimental ways (Bühler et al., 2008; Favre-Bulle et al., 1991; Kadisch et al., 2017). However, the data suggest that it was rate of concentration increase, rather than absolute volumetric concentration of 1-octanol, which was responsible for loss of performance. The fact that low-density cultures could tolerate specific reaction rates which high-density cultures could not, suggests that cells may be affected by the 1-octanol exported by their neighbours, and not only by the intracellular build-up due to their own hydroxylation activity. Alternatively, high density cultures may be experiencing additional limitations or stresses – such as oxygen limitation – which increases cells' vulnerability to substrate and product toxicity.

In previous investigations of toxicity in the CYP153A6 system, some cultures have been doped with up to 20 mM 1-octanol without inhibiting the initial reaction rate (Gudimanchi et al., 2012); but other cultures have demonstrated lower product formation rates following 1-octanol doping, with complete loss of hydroxylation activity when doping exceeded 30 mM 1-octanol. These toxicity effects were markedly less severe when the 1-octanol was generated by biocatalytic activity over an extended timeframe (Olaofe, 2013). Potentially, the lower volumetric production rates in low-density cultures allowed for more effective *in-situ* extraction of 1-octanol into a separate organic phase, while in the high-density cultures, some of the excreted 1-octanol diffused into the membranes of neighbouring cells before it could be extracted. Mechanically broken cells (whose membranes were already disrupted) may have been less vulnerable to the detrimental effects of hydrocarbon partitioning. Having a lower barrier to passive transport could also have enhanced *in situ* extraction rates. Conversely, Triton X-100 may have discouraged the partitioning of 1-octanol into the organic phase, exposing cells to higher concentrations of the toxin.

The overall physiological state of the cell has been postulated to be a key determinant of enzyme performance, and this physiology is vulnerable to substrate and product toxicity (Cornelissen et al., 2011; Olaofe et al., 2013). This study confirms that the stability of the CYP153A6 whole-cell system is linked to the accumulation of 1-octanol in the aqueous phase. It is shown that the rate at which the 1-octanol is produced, as well as the manner and degree to which the cells are permeabilised, determines the damage that the product will inflict on the cells.

5.5.3 By-product formation

The decision to compare cultures in terms of their total product formation, and not exclude the formation of octyl acetate by-product, was based on the fact that octyl acetate was not detected in biotransformations involving cells without the pACYCDuet plasmid, and besides the fact that CYP153A6 has been shown to be highly regioselective, displaying over 95% affinity for terminal carbon oxidation (Funhoff et al., 2006), the formation of octyl acetate did not match the expected metabolic pathway for alkane degradation, in which linear alkanes are oxidised to form primary alcohols, which are then converted into aldehydes, and finally into fatty acids suitable for catabolism via β -oxidation (Rojo, 2009). It therefore seemed unlikely that this by-product arose from over-oxidation of 1-octanol by CYP153A6. It may have been an artefact of the expression system: pACYCDuet contains a chloramphenicol resistance marker, expressing chloramphenicol acetyltransferase (CAT), which targets one of the hydroxyl groups present on the chloramphenicol molecule. CAT has been shown to acetylate perillyl alcohol resulting from the hydroxylation of limonene by a cytochrome P450 (Alonso-Gutierrez et al., 2013), so it is a likely agent in the acetylation of 1-octanol.

Octyl acetate was detected in all cultures of cells carrying the pACYCDuet plasmid; however, the extent of by-product formation varied depending on the type of membrane permeabilization treatment that had been applied. The extent of by-product formation in the various systems can be seen in Figure 5.19. Over two days, untreated whole cells without additional dehydrogenase produced 0.938 ± 0.019 moles of octyl acetate for every mole of 1-octanol produced. The co-expression of dehydrogenase encouraged the formation of by-product in untreated whole-cell systems, leading to product ratios of 1.434 ± 0.092 moles of octyl acetate per mole of 1-octanol.

However, cells with compromised membranes demonstrated reduced by-product formation. Ruptured cells displayed product ratios of $0.333 \pm 0.017 \text{ mol}_{\text{OA}} \cdot \text{mol}_{\text{C8OH}}^{-1}$ without additional dehydrogenase, and $0.190 \pm 0.018 \text{ mol}_{\text{OA}} \cdot \text{mol}_{\text{C8OH}}^{-1}$ with additional dehydrogenase. Disintegrated cell (with GLD) achieved product ratios of $0.145 \pm 0.023 \text{ mol}_{\text{OA}} \cdot \text{mol}_{\text{C8OH}}^{-1}$. Cell free extract displayed product ratios of $0.0478 \pm 0.0059 \text{ mol}_{\text{OA}} \cdot \text{mol}_{\text{C8OH}}^{-1}$ (without GLD) and $0.0644 \pm 0.0050 \text{ mol}_{\text{OA}} \cdot \text{mol}_{\text{C8OH}}^{-1}$ (with GLD). Exposure to acetone led to ratios of $0.865 \pm 0.023 \text{ mol}_{\text{OA}} \cdot \text{mol}_{\text{C8OH}}^{-1}$ (without GLD) and $0.820 \pm 0.073 \text{ mol}_{\text{OA}} \cdot \text{mol}_{\text{C8OH}}^{-1}$ (with GLD), while cells exposed to Triton X-100 (with GLD) produced $0.833 \pm 0.012 \text{ mol}_{\text{OA}} \cdot \text{mol}_{\text{C8OH}}^{-1}$.

The formation of octyl acetate provides an indication of how easily hydrocarbons could enter and leave the cell. Difficulty of hydrocarbon efflux would have resulted in the 1-octanol produced by the P450 remaining in the cytoplasm for longer periods, increasing its chances of coming into contact with chloramphenicol acetyl transferase. Mechanically broken cells showed much less by-product formation than whole cells. The low by-product formation by ruptured and disintegrated cells is particularly significant, since these cells produced the highest titres, and selectivity in biotransformations typically decreases as titres increase (Tieves et al., 2016). Exposure to acetone or Triton X-100 also reduced by-product formation slightly. The results support the hypothesis that by-product formation is driven by accumulation of product within the cytoplasm, as has been suggested for the case of 1-dodecanol over-oxidation (Grant et al., 2012, 2011).

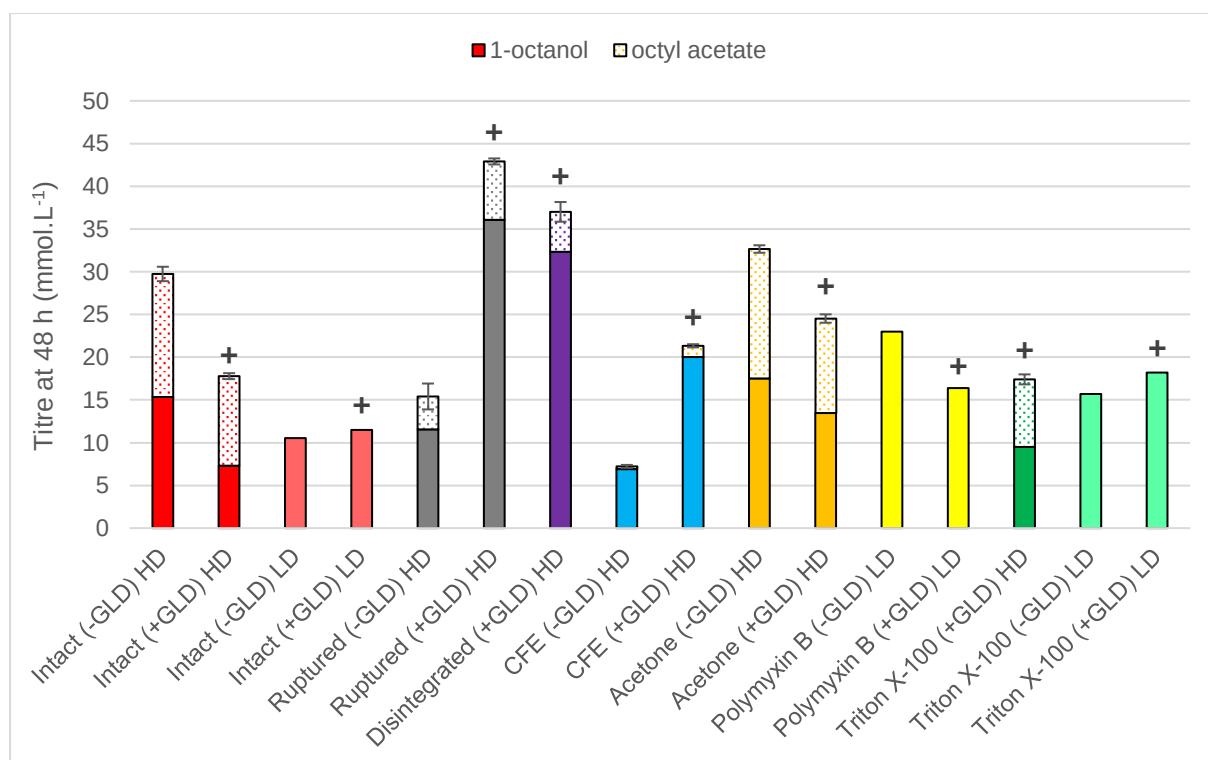


Figure 5.19: Volumetric titre following two days of biotransformation with various cell preparations. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. Crosses above data bars indicate that cells were co-expressing glycerol dehydrogenase (+GLD); cells in other cultures carried the empty Duet vector (-GLD). Biotransformations were carried out with 1 mL of resting cell suspension and 200 μ L of n-octane in 40 mL amber vials. Vials were sacrificed for sampling at each time point; error bars represent the standard deviation across at least two vials. HD: high density (31.6 ± 4.2 g_{DCW}.L⁻¹). LD: low density (4.55 ± 0.13 g_{DCW}.L⁻¹). High-density cultures were expressing GLD from the pACYCDuet plasmid; low-density cultures were expressing GLD from the pCDFDuet plasmid.

5.6 Conclusions from chemically and mechanically permeabilised cells

In literature, permeabilization of the outer membrane has been shown to result in improved biotransformation of various hydrophobic substrates (Call et al., 2016; Canovas et al., 2005; Funhoff et al., 2006; Grant et al., 2012, 2011; Julsing et al., 2012; Kadisch et al., 2016; Ladkau et al., 2016; Ni and Chen, 2005; Pennec et al., 2015; Ruff et al., 2016). The co-expression of dehydrogenases for cofactor regeneration has also yielded substantial performance improvements in some cases (Kataoka et al., 1999; Mouri et al., 2006; Schewe et al., 2008). This study tested various combinations of membrane permeabilization and glycerol dehydrogenase co-expression in a recombinant *E. coli* system for hydroxylation of n-octane to 1-octanol by CYP153A6.

When cell density was high and glycerol dehydrogenase was being co-expressed, a rapid cessation of product formation was observed between 10 h and 23 h for intact cells, acetone-washed cells, and cells mixed with Triton X-100. This contributed to lower final titres, relative to their counterparts without GLD. On the other hand, when GLD was *not* present, ruptured cells achieved significantly lower rates of product formation than intact cells. Membrane breakage and co-expression of glycerol dehydrogenase were both harmful when applied singly, yet ruptured or disintegrated cells co-expressing GLD produced higher titres after 48 h than any other system tested, largely due to them having more stable rates of product formation relative to other high-density cultures. This highlights the importance of a *correctly balanced* cofactor pool. When cells did not have sufficient access to NADH, biotransformation was impeded (as happened with mechanically broken cells without GLD, or with cell free extracts). Co-expression of GLD was necessary in highly permeabilised cells, in order to meet the demand arising from increased access to substrate, while also compensating for NADH lost via diffusion or the disruption of cell metabolism. However, when cells were not sufficiently permeabilised, GLD

co-expression decreased the stability of the whole-cell biocatalyst (as happened with intact cells and acetone-washed cells).

The loss of stability in the biocatalytic system was not due to loss of active CYP153A6, since intact and chemically permeabilised cells maintained stable P450 concentrations when GLD was present. Nor was the instability due to product accumulation, either volumetric or intracellular. Intact cells with and without GLD followed an almost-identical course of reaction between 0 h and 10 h, yet cells with GLD lost activity much more quickly thereafter. Acetone-washed cells accumulated product slightly slower when GLD was present, but this did not prevent them from ceasing production after 23 h. The results suggest that the additional glycerol dehydrogenase was directly responsible for the instability of intact or poorly-permeabilised cells. Possibly, the enhanced capacity for NADH regeneration skewed the natural balance of the cell's cofactor pool, impairing cellular functions or leading the cell to increase its rates of oxygen consumption beyond the capacity of the vial system. Too much NADH proved as harmful as too little.

Low-density systems generally produced lower titres and space time yields. However, when biomass concentration decreased by a factor of seven, titres decreased by only three-fold, suggesting that individual cells were twice as productive in low-density systems. They were also much more stable biocatalysts, even when co-expressing glycerol dehydrogenase. Loss of biocatalytic activity was strongly linked to the volumetric rate of 1-octanol production. High-density and low-density cultures of intact cells achieved similar rates of product formation per unit of biomass, yet only the high-density cultures experienced significant instability. On the other hand, high-density cultures of permeabilised cells were able to accumulate high titres, indicating that the reduced stability of other high-density cultures was not directly proportional to product concentration. This suggests that it is necessary to strike a balance between maximising biocatalyst productivity and maximising product titre.

Significant variations in the concentration and stability of CYP153A6 had no effect on biocatalytic performance, indicating that CYP153A6 was still above its reaction-limiting concentration in these biotransformations, even after the transport of substrate and product and the regeneration of NADH had been enhanced. In this system, limitations in transport were found to be more critical than limitations in cofactor. This is evident from the high titres obtained using mechanically broken cells, as well as the general ineffectiveness of glycerol dehydrogenase co-expression in intact or chemically permeabilised systems. Overcoming the physical barrier of the cell envelope proved to be the most effective means of improving the stability of the biocatalytic system and achieving high product titres.

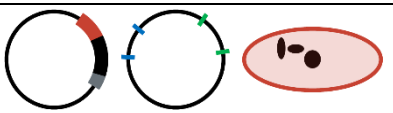
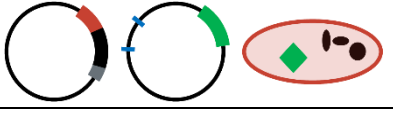
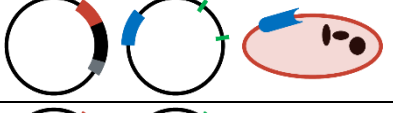
5.7 Expression of the membrane channel protein AlkL

From a process perspective, the use of mechanically broken cells would necessitate additional upstream processing steps, while the addition of chemical permeabilising agents would add significantly to raw materials costs, and might complicate downstream processing. Consequently, finding a biological approach to adjust membrane integrity is highly desirable. In the alkane-metabolising *Pseudomonas oleovorans*, the regulatory protein AlkS induces transcription of the genes necessary for the transformation of alkanes into fatty acids, along with other genes not directly associated with alkane catabolism, but shown to be beneficial for growth on alkanes (van Beilen et al., 2001). One of these, known as AlkL, was shown to be a membrane channel protein that boosts the transport of hydrophobic substrates (Julsing et al., 2012). Since then, AlkL has been successfully applied in numerous heterologous systems carrying out biotransformation of hydrophobic substrates (Cornelissen et al., 2013; Grant et al., 2014; Ladkau et al., 2016; Scheps et al., 2013; Schrewe et al., 2014; van Nuland et al., 2016). The effects of AlkL on this study's biotransformation system were therefore tested; the experiments also investigated whether enhanced cofactor regeneration capacity would be beneficial in the presence of AlkL.

Four heterologous *E. coli* strains were tested. All strains contained the CYP operon, consisting of CYP153A6 and its native ferredoxin and ferredoxin reductase, on pET28b+; alongside the pCDFDuet vector. In the control strain, designated C, pCDFDuet was empty; in the strain designated CG,

pCDFDuet contained glycerol dehydrogenase; in the strain designated CA, pCDFDuet contained AlkL; and in the strain designated CAG, pCDFDuet contained both glycerol dehydrogenase and AlkL.

Strains used in this section of work:

<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF		C
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(GLD)		CG
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(AlkL)		CA
<i>E. coli</i> BL21(DE3) containing pET(CYP) and pCDF(AlkL+GLD)		CAG

Four separate biological replicates were performed to compare the performance of the four strains. Two of these biotransformations ran for two days, and two more were extended over five days.

5.7.1 Variability in enzyme expression and stability between strains

As discussed in Section 4.2, the co-expression of glycerol dehydrogenase and AlkL depressed the expression of active CYP153A6. In order to achieve similar intracellular P450 concentrations across the four biocatalyst strains, the availability of δ -aminolevulinic acid during growth and expression was modified for each culture. The culture medium for strain CAG contained 1 mM δ -ALA, compared to 0.1 mM for strains CA and CG, and 0.05 mM δ -ALA for strain C. Nevertheless, at the start of the biotransformation period, the intracellular P450 concentration in strain CA was lower than in strain C by a factor of 1.8, and the intracellular P450 concentration in strain CAG was lower than in strain CG by a factor of 1.7. The average concentrations of active CYP153A6 across the experiments are summarised in Table 5.6. Starting concentrations were above $0.06 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ but below $0.18 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$, so while biotransformation is unlikely to have been limited by enzyme availability, such limitations cannot be ruled out (see Section 4.3).

In the case of the two-day experiments, the final concentration of active CYP153A6 had an average value of $0.093 \pm 0.030 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$. This meant that P450 concentrations were relatively stable in the strains with the lowest starting concentrations (CA and CAG), while strains C and CG lost roughly 50% of their active enzyme over the two-day period, as all strains converged towards $0.1 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$.

P450 concentrations measured after 121 h of biotransformation showed that cells lost 60 – 75 % of their active enzyme over the five-day period, with larger losses in strains co-expressing glycerol dehydrogenase or AlkL. Final P450 concentrations had dropped to below $0.06 \mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ for the co-expressing strains, meaning that the five-day biotransformations were likely to have been limited by enzyme availability in their final stages.

Table 5.6: Active CYP153A6 concentration at the beginning and end of the biotransformation period, for systems with varying levels of cofactor regeneration capacity and alkane transport capacity. Values at 0 h are the average of all four biological replicates. Values at 49 h are the average of biological replicates A and B. Values at 121 h are taken from biological replicate D. Each biological replicate included 2-3 sample replicates.

Strain	Average starting concentrations		Average concentrations after 49 ± 1 h		Average concentrations after 121 ± 1 h	
	μM	μmol _{P450} ·g _{DCW} ⁻¹	μM	μmol _{P450} ·g _{DCW} ⁻¹	μM	μmol _{P450} ·g _{DCW} ⁻¹
C	0.797 ±0.036	0.178 ±0.012	0.355 ±0.076	0.094 ±0.020	0.372 ±0.033	0.071 ±0.007
CG	0.616 ±0.012	0.138 ±0.013	0.345 ±0.001	0.087 ±0.013	0.189 ±0.072	0.049 ±0.019
CA	0.368 ±0.015	0.099 ±0.006	0.272 ±0.089	0.099 ±0.033	0.179 ±0.013	0.038 ±0.003
CAG	0.299 ±0.021	0.082 ±0.009	0.242 ±0.139	0.091 ±0.0550	0.107 ±0.040	0.022 ±0.008

5.7.2 Production of 1-octanol

Key performance indicators for each strain are presented in Figure 5.20, in the form of average space time yields and yields on biomass after one, two or five days of biotransformation. As in other experiments with low-density cultures, the co-expression of glycerol dehydrogenase was not harmful to biocatalyst stability, and actually improved performance slightly, relative to unmodified whole cells. Strain CG achieved an average space time yield of 0.299 ± 0.032 mmol_{product}·L⁻¹·h⁻¹ over the first day of biotransformation, equivalent to a yield on biomass of 1.71 ± 0.19 mmol·g_{DCW}⁻¹. This was an increase of 11-13% relative to strain C. The advantage was maintained throughout the biotransformation, with both strains demonstrating comparable stability, as shown by the comparable decreases in space time yield shown in Figure 5.20.

The co-expression of AlkL (strain CA) provided consistent performance enhancements relative to both unmodified whole cells (strain C) and cells co-expressing dehydrogenase (CG). At the one-day mark, strain CA achieved a space time yield of 0.374 ± 0.026 mmol·L⁻¹·h⁻¹, 25% more than strain CG, and 39% more than strain C. The results were even more impressive when the slightly lowered cell density of the CA culture was taken into account. After 26 h, strain CA achieved a yield on biomass of 2.57 ± 0.20 mmol·g_{DCW}⁻¹; 51% more than strain CG, and 70% more than strain C. However, strain CA was slightly less stable than strains C or CG. Thus, the advantages of AlkL co-expression were more muted after two or five days of biotransformation, although strain CA outperformed strains C and CG at both time points.

Both AlkL co-expression and glycerol dehydrogenase co-expression had improved biocatalyst performance when applied separately, but the simultaneous co-expression of AlkL and glycerol dehydrogenase was not beneficial. Relative to strain C, strain CAG produced roughly the same yield on biomass after either one, two, or five days. Because the CAG cultures typically had a slightly lower cell density than the C cultures, their space time yields were 12-16% lower on average. However, strains C and CAG demonstrated comparable stability across five days of biotransformation.

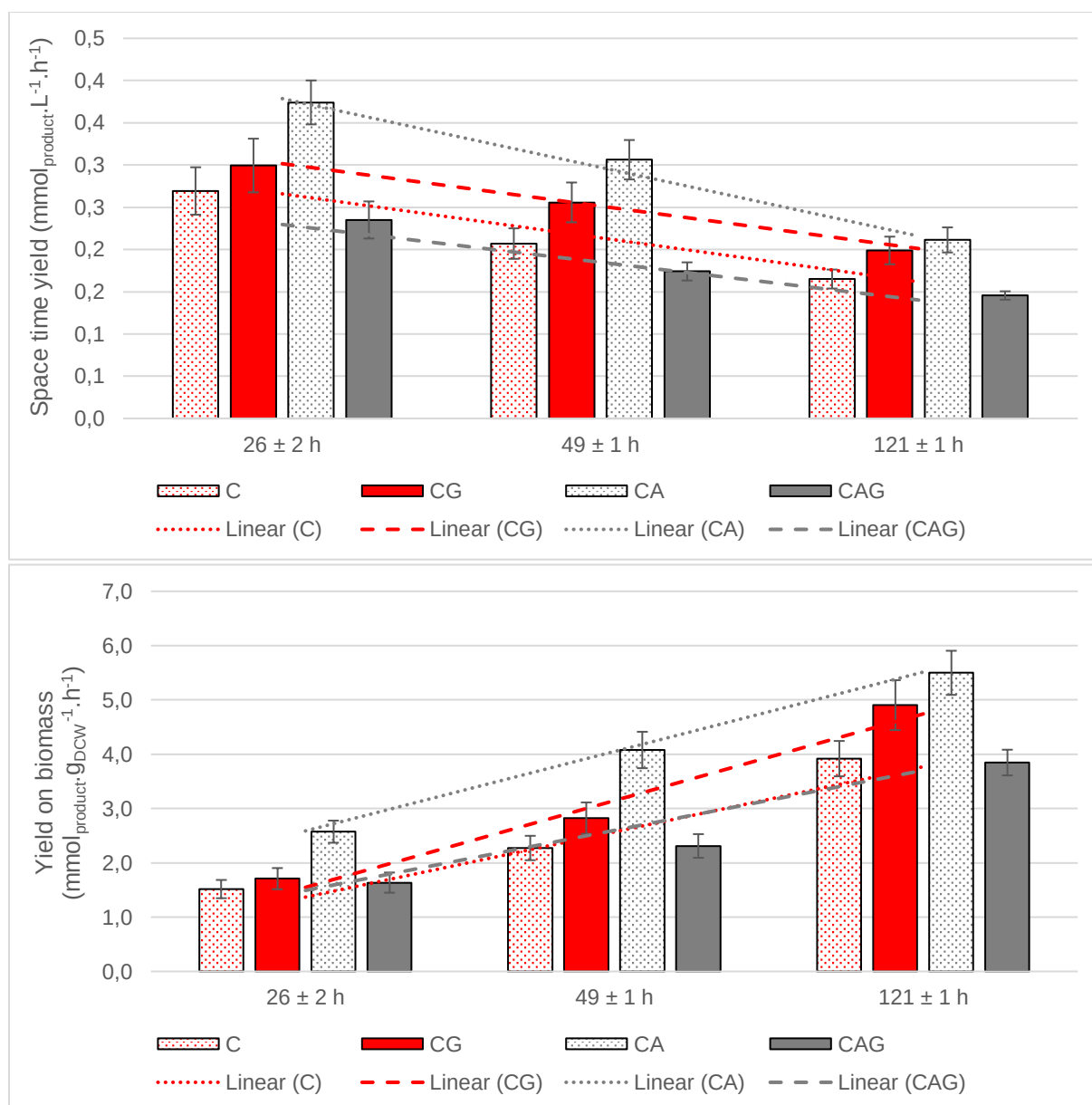


Figure 5.20: Space time yields and yields on biomass over two to five days of biotransformation, for four strains expressing different combinations of heterologous proteins. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. Strain C was also carrying the empty pCDFDuet vector, Strain CG was co-expressing glycerol dehydrogenase on pCDFDuet, Strain CA was co-expressing AlkL on pCDFDuet, and Strain CAG was co-expressing both glycerol dehydrogenase and AlkL on pCDFDuet. The concentrations of δ -ALA and FeCl_3 in the growth and expression media were used to control the intracellular concentration of CYP153A6 to comparable levels across all strains. Biotransformations were carried out in 40 mL vials at 20°C and 200 rpm, using 1 mL of cells suspended in phosphate buffer (average cell density $4.07 \pm 1.04 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$), 0.2 mL of n-octane and 0.1 mL of BEHP. Vials were sacrificed at each time point for sampling, with three vials per strain per sample point. Values at 26 h and 49 h are the average of all four biological replicates. Values at 121 h are the average of two biological replicates.

Figure 5.21 and Figure 5.22 present the data for each of the four biological replicates, in the form of yield on biomass over the course of the biotransformation period. There was some variability in performance between biological replicates. Strain CA was consistently the best performer, but its yields on biomass after two days varied from $3.13 \pm 0.15 \text{ mmol} \cdot \text{g}_{\text{DCW}}^{-1}$ to $5.37 \pm 0.32 \text{ mmol} \cdot \text{g}_{\text{DCW}}^{-1}$. Yields on biomass for strains C, CG, and CAG were consistently around $2 - 3 \text{ mmol} \cdot \text{g}_{\text{DCW}}^{-1}$; sometimes these three strains behaved very similarly over the first two days, and sometimes their yields on biomass diverged.

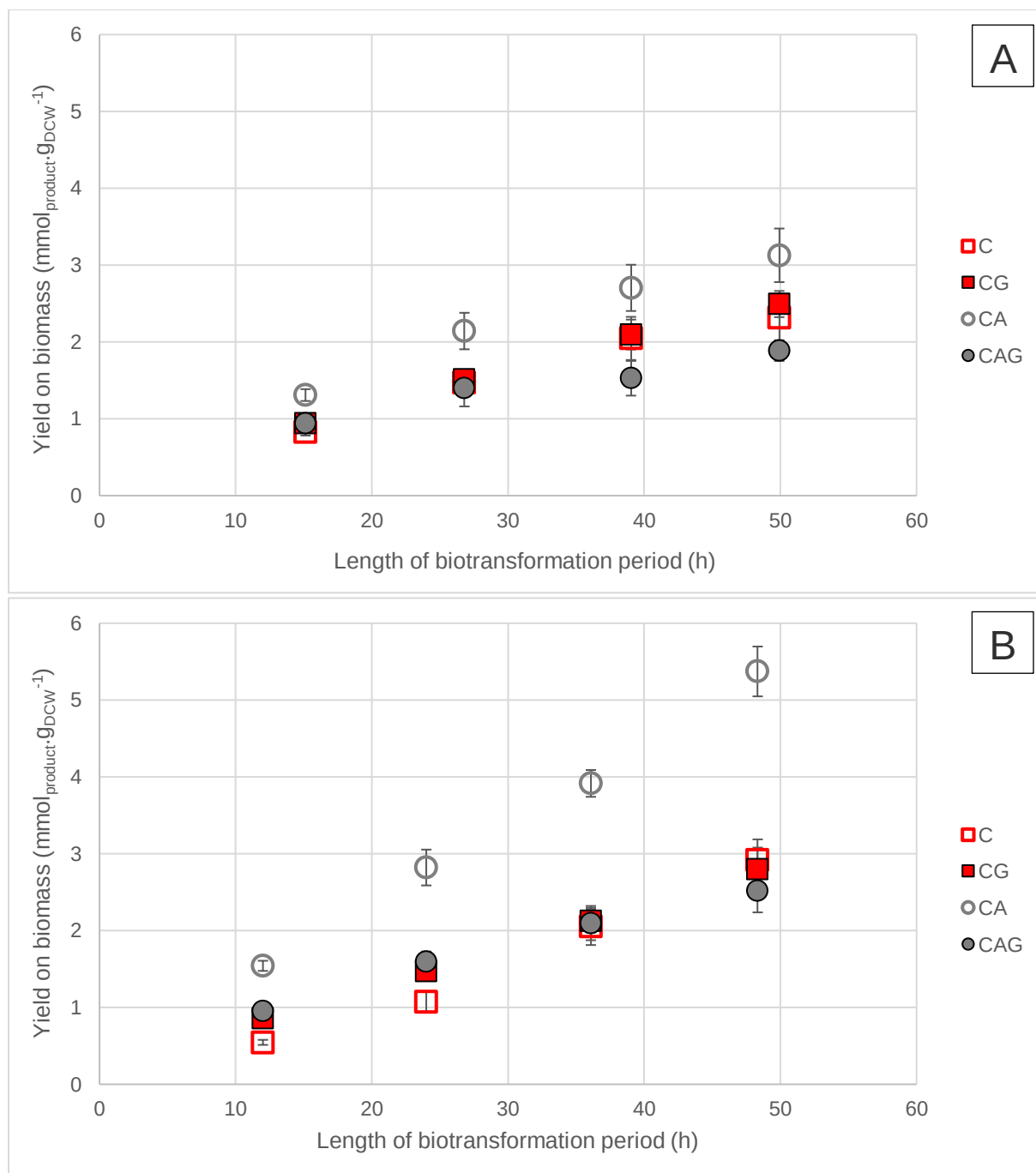


Figure 5.21: Effects of AlkL and glycerol dehydrogenase co-expression on 1-octanol formed per unit of biomass over two days of biotransformation, for four strains expressing different combinations of heterologous proteins. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. Strain C was also carrying the empty pCDFDuet vector, Strain CG was co-expressing glycerol dehydrogenase on pCDFDuet, Strain CA was co-expressing AlkL on pCDFDuet, and Strain CAG was co-expressing both glycerol dehydrogenase and AlkL on pCDFDuet. The concentrations of δ -ALA and FeCl_3 in the growth and expression media were used to control the intracellular concentration of CYP153A6 to comparable levels across all strains. Biotransformations were carried out in 40 mL vials at 20°C and 200 rpm, using 1 mL of cells suspended in phosphate buffer (average cell density $4.07 \pm 1.04 \text{ g}_{\text{DCW}} \cdot \text{L}^{-1}$), 0.2 mL of n-octane and 0.1 mL of BEHP. Vials were sacrificed at each time point for sampling. Error bars represent the standard deviation in three sample replicates (i.e. three vials).

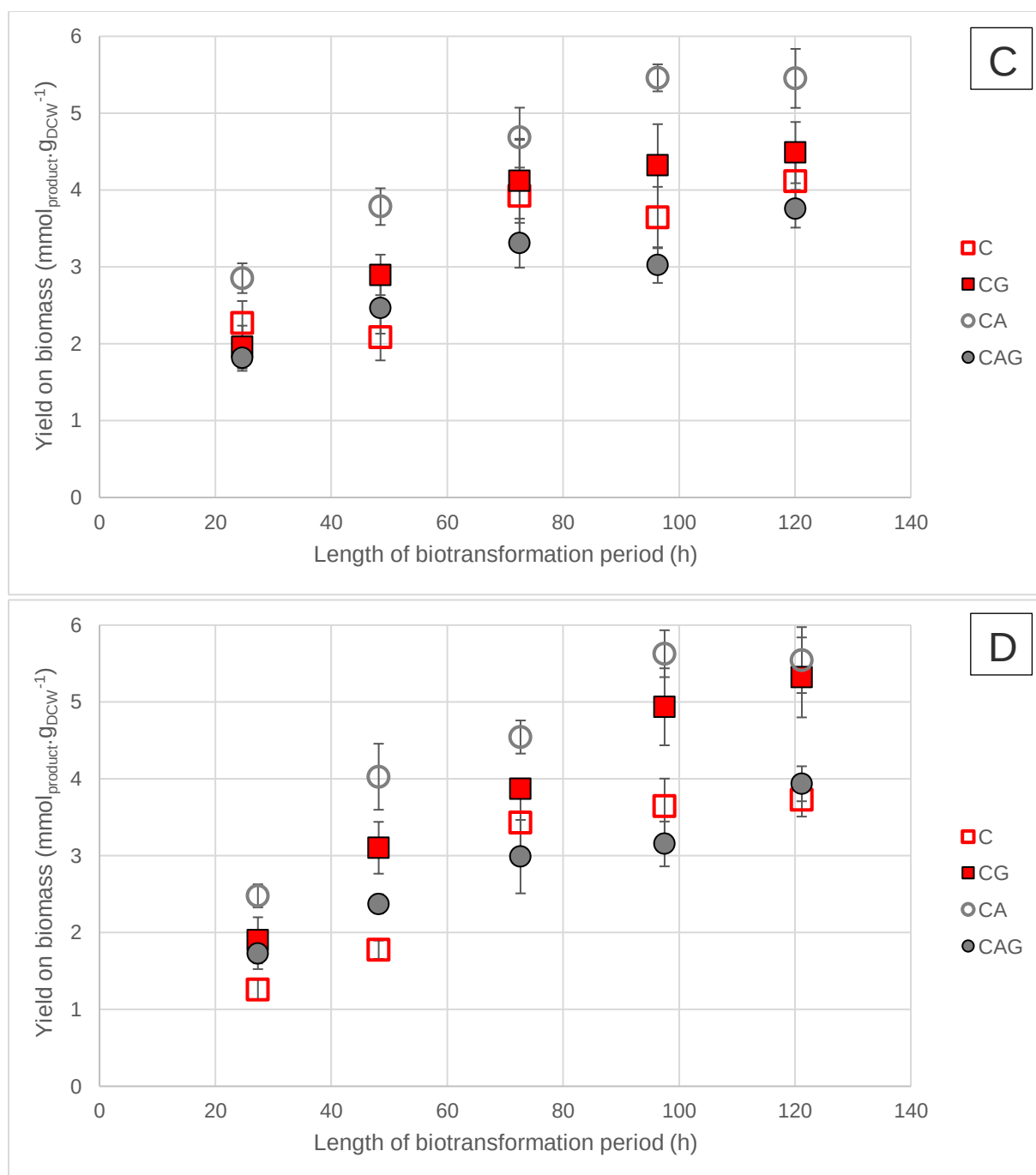


Figure 5.22: Effects of AlkL and glycerol dehydrogenase co-expression on 1-octanol formed per unit of biomass over five days of biotransformation, for four strains expressing different combinations of heterologous proteins. All cells were expressing CYP153A6, FdR, and Fdx from the pET28b+ plasmid. Strain C was also carrying the empty pCDFDuet vector, Strain CG was co-expressing glycerol dehydrogenase on pCDFDuet, Strain CA was co-expressing AlkL on pCDFDuet, and Strain CAG was co-expressing both glycerol dehydrogenase and AlkL on pCDFDuet. The concentrations of δ -ALA and FeCl₃ in the growth and expression media were used to control the intracellular concentration of CYP153A6 to comparable levels across all strains. Biotransformations were carried out in 40 mL vials at 20°C and 200 rpm, using 1 mL of cells suspended in phosphate buffer (average cell density 4.07 ± 1.04 g_{DCW} · L⁻¹), 0.2 mL of n-octane and 0.1 mL of BEHP. Vials were sacrificed at each time point for sampling. Error bars represent the standard deviation in three sample replicates (i.e. three vials).

The variation between biological replicates cannot be attributed to differences in the concentration of active CYP153A6. The initial and final intracellular P450 concentrations for each strain were the same between Replicates A and B (within error), yet all four strains performed better in Replicate B. While it is not clear what led to the improved performance, it is clear that strain CA was able to derive the most

advantage from it – its yield on biomass improved by 72% between replicates A and B, as opposed to improvements of 12% – 33% for other strains. The differences in performance can most likely be attributed to small differences in the metabolic state of the cells between replicates. For example, Gudiminch et al. (2012, 2011) have shown that differences in the cell's starting cofactor pool affect the initial activity of whole cell biocatalysts. In Replicate B, strain CA might have harnessed an improved cofactor pool to counter the negative side-effects associated with AlkL, while the enhanced cofactor regeneration capacity of strains CG and CAG meant that these cells had less to gain from improvements in the starting cofactor pool. Similarly, a reduced starting cofactor pool in Replicate D might explain why strain CG outperformed strain C in this case, where previously the two strains had performed very similarly.

The co-expression of AlkL has been shown to improve the hydroxylation activity of AlkB on n-octane by up to tenfold in *E. coli* (Grant et al., 2014; Julsing et al., 2012). For members of the CYP153 family expressed in *E. coli*, hydroxylation of limonene could be increased 5-fold (Cornelissen et al., 2013) and ω -oxidation of C₁₂ fatty acid methyl ester could be increased 3-fold (Scheps et al., 2013). In this study, the co-expression of AlkL alongside CYP153A6 in *E. coli* increased the hydroxylation of n-octane by 40 – 80 % relative to unmodified whole cells. On a volumetric basis, the benefits of AlkL were somewhat attenuated, but this was mainly due to the fact that culture densities were slightly lower in strains CA and CAG, compared to strains C and CG.

As in previous experiments, the co-expression of glycerol dehydrogenase could lead to performance improvements in cells without AlkL, but the benefits of dehydrogenase co-expression varied considerably between biological replicates, with dehydrogenase providing no significant benefit in some experiments.

Strain CAG performed very poorly, suggesting that the metabolic burden of expressing so many heterologous proteins left cells ill-equipped to carry out biotransformation. Studies on several other *in vivo* alkane hydroxylase systems suggest that the heterologous expression of AlkL has detrimental effects on biocatalyst stability (Call et al., 2016; Grant et al., 2014; Kadisch et al., 2016; Ladkau et al., 2016). However, strain CAG did not appear to be substantially less stable than strains C or CG, judging by changes in space time yields between one, two, and five days. The poor performance of strain CAG may be due to limitations in enzyme availability, as the expression of active CYP153A6 in strain CAG was consistently the lowest of all the strains.

It is particularly interesting to note that strain CA was able to achieve yields on biomass similar to those achieved by low-density cells mixed with Triton X-100, especially since AlkL co-expression reduced the availability of active CYP153A6, probably to limiting concentrations. This strongly suggests that cross-membrane transport of n-octane is a more serious limitation than interphase transport, even in vial systems where mixing characteristics are sub-optimal.

Given that proper control of AlkL expression has allowed systems to maintain high cell densities, with associated increases in overall yields (Grant et al., 2014), it stands to reason that there may be considerable room to improve the performance of the CA strain via genetic engineering. Furthermore, the benefits of AlkL have been shown to become more substantial as substrate chain length increases (Grant et al., 2014; Julsing et al., 2012; van Nuland et al., 2016). Altogether, this suggests that the use of AlkL in the CYP153A6 whole-cell system is worth exploring further.

6 Concluding Remarks

6.1 Summary of key results

Whole-cell systems present numerous potential choke points to a biotransformation process; this is especially true when considering the multi-component heterologous systems necessary for hydroxylation of linear alkanes. The system investigated in this study consisted of resting *Escherichia coli* BL21(DE3) cells expressing the CYP operon from *Mycobacterium* sp. HXN-1500, which includes the alkane hydroxylase CYP153A6, the ferredoxin Fdx, and the ferredoxin reductase FdR. The concentrations of CYP153A6, Fdx, and FdR; the ease of access these enzymes have to one another and to the cellular cofactor NADH; the regeneration of NADH; the supply of molecular oxygen; and the transport of hydrocarbon substrate and products across both the cell membrane and the aqueous-organic phase interface – any or all these factors could plausibly limit the hydroxylation reaction. This study focused on CYP153A6 expression, oxygen supply, cofactor regeneration capacity, and cross-membrane transport of hydrocarbons.

6.1.1 Expression and efficacy of CYP153A6 in intact whole cells

The intracellular concentration of active CYP153A6 could be controlled by varying the amounts of ferric chloride (FeCl_3 , a source of iron for incorporation into haem) and aminolevulinic acid (δ -ALA, a precursor molecule in the haem formation pathway) added to the growth and expression medium. Increasing the concentration of either chemical led to small improvements in the level of active CYP153A6 expressed, but a simultaneous increase in the concentrations of both chemicals was necessary to obtain maximum expression. The two precursors interacted synergistically up to concentrations of 100 μM FeCl_3 and 0.5 – 0.6 mM δ -ALA; further addition of FeCl_3 was detrimental to enzyme expression, while further addition of δ -ALA up to 1 mM continued to be effective, but offered diminishing returns. It appeared that excess precursors were being channelled into the formation of inactive forms of the protein.

Strains co-expressing additional heterologous proteins (glycerol dehydrogenase, GLD, and/or AlkL) demonstrated reduced expression of CYP153A6 at equivalent levels of precursor addition, and an increased tendency for CYP153A6 to be expressed in the inactive form. The negative effect of AlkL was greater than that of GLD, with the most severe impact arising in cells expressing both AlkL and GLD alongside the CYP153A6/FdR/Fdx.

Ultimately, however, intracellular P450 concentration in the range of 0.18 – 3.13 $\mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$ had no significant impact on the biocatalytic performance of resting whole cells under the biotransformation conditions explored. Six cultures of intact resting cells were tested at the same time and under identical conditions, except for the amount of FeCl_3 and δ -ALA added to the growth and expression medium. The six cultures therefore displayed a range of intracellular CYP153A6 concentrations: the highest levels of P450 expression were produced by cells grown with 0.5 mM δ -ALA and 100 μM FeCl_3 , and ranged from 0.31 – 3.13 $\mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$; cells which received no supplementation with haem precursors produced the lowest levels of P450, ranging from 0.18 – 1.15 $\mu\text{mol}_{\text{P450}}.\text{g}_{\text{DCW}}^{-1}$. No significant differences were observed in volumetric titre, space time yield, or yield on biomass between any of the six cultures. Apparent turnover frequencies in all six cultures, at every time point measured, were well below their theoretical maximum of 90 min^{-1} (Funhoff et al., 2006) and showed no signs of reaching a plateau indicative of limitations in enzyme loading.

6.1.2 Oxygen supply

Prior research has demonstrated high rates of interphase oxygen transfer in the vial apparatus used in this study, especially in the presence of alkanes (Meissner, 2013). To confirm that the biotransformation was not limited by oxygen limitations, the quantity of oxygen available was varied in one of two ways: by periodically replenishing the headspace of selected vials, or by varying the size of the vials in which biotransformations were carried out (40 mL vs 60 mL total volume, keeping cross-sectional area constant). In both cases, vials with additional oxygen supply did not outperform those without.

6.1.3 Regeneration of NADH

To determine whether biotransformation was limited by the supply of NADH cofactor, the NADH regeneration capacity of the host cells was increased via co-expression of glycerol dehydrogenase (GLD). Strains with and without additional dehydrogenase were applied as cultures of resting whole cells. Dehydrogenase co-expression did not improve the initial rates of 1-octanol production in intact cells. The co-expression of GLD did lead to minor improvements in the stability of the biocatalytic system in cultures where cell density was low, but stability was significantly reduced in cultures where cell density was high, with biotransformation ceasing entirely between 10 and 24 h.

6.1.4 Cross-membrane transport of hydrocarbons

Cross-membrane transport of hydrocarbons was facilitated via mechanical breakage of the cell wall, exposure to permeabilising chemicals, or co-expression of the membrane channel protein AlkL. Mechanical breakage did not improve initial rates of product formation relative to intact cells, but led to substantial improvements in biocatalyst stability in strains which were co-expressing glycerol dehydrogenase. High-density cultures of mechanically broken cells were still producing 1-octanol after 72 h of biotransformation, while intact or chemically permeabilised cells had lost most or all of their biocatalytic activity by 48 h. Consequently, ruptured cells co-expressing GLD produced the highest titres of any system tested (52.2 mM). Cell free extract also outperformed intact cells in strains with enhanced cofactor regeneration capacity, but underperformed relative to intact cells in strains without additional dehydrogenase.

To further investigate cross-membrane transport effects, cells were permeabilised via acetone washing, or via exposure to Polymyxin B or Triton X-100 in the biotransformation buffer. Chemical permeabilization consistently improved biocatalyst performance relative to intact cells of the same strain. High-density cultures treated with Triton X-100 achieved higher space time yields than any other system tested ($4.30 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$), although these cells lost activity rapidly and thus could not match the titres achieved by mechanically broken cells co-expressing GLD. As with intact cells, additional glycerol dehydrogenase was typically associated with reductions in biocatalyst stability in high-density cultures of chemically permeabilised cells; in low-density cultures, co-expression of GLD was not necessarily harmful, but also did not provide significant benefits.

The co-expression of AlkL significantly boosted initial hydroxylation rates per cell, despite suppressing CYP153A6 expression substantially. Furthermore, AlkL co-expression had no adverse effects on the stability of the biocatalytic system. This resulted in the highest activities ($21.9 \text{ mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$) and yields on biocatalyst ($5.46 \text{ mmol}_{\text{product}} \cdot \text{g}_{\text{DCW}}^{-1}$) of any system tested.

6.2 Re-examination of hypotheses

Hypothesis 1: The intracellular concentration of CYP153A6 will increase in proportion to the concentrations of haem precursors that are added to the medium during the growth and expression phase. However, due to limitations in substrate and cofactor availability, increasing concentrations of CYP153A6 will not improve biocatalyst activity. Meanwhile, the expression of additional proteins will increase the metabolic burden on the cell. Consequently, biocatalyst performance in terms of maximum titre, yield on biocatalyst, and space time yield will decrease with increasing CYP153A6 concentration above a critical value.

The increased concentration of CYP153A6 with increasing haem precursors was confirmed to be the case for haem precursor concentrations up to 1 mM δ -ALA and 100 μM FeCl_3 , provided the supply of both precursors was increased in tandem. Results also showed that cultures of intact cells with enzyme concentrations ranging from 0.18 – 3.13 $\mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$ achieved similar titres and yields on biomass regardless of intracellular enzyme concentration, confirming that excess expression of CYP153A6 did not augment activity.

Hypothesis 2: When a heterologous glycerol dehydrogenase is co-expressed with CYP153A6, NADH regeneration rates will increase, but unaddressed transport limitations will prevent the biocatalyst from utilising the additional cofactor effectively. Meanwhile, the expression of additional proteins will increase

the metabolic burden on the cell. Consequently, cells co-expressing additional glycerol dehydrogenase will not outperform intact cells expressing only CYP153A6 with its associated FdR and Fdx in terms of initial hydroxylation rate, and will underperform in terms of final titre, yield on biocatalyst, and space time yield.

This was found to be the case for cultures with high cell densities ($\pm 30 \text{ g}_{\text{DCW}}\cdot\text{L}^{-1}$), but low-density cultures ($4.5 \text{ g}_{\text{DCW}}\cdot\text{L}^{-1}$) were not negatively affected by the co-expression of dehydrogenase, and may actually have derived minor benefits in the form of enhanced CYP153A6 stability.

Hypothesis 3: Membrane permeabilization techniques will allow for enhanced transport of both n-octane and 1-octanol, leading to higher initial hydroxylation rates. However, compromising membrane integrity will cause the cell to lose important components and make it more vulnerable to toxicity. Consequently, permeabilised cells will be unable to sustain their initial high rates, and will underperform relative to non-permeabilised cells, in terms of final titre, yield on biocatalyst, and space time yield.

Hypothesis 4: Permeabilised cells co-expressing glycerol dehydrogenase alongside CYP153A6 will experience increased metabolic burden as well as detrimental effects associated with loss of membrane integrity. However, they will have access to additional substrate as well as the cofactors necessary to activate it. Consequently, initial hydroxylation rates will increase, as will maximum space time yield, but the biocatalyst will be unable to accumulate high titres of 1-octanol due to loss of stability.

Very different results were obtained depending on whether cells were chemically permeabilised or mechanically broken. Mechanical breakage of the cell envelope reduced initial hydroxylation rates in strains relying on natural cofactor regeneration capacity; in strains co-expressing glycerol dehydrogenase, initial rates were unchanged. Furthermore, mechanically permeabilised cells proved to be considerably more stable than intact cells, even at high cell density. Thus, mechanically broken cells co-expressing dehydrogenase produced high final titres, space time yields and yields on biocatalyst. Meanwhile, chemical permeabilization led to higher initial reaction rates. Stability of low-density systems was good, regardless of whether or not dehydrogenase was co-expressed, and chemically permeabilised cells outperformed intact cells. In high-density systems, chemically permeabilised cells lost activity rapidly, performing slightly better than intact cells, but not nearly as well as mechanically broken cells.

Hypothesis 5: The co-expression of the membrane transport protein AlkL alongside CYP153A6 will increase n-octane availability without compromising membrane integrity. Consequently, the initial hydroxylation rate will increase. However, as with other permeabilization techniques, the biocatalyst will not be able to sustain these hydroxylation rates due to insufficient regeneration of NADH cofactor. Consequently, cells co-expressing AlkL will not outperform cells expressing only CYP153A6 in terms of final titre, yield on biocatalyst, or average space time yield.

The co-expression of AlkL increased initial hydroxylation rates by up to 2.8-fold. Furthermore, the biocatalytic system could sustain reasonably high levels of activity for up to four days. Hence, contrary to expectation, intact cells co-expressing AlkL demonstrated higher activities and yields on biocatalyst than any other system tested.

Hypothesis 6: The combination of CYP153A6, enhanced n-octane transport due to co-expression of AlkL, and enhanced NADH regeneration due to co-expression of glycerol dehydrogenase, will provide the biocatalyst with a continuous supply of both substrate and cofactor. These benefits will outweigh the detrimental effects of high metabolic burden associated with expressing multiple heterologous proteins. Consequently, the hydroxylation rate, final titre, yield on biocatalyst, and space time yield will increase relative to cells expressing only CYP153A6.

The co-expression of both glycerol dehydrogenase and AlkL was found to overburden the cell, reducing the expression of active CYP153A6 to below $0.1 \mu\text{molP}_{450}\cdot\text{g}_{\text{DCW}}^{-1}$. Space time yields and yields on biomass were substantially reduced relative to strains co-expressing AlkL but not GLD, and somewhat reduced relative to strains expressing only A6/FdR/Fdx. Strains co-expressing AlkL and GLD exhibited the highest turnover frequencies after five days of biotransformation (relative to other strains), but this may have been partially due to CYP153A6 concentrations being within a limiting range.

Ultimately, cross-membrane transport emerged as the primary limitation within the system under consideration. Co-expression of glycerol dehydrogenase was beneficial only in the case of mechanically broken cells or cell free extract, where it presumably compensated for cofactor leakage and the disruption of metabolic processes associated with the cell membrane. Initial hydroxylation rates increased noticeably in most chemically permeabilised systems and in strains co-expressing AlkL, regardless of whether the strain was co-expressing glycerol dehydrogenase, demonstrating that the cell's natural cofactor regeneration capacity is sufficient to support an enhanced rate of reaction. However, initial rates in mechanically broken cells were no higher than those of intact cells, which again would point to cofactor becoming a limiting factor only if cell containment is breached. The positive effect of membrane breakage on the stability of the biocatalytic system is notable. Together with a strong correlation between biocatalyst stability and volumetric rate of 1-octanol production, it suggests that efficient in-situ extraction is key to the performance of whole-cell biocatalysts.

6.3 Literature Context

Table 6.1 summarises a variety of whole-cell catalysts reported in the literature for the activation of n-octane, covering several different alkane hydroxylases (AlkB, several members of the CYP153 subfamily, CYP154A8, and the artificial fusion construct CYP153A13-RhFRed), *Escherichia coli* and *Pseudomonas putida* hosts, and resting and growing systems. These systems are compared in terms of the final amount of product accumulated per unit of volume, cells, or enzyme; the maximum rate of product formation; and the length of time for which this maximum rate could be maintained.

Several interesting trends can be noted from a comparison of the various systems:

First, that the CYP153A6 system has consistently performed well across a range of indicators, especially in its ability to achieve high titres and competitive space time yields relative to other systems. This speaks both to the potential of the A6 enzyme itself, and to the effectiveness of the process engineering approach that has been applied to this system by various researchers.

Second, that n-octane biotransformations rarely extend past 24 h. In some studies, this may have been chosen as an arbitrary cut-off point for data collection; in other cases, biocatalytic activity had largely ceased by this point. Nevertheless, it is notable that system instability is not linked to biocatalytic activity. The highest recorded activity was $22.0 \text{ mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$ (Pennec et al., 2015); three of the five longest-running systems achieved activities of $21.9 \text{ mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$ (this study), $17.6 \text{ mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$ (Meissner, 2013), and $10.3 \text{ mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$ (this study); the other two systems did not provide activity data. Out of the five highest recorded activities, three were achieved by systems that went on to sustain biotransformation for 48 h or more (Gudimich et al., 2012; Meissner, 2013; this study). This is in line with this study's finding that stability of the whole-cell biocatalyst is independent of the stability or activity of the biocatalytic enzyme itself.

Third, that final titre, stability of the whole-cell biocatalyst, and yield on biocatalyst in the CYP153A6 system could be improved by moving from resting cells to growing cells. The co-expression of AlkL would allow such growing cell systems to benefit from enhanced cross-membrane transport of hydrocarbons, possibly allowing for further improvements in system performance.

Fourth, that the enzyme activities being measured in whole cells are related to, but not entirely dependent on, intracellular enzyme concentration. This is illustrated by the selected data presented in Table 6.2 and Figure 6.1. If intact cell systems are considered as a group, there is a direct correlation between increased intracellular enzyme concentration and decreased biocatalytic activity (excluding the results from Pennec et al. (2015) as an outlier). Permeabilised cell systems show a similar correlation. This supports the findings of this study, that whole-cell biotransformations are primarily limited by factors other than enzyme loading. However, permeabilization also shifts the concentration/activity curve to the left – which would imply that, at any given enzyme loading, the maximum activity that permeabilised cells can achieve is lower than that of intact cells. This may be because disrupting the membrane structure interferes with cell metabolism; the effect does not seem to depend on cell density (which was different in all three cases) or on the co-expression of additional glycerol dehydrogenase (which was present in the case of mechanical and chemical permeabilization,

but not in the case of AlkL co-expression). On the other hand, permeabilised cells are using enzyme more efficiently: they are capable of achieving a given activity target at a lower enzyme loading, presumably due to their enhanced access to substrate.

Table 6.1: Activation of n-octane by alkane hydroxylases in whole-cell biocatalysts. Hydroxylases were expressed with their wild-type electron transfer proteins unless stated otherwise. Cell densities preceded by the symbol $\leq$ indicates that growing cultures were used; all other biotransformations used resting cells. All hydroxylation products (which may include 1-octanol, 2-octanol, octanoic acid, octanediol, or octyl acetate) were included when calculating performance metrics, but specificities for 1-octanol are noted in brackets; octyl acetate was not considered a by-product in the context of this study, as it is an artefact of the expression vectors used. The five highest values reported for each performance indicator are highlighted in red, and the highest reported value is also underlined. (NS: not specified)

Key heterologous proteins	Host cell	Enzyme concentration ($\mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$)	Cell density ($\text{g}_{\text{DCW}} \cdot \text{L}^{-1}$)	Length of biotransformation (h)	Maximum titre (mM) [% 1-octanol]	Maximum activity ($\text{mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$)	Maximum yield on biocatalyst ($\text{mmol}_{\text{product}} \cdot \text{g}_{\text{DCW}}^{-1}$)	Maximum space time yield ($\text{mmol}_{\text{product}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$)	Average space time yield over 24 h ($\text{mmol}_{\text{product}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$)	Reference
AlkB + AlkL	<i>E. coli</i> W3110	NS	≤ 2	20	5.9 [NS]	-	6.5	0.42	-	Favre-Bulle et al. (1991)
AlkB	<i>P. putida</i> PpS81	NS	≤ 2	24	7.7 [>99%]	-	15.4	0.45	0.32	Bosetti et al. (1992)
CYP153Aci	<i>E. coli</i> BL21(DE3)	-	NS	24	19.0 [90%]	4.4	-	0.79	0.79	Fujii et al. (2006)
CYP153A13-RhFRed fusion	<i>E. coli</i> BL21(DE3)	NS	NS	24	6.1 [NS]	-	-	1.54	0.26	Nodate et al. (2006)
CYP153Aci + CamAB reductase/mediator	<i>E. coli</i> BL21(DE3)	-	NS	24	5.2 [94%]	11.8	-	0.22	0.22	Fujita et al. (2009)
AlkB	<i>E. coli</i> BL21(DE3)	NS	3.5	1	1.1 [91%]	-	0.32	1.12	-	Koch et al. (2009)
CYP153A6		-	NS		0.1 [97%]	6.2	-	0.08		
CYP153A13-RhFRed fusion	<i>E. coli</i> BL21(DE3)	NS	7.5	5	3.5 [> 95%]	-	0.47	1.76	-	Bordeaux et al. (2011)
AlkB + AlkL	<i>E. coli</i> GEC137	NS	2.4	24	8.8 [64%]	-	6.9	0.88	0.37	Grant et al. (2011)
CYP153A6	<i>E. coli</i> BL21(DE3)	0.45	7	18	15.4 [NS]	6.9	2.2	1.30	-	Gudiminch et al. (2011)
CYP153A6	<i>E. coli</i> BL21(DE3)	0.18	11	48	67.6 [> 90%]	20.5	6.1	2.46	<u>2.46</u>	Gudiminch et al. (2012)
AlkB + AlkL	<i>E. coli</i> W3110	NS	0.5	0.1	0.3 [NS]	-	0.6	3.00	-	Julsing et al. (2012)
CYP153A6	<i>E. coli</i> BL21(DE3)	0.26	≤ 7	72	68.3 [> 99%]	17.6	14.0	1.61	1.44	Meissner (2013)
CYP153A6	<i>E. coli</i> BL21(DE3)	NS	5.4	60	49.9 [> 99%]	-	9.2	1.23	1.23	Olaofe et al. (2013)

Table 6.1 cont.: Activation of n-octane by alkane hydroxylases in whole-cell biocatalysts. Hydroxylases were expressed with their wild-type electron transfer proteins unless stated otherwise. Cell densities preceded by the symbol $\leq$ indicates that growing cultures were used; all other biotransformations used resting cells. All hydroxylation products (which may include 1-octanol, 2-octanol, octanoic acid, octanediol, or octyl acetate) were included when calculating performance metrics, but specificities for 1-octanol are noted in brackets; octyl acetate was not considered a by-product in the context of this study, as it is an artefact of the expression vectors used. The five highest values reported for each performance indicator are highlighted in red, and the highest reported value is also underlined. (NS: not specified)

Key heterologous proteins	Host cell	Enzyme concentration ($\mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$)	Cell density ($\text{g}_{\text{DCW}} \cdot \text{L}^{-1}$)	Length of biotransformation (h)	Maximum titre (mM) [% 1-octanol]	Maximum activity ($\text{mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$)	Maximum yield on biocatalyst ($\text{mmol}_{\text{product}} \cdot \text{g}_{\text{DCW}}^{-1}$)	Maximum space time yield ($\text{mmol}_{\text{product}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$)	Average space time yield over 24 h ($\text{mmol}_{\text{product}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$)	Reference
CYP153A6	<i>E. coli</i> BL21(DE3)	NS	≤ 7	96	<u>119.0</u> [$> 99\%$]	-	16.9	2.30	2.30	Olaofe (2013)
AlkB + ω -transaminase CV2025	<i>E. coli</i> BL21(DE3)	NS	1.3	0.1	0.3 [51%]	-	0.2	4.0	-	Schrewe et al. (2013b)
CYP153A16	<i>P. putida</i> KT2440(MmarAlk)	NS	18	1.75	2.4 [$> 99\%$]	-	0.1	1.4	-	Vallon et al. (2013)
AlkB	<i>E. coli</i> DH1	NS	NS	20	[11%]	-	2.1	-	-	Grant et al. (2014)
AlkB + AlkL					[$> 95\%$]		0.2			
CYP153A (<i>Polaromonas</i> JS666) + hydrogenase (<i>R. eutropha</i>)	<i>P. putida</i> KT2440	NS	10	24	1.25 [61%]	-	0.1	0.16	0.05	Lonsdale et al. (2015)
CYP153A6	<i>E. coli</i> BL21(DE3)	0.65	10	8	23 [95%]	22.0	2.3	2.9	-	Pennec et al. (2015)
AlkB + AlkL	<i>E. coli</i> HB101	NS	NS	1	- [75%]	-	7.1	-	-	Call et al. (2016)
CYP154A8	<i>P. putida</i> DSM12264	0.30	11.9	48	17.6 [78%]	13.7	1.5	1.58	0.73	Tieves et al. (2016)
CYP153A6 + GLD	<i>E. coli</i> BL21(DE3) High cell density, ruptured	0.29	17.4	72	52.2 [$> 99\%$]	10.3	3.00	3.11	1.23	This study
CYP153A6 + GLD	<i>E. coli</i> BL21(DE3) High cell density, Triton X-100	0.14	34.6	10	18.0 [$> 99\%$]	13.9	0.52	4.30	0.78	
CYP153A6 + AlkL	<i>E. coli</i> BL21(DE3) Low cell density	0.09	4.6	96	25.1 [$> 99\%$]	21.9	5.46	0.53	0.53	

Table 6.2: Selected data from Table 6.1. Unless noted otherwise, the host cells were resting cultures of intact *Escherichia coli*, co-expressing a P450 and its native redox proteins

Enzyme concentration ($\mu\text{mol}_{\text{P450}} \cdot \text{g}_{\text{DCW}}^{-1}$)	Maximum activity ($\text{mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$)	Notes	Reference
0.09	21.9	Permeabilised (AlkL)	This study
0.14	13.9	Permeabilised (Triton X-100) (Co-expressing GLD)	This study
0.18	20.5		Gudiminchi et al. (2012)
0.26	17.6	Growing culture	Meissner (2013)
0.29	10.3	Permeabilised (ruptured) (Co-expressing GLD)	This study
0.30	13.7	<i>Pseudomonas putida</i> host	Tieves et al. (2016)
0.45	6.9		Gudiminchi et al. (2011)
0.65*	22		Pennec et al. (2015)

* Calculated based on cell density values given in terms of wet cell weight, assuming $\text{g}_{\text{DCW}} \cdot \text{L}^{-1} \approx 20\% \text{ g}_{\text{WCW}} \cdot \text{L}^{-1}$

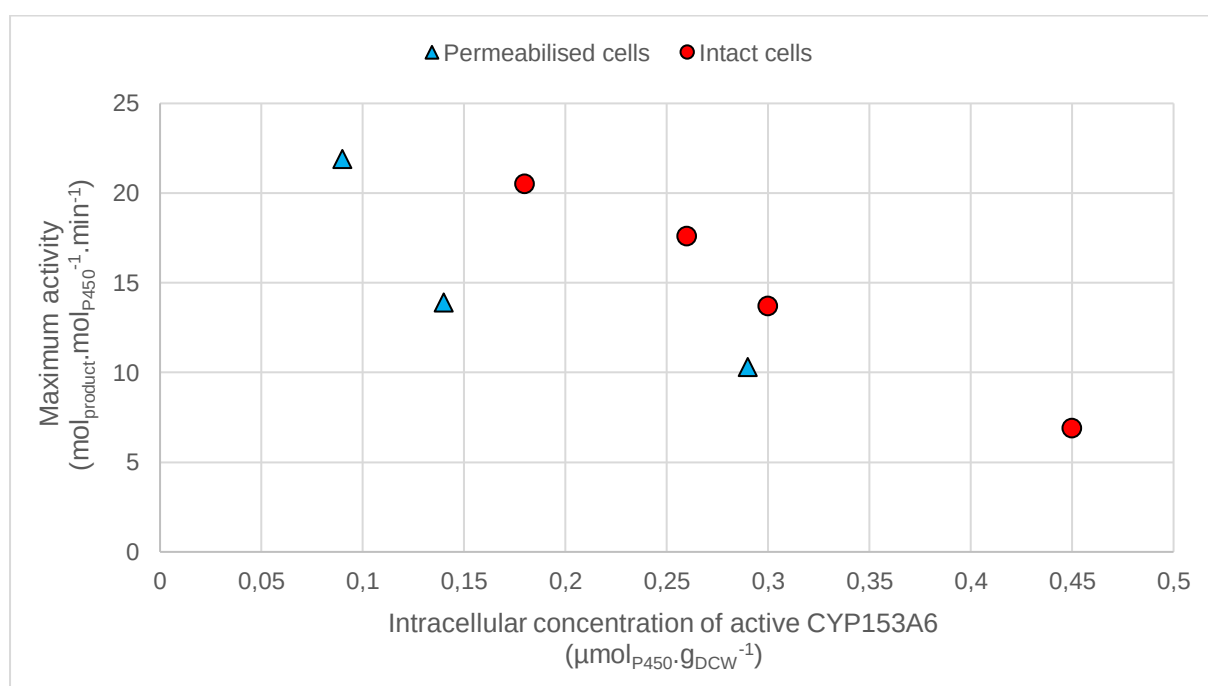


Figure 6.1: Graphic representation of maximum activity as a function of intracellular enzyme concentration, using data presented in Table 6.2 above. Pennec et al. (2015) has been excluded as an outlier.

6.4 Recommendations for future work

This study has demonstrated the key role that cross-membrane transport can play in enhancing the reaction rates of whole-cell P450 biocatalysts – provided that other system components, most notably NADH, are correctly balanced. However, it is not helpful for research into biocatalysis to focus on activity but neglect stability, which also plays a key role: biotransformations must achieve not only high space time yields but also high final titres. This study has demonstrated some interesting points regarding the stability of whole-cell P450-based biocatalysts. The stability of the whole-cell biocatalytic system was found to be independent from the stability of the CYP153A6, under the biotransformation conditions tested; presumably, this was due to active CYP153A6 being present in excess of stoichiometric requirements. Furthermore, the results of this study, along with those in the literature, suggest that increasing the rate of production per enzyme (or per cell) does not undermine overall system stability.

Instead, loss of overall system stability correlated strongly with the volumetric rate of product formation, and low-density systems proved to be more stable than high-density systems. This suggests that biomass loading may be the key determinant of stability in the whole-cell P450-based system.

On the basis of the results generated by this study, as well as the limitations in the data, several avenues of further research present themselves.

Genetic engineering was not a major focus of this study. The enzymes necessary for biocatalysis (CYP153A6, FdR, and Fdx) were cloned into pET28b+ as a single operon, expressed from a single promoter. The relative expression levels of the CYP153A6, Fdx, and FdR were not investigated. Given that other work suggests that the optimal concentration of Fdx is a factor of 10 – 30 greater than the concentrations of CYP153A6 and FdR (Arnold et al., 2012; Kochius et al., 2018), large improvements in biocatalytic performance could presumably be achieved by tuning the relative expression levels of these three components. Inadequate expression of Fdx might explain why even the highest activities reported for CYP153A6 in whole cells ($22 \text{ mol}_{\text{product}} \cdot \text{mol}_{\text{P450}}^{-1} \cdot \text{min}^{-1}$) are four-fold lower than the maximum theoretical activity.

At the same time, future work will need to address the highly inefficient expression of active CYP153A6 in the presence of AlkL, since it is desirable to overcome cross-membrane transport limitations without depressing intracellular P450 concentration to limiting levels.

Passive membrane transporters are known to be most beneficial in the case of larger, more sterically demanding substrates (Ruff et al., 2016; van Nuland et al., 2016). As such, it would be interesting to investigate how the biocatalyst handles longer chain length alkanes, with and without the co-expression of AlkL.

The most promising systems identified by this study were all permeabilised: mechanically ruptured cells (co-expressing glycerol dehydrogenase), cells exposed to Triton X-100 or Polymyxin B, and cells co-expressing AlkL. Since the results suggested a trade-off between cell density and system stability, it would be worth determining the optimal cell density in these permeabilised, resting cell systems. Cells co-expressing AlkL could further be applied as growing cells, under the same conditions as those of Olaofe (2013), to determine whether AlkL can improve biocatalytic performance in growing cell systems.

Overall, improvements in cross-membrane transport substantially improved the performance of resting whole-cell biocatalysts. Ensuring a sufficient supply of NADH cofactor was found to be essential but, except in the case of mechanically broken cells, the native cofactor pool was sufficient to support extended biotransformations at the levels of activity supported by these biotransformation conditions. A comparison between this study and the results reported in the literature suggests that the potential of the CYP153A6 system can still be extended further, through the optimisation of operating conditions in permeabilised systems.

References

- Abbasian, F., Lockington, R., Mallavarapu, M., Naidu, R., 2015. *A comprehensive review of aliphatic hydrocarbon biodegradation by bacteria*. Appl. Biochem. Biotechnol. 176(3), 670–699. doi:10.1007/s12010-015-1603-5
- Alonso-Gutierrez, J., Chan, R., Batth, T.S., Adams, P.D., Keasling, J.D., Petzold, C.J., Lee, T.S., 2013. *Metabolic engineering of Escherichia coli for limonene and perillyl alcohol production*. Metab. Eng. 19, 33–41. doi:10.1016/j.ymben.2013.05.004
- Andersen, K.B., von Meyenburg, K., 1980. Are growth rates of *Escherichia coli* in batch cultures limited by respiration? J. Bacteriol. 144(1), 114–123. doi:10.1128/J. Bacteriol. 144.1: 114-123
- Arnold, F.H., 1998. *Design by directed evolution*. Acc. Chem. Res. 31(3), 125–131. doi:10.1021/ar960017f
- Arnold, F.H., Brustad, E., Lewis, J., Chen, M., Komor, R., Coelho, P., 2012. DOE Award No. DE-FG02-06ER15762 *Structural and kinetic studies of novel cytochrome P450 small-alkane hydroxylases*. Pasadena. doi:10.2172/1035499
- Arnold, P.L., McMullon, M.W., Rieb, J., Kühn, F.E., 2015. *C-H bond activation by f-block complexes*. Angew. Chem. Int. Ed. Engl. 54(1), 82–100. doi:10.1002/anie.201404613
- Ayala, M., Torres, E., 2004. *Enzymatic activation of alkanes: constraints and prospective*. Appl. Catal. A Gen. 272(1), 1–13. doi:10.1016/j.apcata.2004.05.046
- Bailey, J.E., Sburlati, A., Hatzimanikatis, V., Lee, K., Renner, W.A., Tsai, P.S., 1996. *Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes*. Biotechnol. Bioeng. 52(1), 109–121. doi:10.1002/(SICI)1097-0290(19961005)52:1<109::AID-BIT11>3.0.CO;2-J
- Baneyx, F., 1999. *Recombinant protein expression in Escherichia coli*. Curr. Opin. Biotechnol. 10(5), 411–421. doi:10.1016/S0958-1669(99)00003-8
- Becker, J., Wittmann, C., 2015. *Advanced biotechnology: Metabolically engineered cells for the bio-based production of chemicals and fuels, materials, and health-care products*. Angew. Chem. Int. Ed. Engl. 54(11), 3328–3350. doi:10.1002/anie.201409033
- Bell, S., Stevenson, J., Boyd, H., Campbell, S., Riddle, A., Orton, E., Wong, L., 2002. *Butane and propane oxidation by engineered cytochrome P450cam*. Chem. Commun. (Camb). 5, 490–491. doi:10.1039/B110957J
- Bergman, R.G., 2007. *Organometallic chemistry: C–H activation*. Nature 446(7134), 391–393. doi:10.1038/446391a
- Berla, B.M., Saha, R., Maranas, C.D., Pakrasi, H.B., 2015. *Cyanobacterial alkanes modulate photosynthetic cyclic electron flow to assist growth under cold stress*. Sci. Rep. 5, 14894. doi:10.1038/srep14894
- Bernhardt, R., 2006. *Cytochromes P450 as versatile biocatalysts*. J. Biotechnol. 124(1), 128–145. doi:10.1016/j.jbiotec.2006.01.026
- Bernhardt, R., Urlacher, V.B., 2014. *Cytochromes P450 as promising catalysts for biotechnological application: Chances and limitations*. Appl. Microbiol. Biotechnol. 98(14), 6185–6203. doi:10.1007/s00253-014-5767-7
- Blank, L.M., Ebert, B.E., Bühler, B., Schmid, A., 2008. *Metabolic capacity estimation of Escherichia coli as a platform for redox biocatalysis: Constraint-based modeling and experimental verification*. Biotechnol. Bioeng. 100(6), 1050–1065. doi:10.1002/bit.21837
- Bordeaux, M., de Girval, D., Rullaud, R., Subileau, M., Dubreucq, E., Drone, J., 2014. *High-cell-density cultivation of recombinant Escherichia coli, purification and characterization of a self-sufficient biosynthetic octane ω -hydroxylase*. Appl. Microbiol. Biotechnol. 98(14), 6275–6283. doi:10.1007/s00253-014-5671-1

- Bordeaux, M., Galarneau, A., Drone, J., 2012. *Catalytic, mild, and selective oxyfunctionalization of linear alkanes: Current challenges*. *Angew. Chem. Int. Ed. Engl.* 51(43), 10712–10723. doi:10.1002/anie.201203280
- Bordeaux, M., Galarneau, A., Fajula, F., Drone, J., 2011. *A regioselective biocatalyst for alkane activation under mild conditions*. *Angew. Chem. Int. Ed. Engl.* 50(9), 2075–2079. doi:10.1002/anie.201005597
- Bosetti, A., Beilen, J.B. Van, Preusting, H., Lageveen, R.G., Witholt, B., 1992. *Production of primary aliphatic alcohols with a recombinant Pseudomonas strain, encoding the alkane hydroxylase enzyme system*. *Enzyme Microb. Technol.* 14(9), 702–708. doi:10.1016/0141-0229(92)90109-2
- Bouchez-Naïtali, M., Rakatozafy, H., Marchal, R., Leveau, J.-Y., Vandecasteele, J.-P., 1999. *Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake*. *J. Appl. Microbiol.* 86(3), 421–428. doi:10.1046/j.1365-2672.1999.00678.x
- Boyarskiy, S., Tullman-Ercek, D., 2015. *Getting pumped: Membrane efflux transporters for enhanced biomolecule production*. *Curr. Opin. Chem. Biol.* 28, 15–19. doi:10.1016/j.cbpa.2015.05.019
- Bühler, B., Park, J.B., Blank, L.M., Schmid, A., 2008. *NADH availability limits asymmetric biocatalytic epoxidation in a growing recombinant Escherichia coli strain*. *Appl. Environ. Microbiol.* 74(5), 1436–1446. doi:10.1128/AEM.02234-07
- Bühler, B., Schmid, A., 2004. *Process implementation aspects for biocatalytic hydrocarbon oxyfunctionalization*. *J. Biotechnol.* 113(1-3), 183–210. doi:10.1016/j.jbiotec.2004.03.027
- Call, T.P., Akhtar, M.K., Baganz, F., Grant, C., 2016. *Modulating the import of medium-chain alkanes in E. coli through tuned expression of FadL*. *J. Biol. Eng.* 10, 5. doi:10.1186/s13036-016-0026-3
- Canovas, M., Torroglosa, T., Iborra, J.L., 2005. *Permeabilization of Escherichia coli cells in the biotransformation of trimethylammonium compounds into L-carnitine*. *Enzyme Microb. Technol.* 37(3), 300–308. doi:10.1016/j.enzmictec.2004.07.023
- Carneiro De Melo, A.M.S., Cook, G.M., Miles, R.J., Poole, R.K., 1996. *Nisin stimulates oxygen consumption by Staphylococcus aureus and Escherichia coli*. *Appl. Environ. Microbiol.* 62(5), 1831–1834. doi:10.1128/AEM.62.5.1831-1834.1996
- Ceccoli, R.D., Bianchi, D.A., Rial, D. V., 2014. *Flavoprotein monooxygenases for oxidative biocatalysis: Recombinant expression in microbial hosts and applications*. *Front. Microbiol.* 5, 25. doi:10.3389/fmicb.2014.00025
- Chen, X., Li, S., Liu, L., 2014. *Engineering redox balance through cofactor systems*. *Trends Biotechnol.* 32(6), 337–343. doi:10.1016/j.tibtech.2014.04.003
- Cheng, F., Zhu, L., Schwaneberg, U., 2015. *Directed evolution 2.0: Improving and deciphering enzyme properties*. *Chem. Commun. (Camb)*. 51(48), 9760–9772. doi:10.1039/C5CC01594D
- Choi, J.M., Han, S.S., Kim, H.S., 2015. *Industrial applications of enzyme biocatalysis: Current status and future aspects*. *Biotechnol. Adv.* 33(7), 1443–1454. doi:10.1016/j.biotechadv.2015.02.014
- Choi, S., Song, C.W., Shin, J.H., Lee, S.Y., 2015. *Biorefineries for the production of top building block chemicals and their derivatives*. *Metab. Eng.* 28, 223–239. doi:10.1016/j.ymben.2014.12.007
- Chubukov, V., Sauer, U., 2014. *Environmental dependence of stationary-phase metabolism in Bacillus subtilis and Escherichia coli*. *Appl. Environ. Microbiol.* 80(9), 2901–2909. doi:10.1128/AEM.00061-14
- Chun, Y., Shimada, T., Sanchez-Ponce, R., Martin, M. V, Lei, L., Zhao, B., Kelly, S.L., Waterman, M.R., Lamb, D.C., Guengerich, F.P., 2007. *Electron transport pathway for a Streptomyces cytochrome P450: Cytochrome P450 105D5-catalyzed fatty acid hydroxylation in Streptomyces coelicolor A3(2)*. *J. Biol. Chem.* 282(24), 17486–17500. doi:10.1074/jbc.M700863200

- Ciaramella, A., Minerdi, D., Gilardi, G., 2017. *Catalytically self-sufficient cytochromes P450 for green production of fine chemicals*. Rend. Fis. Acc. Lincei 28, 169–181. doi:10.1007/s12210-016-0581-z
- Cornelissen, S., Julsing, M.K., Volmer, J., Riechert, O., Schmid, A., Bühler, B., 2013. *Whole-cell-based CYP153A6-catalyzed (S)-limonene hydroxylation efficiency depends on host background and profits from monoterpene uptake via AlkL*. Biotechnol. Bioeng. 110(5), 1282–1292. doi:10.1002/bit.24801
- Cornelissen, S., Liu, S., Deshmukh, A.T., Schmid, A., Bühler, B., 2011. *Cell physiology rather than enzyme kinetics can determine the efficiency of cytochrome P450-catalyzed C-H-oxygenation*. J. Ind. Microbiol. Biotechnol. 38(9), 1359–1370. doi:10.1007/s10295-010-0919-y
- Cserjan-Puschmann, M., Kramer, W., Duerrschmid, E., Striedner, G., Bayer, K., 1999. *Metabolic approaches for the optimisation of recombinant fermentation processes*. Appl. Microbiol. Biotechnol. 53, 43–50. doi:10.1007/s002530051612
- Curran, A., Swainston, N., Day, J., Kell, D.B., 2015. *Synthetic biology for the directed evolution of protein biocatalysts: Navigating sequence space intelligently*. Chem. Soc. Rev. 44(5), 1172–1239. doi:10.1039/C4CS00351A
- da Silva, A.J., Horta, A.C.L., Velez, A.M., Iemma, M.R.C., Sargo, C.R., Giordano, R.L., Novo, M.T.M., Giordano, R.C., Zangirolami, T.C., 2013. *Non-conventional induction strategies for production of subunit swine erysipelas vaccine antigen in E. coli fed-batch cultures*. Springerplus 2, 322. doi:10.1186/2193-1801-2-322
- Dai, Z., Nielsen, J., 2015. *Advancing metabolic engineering through systems biology of industrial microorganisms*. Curr. Opin. Biotechnol. 36, 8–15. doi:10.1016/j.copbio.2015.08.006
- de Carvalho, C.C.C.R., 2017. *Whole cell biocatalysts: Essential workers from nature to the industry*. Microb. Biotechnol. 10(2), 250–263. doi:10.1111/1751-7915.12363
- Durairaj, P., Hur, J.S., Yun, H., 2016. *Versatile biocatalysis of fungal cytochrome P450 monooxygenases*. Microb. Cell Fact. 15(1), 125. doi:10.1186/s12934-016-0523-6
- Farmer, I.S., Jones, C.W., 1976. *The effect of temperature on the molar growth yield and maintenance requirement of Escherichia coli W during aerobic growth in continuous culture*. FEBS Lett. 67(3), 359–363. doi:10.1016/0014-5793(76)80564-9
- Fasan, R., Chen, M.M., Crook, N.C., Arnold, F.H., 2007. *Engineered alkane-hydroxylating cytochrome P450BM3 exhibiting natively catalytic properties*. Angew. Chem. Int. Ed. Engl. 46(44), 8414–8418. doi:10.1002/anie.200702616
- Fasan, R., Crook, N.C., Peters, M.W., Meinhold, P., Buelter, T., Landwehr, M., Cirino, P.C., Arnold, F.H., 2011. *Improved product-per-glucose yields in P450-dependent propane biotransformations using engineered Escherichia coli*. Biotechnol. Bioeng. 108(3), 500–510. doi:10.1002/bit.22984
- Favre-Bulle, O., Schouten, T., Kingma, J., Witholt, B., 1991. *Bioconversion of n-octane to octanoic acid by a recombinant Escherichia coli cultured in a two-liquid phase bioreactor*. Biotechnology 9(4), 367–371. doi:10.1038/nbt0491-367
- Fiorentini, F., Hatzl, A., Schmidt, S., Savino, S., Glieder, A., Mattevi, A., 2018. *The extreme structural plasticity in the CYP153 subfamily of P450s directs development of designer hydroxylases*. Biochemistry 57(48), 6701–6714. doi:10.1021/acs.biochem.8b01052
- Fisher, M., Zheng, Y., Rettie, A., 1998. *Positional specificity of rabbit CYP4B1 for omega-hydroxylation of short-medium chain fatty acids and hydrocarbons*. Biochem. Biophys. Res. Commun. 248(2), 352–355. doi:10.1006/bbrc.1998.8842
- Foo, J.L., Leong, S.S., 2013. *Directed evolution of an E. coli inner membrane transporter for improved efflux of biofuel molecules*. Biotechnol. Biofuels 6(1), 81. doi:10.1186/1754-6834-6-81

- Fujii, T., Narikawa, T., Sumisa, F., Arisawa, A., Takeda, K., Kato, J., 2006. *Production of α,ω - alkanediols using Escherichia coli expressing a cytochrome P450 from Acinetobacter sp. OC4*. Biosci. Biotechnol. Biochem. 70(6), 1379–1385. doi:10.1271/bbb.50656
- Fujita, N., Sumisa, F., Shindo, K., Kabumoto, H., Arisawa, A., Ikenaga, H., Misawa, N., 2009. *Comparison of two vectors for functional expression of a bacterial cytochrome P450 gene in Escherichia coli using CYP153 genes*. Biosci. Biotechnol. Biochem. 73(8), 1825–1830. doi:10.1271/bbb.90199
- Funhoff, E.G., Bauer, U., García-Rubio, I., Witholt, B., van Beilen, J.B., 2006. *CYP153A6, a soluble P450 oxygenase catalyzing terminal-alkane hydroxylation*. J. Bacteriol. 188(14), 5220–5227. doi:10.1128/JB.00286-06
- Funhoff, E.G., Van Beilen, J.B., 2007. *Alkane activation by P450 oxygenases*. Biocatal. Biotransformation 25(2-4), 186–193. doi:10.1080/10242420701379254
- Fürst, M.J., Fiorentini, F., Fraaije, M.W., 2019. *Beyond active site residues: overall structural dynamics control catalysis in flavin-containing and heme-containing monooxygenases*. Curr. Opin. Struct. Biol. 59, 29–37. doi:10.1016/j.sbi.2019.01.019
- Girvan, H.M., Munro, A.W., 2016. *Applications of microbial cytochrome P450 enzymes in biotechnology and synthetic biology*. Curr. Opin. Chem. Biol. 31, 136–145. doi:10.1016/j.cbpa.2016.02.018
- Glick, B.R., 1995. *Metabolic load and heterologous gene expression*. Biotechnol. Adv. 13(2), 247–261. doi:10.1016/0734-9750(95)00004-a
- Grant, C., da Silva Damas Pinto, A.C., Lui, H., Woodley, J.M., Baganz, F., 2012. *Tools for characterizing the whole-cell bio-oxidation of alkanes at microscale*. Biotechnol. Bioeng. 109(9), 2179–2189. doi:10.1002/bit.24512
- Grant, C., Deszcz, D., Wei, Y.-C., Martínez-Torres, R.J., Morris, P., Folliard, T., Sreenivasan, R., Ward, J., Dalby, P., Woodley, J.M., Baganz, F., 2014. *Identification and use of an alkane transporter plug-in for applications in biocatalysis and whole-cell biosensing of alkanes*. Sci. Rep. 4, 5844. doi:10.1038/srep05844
- Grant, C., Woodley, J.M., Baganz, F., 2011. *Whole-cell bio-oxidation of n-dodecane using the alkane hydroxylase system of P. putida GPO1 expressed in E. coli*. Enzyme Microb. Technol. 48(6-7), 480–486. doi:10.1016/j.enzmictec.2011.01.008
- Gricman, Ł., Vogel, C., Pleiss, J., 2015. *Identification of universal selectivity-determining positions in cytochrome P450 monooxygenases by systematic sequence-based literature mining*. Proteins 83(9), 1593–1603. doi:10.1002/prot.24840
- Grogan, G., 2011. *Cytochromes P450: Exploiting diversity and enabling application as biocatalysts*. Curr. Opin. Chem. Biol. 15(2), 241–248. doi:10.1016/j.cbpa.2010.11.014
- Gröger, H., 2014. *Hydroxy functionalization of non-activated C-H and C=C bonds: New perspectives for the synthesis of alcohols through biocatalytic processes*. Angew. Chem. Int. Ed. Engl. 53(12), 3067–3069. doi:10.1002/anie.201308556
- Gudiminch, R.K., Randall, C., Opperman, D.J., Olaofe, O.A., Harrison, S.T.L., Albertyn, J., Smit, M.S., 2012. *Whole-cell hydroxylation of n-octane by Escherichia coli strains expressing the CYP153A6 operon*. Appl. Microbiol. Biotechnol. 96(6), 1507–1516. doi:10.1007/s00253-012-3984-5
- Gudiminch, R.K., Theron, C.W., Albertyn, J., Smit, M., 2011. *Cofactor regeneration during whole-cell biotransformation of n-octane using E. coli cells expressing the CYP153A6 operon*, in: Munro, A.W., Leys, D. (Eds.), 17th International Conference on Cytochrome P450 Biochemistry, Biophysics and Structure. Medimond International Proceedings, Manchester, UK, pp. 61–67.
- Guengerich, F.P., 2019. *Cytochrome P450 research and The Journal of Biological Chemistry*. J. Biol. Chem. 294(5), 1671–1680. doi:10.1074/jbc.TM118.004144

- Guengerich, F.P., Martin, M. V, Sohl, C.D., Cheng, Q., 2009. *Measurement of cytochrome P450 and NADPH-cytochrome P450 reductase*. Nat. Protoc. 4(9), 1245–1251. doi:10.1038/nprot.2009.121
- Guengerich, F.P., Munro, A.W., 2013. *Unusual cytochrome P450 enzymes and reactions*. J. Biol. Chem. 288(24), 17065–17073. doi:10.1074/jbc.R113.462275
- Hama, S., Yamaji, H., Kaieda, M., Oda, M., Kondo, A., Fukuda, H., 2004. *Effect of fatty acid membrane composition on whole-cell biocatalysts for biodiesel-fuel production*. Biochem. Eng. J. 21(2), 155–160. doi:10.1016/j.bej.2004.05.009
- Hannemann, F., Bichet, A., Ewen, K.M., Bernhardt, R., 2007. *Cytochrome P450 systems - biological variations of electron transport chains*. Biochim. Biophys. Acta 1770(3), 330–344. doi:10.1016/j.bbagen.2006.07.017
- Harris, K.L., Thomson, R.E.S., Strohmaier, S.J., Gumulya, Y., Gillam, E.M.J., 2018. *Determinants of thermostability in the cytochrome P450 fold*. Biochim. Biophys. Acta Proteins Proteom. 1866(1), 97–115. doi:10.1016/j.bbapap.2017.08.003
- Hartl, J., Kiefer, P., Meyer, F., Vorholt, J.A., 2017. *Longevity of major coenzymes allows minimal de novo synthesis in microorganisms*. Nat. Microbiol. 2, 17073. doi:10.1038/nmicrobiol.2017.73
- Hempfling, W.P., Mainzer, S.E., 1975. *Effects of varying the carbon source limiting growth on yield and maintenance characteristics of Escherichia coli in continuous culture*. J. Bacteriol. 123(3), 1076–1087. doi:10.1128/JB.123.3.1076-1087.1975
- Holland-staley, C.A., Lee, K., Clark, D.P., Cunningham, P.R., 2000. *Aerobic activity of Escherichia coli alcohol dehydrogenase is determined by a single amino acid*. J. Bacteriol. 182(21), 6049–6054. doi:10.1128/jb.182.21.6049-6054.2000
- Hollmann, F., Arends, I.W.C.E., Buehler, K., Schallmeyer, A., Bühler, B., 2011. *Enzyme-mediated oxidations for the chemist*. Green Chem. 13, 226–265. doi:10.1039/C0GC00595A
- Holtmann, D., Fraaije, M.W., Arends, I.W.C.E., Opperman, D.J., Hollmann, F., 2014. *The taming of oxygen: Biocatalytic oxyfunctionalisations*. Chem. Commun. (Camb). 50(87), 13180–13200. doi:10.1039/c3cc49747j
- Holtmann, D., Hollmann, F., 2016. *The oxygen dilemma: A severe challenge for the application of monooxygenases?* Chembiochem. 17(15), 1391–1398. doi:10.1002/cbic.201600176
- Huijbers, M.M.E., Montersino, S., Westphal, A.H., Tischler, D., Van Berkel, W.J.H., 2014. *Flavin dependent monooxygenases*. Arch. Biochem. Biophys. 544, 2–17. doi:10.1016/j.abb.2013.12.005
- Hummel, W., Gröger, H., 2014. *Strategies for regeneration of nicotinamide coenzymes emphasizing self-sufficient closed-loop recycling systems*. J. Biotechnol. 191, 22–31. doi:10.1016/j.jbiotec.2014.07.449
- Janocha, S., Bernhardt, R., 2013. *Design and characterization of an efficient CYP105A1-based whole-cell biocatalyst for the conversion of resin acid diterpenoids in permeabilized Escherichia coli*. Appl. Microbiol. Biotechnol. 97(17), 7639–7649. doi:10.1007/s00253-013-5008-5
- Ji, J.H., Liu, Y.F., Zhou, L., Mbadinga, S.M., Pan, P., Chen, J., Liu, J.F., Yang, S.Z., Sand, W., Gu, J.D., Mu, B.Z., 2019. *Methanogenic Degradation of long n-alkanes requires fumarate-dependent activation*. Appl. Environ. Microbiol. 85(16), e00985-19. doi:10.1128/AEM.00985-19
- Jiang, H., Morgan, J.A., 2004. *Optimization of an in vivo plant P450 monooxygenase system in Saccharomyces cerevisiae*. Biotechnol. Bioeng. 85(2), 130–137. doi:10.1002/bit.10867
- Joo, H., Lin, Z., Arnold, F.H., 1999. *Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation*. Nature 399(6737), 670–673. doi:10.1038/21395
- Julleson, D., David, F., Pfleger, B., Nielsen, J., 2015. *Impact of synthetic biology and metabolic engineering on industrial production of fine chemicals*. Biotechnol. Adv. 33(7), 1395–1402. doi:10.1016/j.biotechadv.2015.02.011

- Julsing, M.K., Cornelissen, S., Bühler, B., Schmid, A., 2008. *Heme-iron oxygenases: Powerful industrial biocatalysts?* Curr. Opin. Chem. Biol. 12(2), 177–186. doi:10.1016/j.cbpa.2008.01.029
- Julsing, M.K., Schrewe, M., Cornelissen, S., Hermann, I., Schmid, A., Bühler, B., 2012. *Outer membrane protein AlkL boosts biocatalytic oxyfunctionalization of hydrophobic substrates in Escherichia coli.* Appl. Environ. Microbiol. 78(16), 5724–5733. doi:10.1128/AEM.00949-12
- Jung, E., Park, B.G., Ahsan, M.M., Kim, J., Yun, H., Choi, K.Y., Kim, B.G., 2016. *Production of ω -hydroxy palmitic acid using CYP153A35 and comparison of cytochrome P450 electron transfer system in vivo.* Appl. Microbiol. Biotechnol. 100(24), 10375–10384. doi:10.1007/s00253-016-7675-5
- Kadisich, M., Julsing, M.K., Schrewe, M., Jehmlich, N., Scheer, B., von Bergen, M., Schmid, A., Bühler, B., 2016. *Maximization of cell viability rather than biocatalyst activity improves whole-cell oxyfunctionalization performance.* Biotechnol. Bioeng. 144(4), 874–884. doi:10.1002/bit.26213
- Kadisich, M., Willrodt, C., Hillen, M., Bühler, B., Schmid, A., 2017. *Maximizing the stability of metabolic engineering-derived whole-cell biocatalysts.* Biotechnol. J. 12(8). doi:10.1002/biot.201600170
- Kaluzna, I., Schmitges, T., Straatman, H., Van Tegelen, D., Müller, M., Schürmann, M., Mink, D., 2016. *Enabling selective and sustainable P450 oxygenation technology: production of 4-hydroxy- α -isophorone on kilogram scale.* Org. Process Res. Dev. 20(4), 814–819. doi:10.1021/acs.oprd.5b00282
- Kara, S., Schrittwieser, J.H., Hollmann, F., Ansorge-Schumacher, M.B., 2014. *Recent trends and novel concepts in cofactor-dependent biotransformations.* Appl. Microbiol. Biotechnol. 98(4), 1517–1529. doi:10.1007/s00253-013-5441-5
- Kataoka, M., Yamamoto, K., Kawabata, H., Wada, M., Kita, K., Yanase, H., Shimizu, S., 1999. *Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by Escherichia coli transformant cells coexpressing the aldehyde reductase and glucose dehydrogenase genes.* Appl. Microbiol. Biotechnol. 51(4), 486–490. doi:10.1007/s002530051421
- Kawakami, N., Cong, Z., Shoji, O., Watanabe, Y., 2015. *Highly efficient hydroxylation of gaseous alkanes at reduced temperature catalyzed by cytochrome P450BM3 assisted by decoy molecules.* J. Porphyr. Phthalocyanines 19(1-3), 329–334. doi:10.1142/S1088424615500145
- Kelly, S.L., Kelly, D.E., 2013. *Microbial cytochromes P450: Biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us?* Philos. Trans. R. Soc. Lond. B Biol. Sci. 368(1612), 20120476. doi:10.1098/rstb.2012.0476
- King, Z.A., Feist, A.M., 2014. *Optimal cofactor swapping can increase the theoretical yield for chemical production in Escherichia coli and Saccharomyces cerevisiae.* Metab. Eng. 24, 117–128. doi:10.1016/j.ymben.2014.05.009
- Koch, D.J., Chen, M.M., van Beilen, J.B., Arnold, F.H., 2009. *In vivo evolution of butane oxidation by terminal alkane hydroxylases AlkB and CYP153A6.* Appl. Environ. Microbiol. 75(2), 337–344. doi:10.1128/AEM.01758-08
- Kochius, S., Van Marwijk, J., Ebrecht, A.C., Opperman, D.J., Smit, M.S., 2018. *Deconstruction of the CYP153A6 Alkane Hydroxylase System: Limitations and Optimization of In Vitro Alkane Hydroxylation.* Catalysts 8(11), 531. doi:10.3390/catal8110531
- Kok, M., Oldenhuis, R., van der Linden, P.G., Meulenbergh, C.H., Kingma, J., Witholt, B., 1989. *The Pseudomonas oleovorans alkBAC operon encodes two structurally related rubredoxins and an aldehyde dehydrogenase.* J. Biol. Chem. 264(10), 5442–5451. PMID:2647719
- Kroha, K., 2004. *Industrial biotechnology provides opportunities for commercial production of new long-chain dibasic acids.* INFORM 15(9), 568–571. https://www.researchgate.net/profile/Kyle-Kroha/publication/283826458_Industrial_biotechnology_provides_opportunities_for_commercial_production_of_new_long-chain_dibasic_acids/links/5dc48bcb299bf1a47b1f804d/Industrial-biotechnology-provides-opportunities-for-commercial-production-of-new-long-chain-dibasic-acids.pdf

- Ladkau, N., Assmann, M., Schrewe, M., Julsing, M.K., Schmid, A., Bühler, B., 2016. *Efficient production of the Nylon 12 monomer ω -aminododecanoic acid methyl ester from renewable dodecanoic acid methyl ester with engineered Escherichia coli*. *Metab. Eng.* 36, 1–9. doi:10.1016/j.ymben.2016.02.011
- Ladkau, N., Schmid, A., Bühler, B., 2014. *The microbial cell - functional unit for energy dependent multistep biocatalysis*. *Curr. Opin. Biotechnol.* 30, 178–189. doi:10.1016/j.copbio.2014.06.003
- Lin, E.C., Magasanik, B., 1960. *The activation of glycerol dehydrogenase from Aerobacter aerogenes by monovalent cations*. *J. Biol. Chem.* 235, 1820–1823. PMID:14417009
- Liu, S., Li, C., Fang, X., Cao, Z., 2004. *Optimal pH control strategy for high-level production of long-chain α,ω -dicarboxylic acid by Candida tropicalis*. *Enzyme Microb. Technol.* 34(1), 73–77. doi:10.1016/j.enzmictec.2003.09.001
- Lonsdale, T.H., Lauterbach, L., Honda Malca, S., Nestl, B.M., Hauer, B., Lenz, O., 2015. *H₂-driven biotransformation of n-octane to 1-octanol by a recombinant Pseudomonas putida strain co-synthesizing an O₂-tolerant hydrogenase and a P450 monooxygenase*. *Chem. Commun. (Camb)*. 51(90), 16173–16175. doi:10.1039/C5CC06078H
- Lounatmaa, K., Nanninga, N., 1976. *Effect of polymyxin on the outer membrane of Salmonella typhimurium: Freeze-fracture studies*. *J. Bacteriol.* 128(2), 665–667. doi:10.1128/JB.128.2.665-667.1976
- Lu, H., Ma, J., Liu, N., Wang, S., 2010. *Effects of heme precursors on CYP1A2 and POR expression in the baculovirus/Spodoptera frugiperda system*. *J. Biomed. Res.* 24(3), 242–249. doi:10.1016/S1674-8301(10)60034-6
- Lu, H.Y., Qiu, L.L., Yang, X.J., Zhang, X.M., Zhang, Z., Wang, S.L., 2013. *Optimization of heme precursors for the expression of human cytochrome P450 2A13 and its co-expression with oxidoreductase in baculovirus/sf9 system*. *J. Biochem.* 153(6), 555–563. doi:10.1093/jb/mvt018
- Lundemo, M.T., Notonier, S., Striedner, G., Hauer, B., Woodley, J.M., 2016. *Process limitations of a whole-cell P450 catalyzed reaction using a CYP153A-CPR fusion construct expressed in Escherichia coli*. *Appl. Microbiol. Biotechnol.* 100(3), 1197–1208. doi:10.1007/s00253-015-6999-x
- Lundemo, M.T., Woodley, J.M., 2015. *Guidelines for development and implementation of biocatalytic P450 processes*. *Appl. Microbiol. Biotechnol.* 99(6), 2465–2483. doi:10.1007/s00253-015-6403-x
- Mainzer, S.E., Hemphling, W.P., 1976. *Effects of growth temperature on yield and maintenance during glucose-limited continuous culture of Escherichia coli*. *J. Bacteriol.* 126(1), 251–256. doi:10.1128/JB.126.1.251-256.1976
- McIntosh, J.A., Farwell, C.C., Arnold, F.H., 2014. *Expanding P450 catalytic reaction space through evolution and engineering*. *Curr. Opin. Chem. Biol.* 19, 126–134. doi:10.1016/j.cbpa.2014.02.001
- Meissner, M.P., 2013. *Reactor design for the hydroxylation of n-octane using a CYP153A6-based biocatalytic system expressed in Escherichia coli*. University of Cape Town. <http://hdl.handle.net/11427/9121>
- Meunier, B., de Visser, S.P., Shaik, S., 2004. *Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes*. *Chem. Rev.* 104(9), 3947–3980. doi:10.1021/cr020443g
- Michener, J.K., Nielsen, J., Smolke, C.D., 2012. *Identification and treatment of heme depletion attributed to overexpression of a lineage of evolved P450 monooxygenases*. *Proc. Natl. Acad. Sci. U.S.A.* 109(47), 19504–19509. doi:10.1073/pnas.1212287109
- Minerdi, D., Sadeghi, S.J., Di Nardo, G., Rua, F., Castrignanò, S., Allegra, P., Gilardi, G., 2015. *CYP116B5: A new class VII catalytically self-sufficient cytochrome P450 from Acinetobacter radioresistens that enables growth on alkanes*. *Mol. Microbiol.* 95(3), 539–554. doi:10.1111/mmi.12883

- Moreno, R., Rojo, F., 2019. *Genetic features and regulation of n -alkane metabolism in bacteria*, in: Rojo, F. (Ed.), *Aerobic Utilization of Hydrocarbons, Oils, and Lipids; Handbook of Hydrocarbon and Lipid Microbiology*. Springer Nature, Switzerland, pp. 521–542. https://link.springer.com/referenceworkentry/10.1007/978-3-319-50418-6_23
- Mouri, T., Michizoe, J., Ichinose, H., Kamiya, N., Goto, M., 2006. *A recombinant Escherichia coli whole cell biocatalyst harboring a cytochrome P450cam monooxygenase system coupled with enzymatic cofactor regeneration*. Appl. Microbiol. Biotechnol. 72(3), 514–520. doi:10.1007/s00253-005-0289-y
- Mueller, T.J., Grisewood, M.J., Nazem-Bokaei, H., Gopalakrishnan, S., Ferry, J.G., Wood, T.K., Maranas, C.D., 2014. *Methane oxidation by anaerobic archaea for conversion to liquid fuels*. J. Ind. Microbiol. Biotechnol. 42(3), 391–401. doi:10.1007/s10295-014-1548-7
- Munro, A.W., Girvan, H.M., Mason, A.E., Dunford, A.J., McLean, K.J., 2013. *What makes a P450 tick?* Trends Biochem. Sci. 38(3), 140–150. doi:10.1016/j.tibs.2012.11.006
- Muraki, N., Kitatsuji, C., Aono, S., 2015. *A new biological function of heme as a signalling molecule*. J. Porphyr. Phthalocyanines 19(1-3), 9–20. doi:10.1142/S1088424614501090
- Ni, Y., Chen, R.R., 2005. *Lipoprotein mutation accelerates substrate permeability-limited toluene dioxygenase-catalyzed reaction*. Biotechnol. Prog. 21(3), 799–805. doi:10.1021/bp049575g
- Nie, Y., Chi, C., Fang, H., Liang, J., Lu, S., Lai, G., Tang, Y., Wu, X., 2014. *Diverse alkane hydroxylase genes in microorganisms and environments*. Sci. Rep. 4, 4968. doi:10.1038/srep04968
- Nielsen, J., Keasling, J.D., 2016. *Engineering cellular metabolism*. Cell 164(6), 1185–1197. doi:10.1016/j.cell.2016.02.004
- Nodate, M., Kubota, M., Misawa, N., 2006. *Functional expression system for cytochrome P450 genes using the reductase domain of self-sufficient P450RhF from Rhodococcus sp. NCIMB 9784*. Appl. Microbiol. Biotechnol. 71(4), 455–462. doi:10.1007/s00253-005-0147-y
- Olaofe, O.A., 2013. *A bioprocess approach for enhanced biocatalytic activity and efficiency of whole cell Escherichia coli expressing alkane hydroxylase CYP153A6 for terminal hydroxylation of n-octane*. University of Cape Town. <http://hdl.handle.net/11427/21321>
- Olaofe, O.A., Fenner, C.J., Gudimich, R.K., Smit, M.S., Harrison, S.T.L., 2013. *The influence of microbial physiology on biocatalyst activity and efficiency in the terminal hydroxylation of n-octane using Escherichia coli expressing the alkane hydroxylase, CYP153A6*. Microb. Cell Fact. 12, 8. doi:10.1186/1475-2859-12-8
- Omura, T., Sato, R., 1964. *A new cytochrome in liver microsomes*. J. Biol. Chem. 237, 1375–1376.
- Pennec, A., Jacobs, C.L., Opperman, D.J., Smit, M.S., 2015. *Revisiting cytochrome P450-mediated oxyfunctionalization of linear and cyclic alkanes*. Adv. Synth. Catal. 357(1), 118–130. doi:10.1002/adsc.201400410
- Peter, S., Kinne, M., Wang, X., Ullrich, R., Kayser, G., Groves, J., Hofrichter, M., 2011. *Selective hydroxylation of alkanes by an extracellular fungal peroxygenase*. FEBS J. 278(19), 3667–3675. doi:10.1111/j.1742-4658.2011.08285.x
- Peters, M., Meinhold, P., Glieder, A., Arnold, F.H., 2003. *Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3*. J. Am. Chem. Soc. 125(44), 13442–13450. doi:10.1021/ja0303790
- Posokhov, Y.O., Kyrychenko, A., 2013. *Effect of acetone accumulation on structure and dynamics of lipid membranes studied by molecular dynamics simulations*. Comput. Biol. Chem. 46, 23–31. doi:10.1016/j.compbiolchem.2013.04.005
- Ramesh, H., Zajkoska, P., Rebros, M., Woodley, J.M., 2016. *The effect of cultivation media and washing whole-cell biocatalysts on monoamine oxidase catalyzed oxidative desymmetrization of 3-azabicyclo[3,3,0]octane*. Enzyme Microb. Technol. 83, 7–13. doi:10.1016/j.enzmictec.2015.11.005

- Rauch, M.C.R., Huijbers, M.M.E., Pabst, M., Paul, C.E., Pešić, M., Arends, I.W.C.E., Hollmann, F., 2020. *Photochemical regeneration of flavoenzymes – An Old Yellow Enzyme case-study*. *Biochim. Biophys. Acta Proteins Proteom.* 1868(1), 140303. doi:10.1016/j.bbapap.2019.140303
- Rebroš, M., Lipták, L., Rosenberg, M., Bučko, M., Gemeiner, P., 2014. *Biocatalysis with Escherichia coli-overexpressing cyclopentanone monooxygenase immobilized in polyvinyl alcohol gel*. *Lett. Appl. Microbiol.* 58(6), 556–563. doi:10.1111/lam.12227
- Rice, C.W., Hempfling, W.P., 1978. *Oxygen-limited continuous culture and respiratory energy conservation in Escherichia coli*. *J. Bacteriol.* 134(1), 115–124. doi:10.1128/JB.134.1.115-124.1978
- Rojo, F., 2009. *Degradation of alkanes by bacteria*. *Environ. Microbiol.* 11(10), 2477–2490. doi:10.1111/j.1462-2920.2009.01948.x
- Rosano, G.L., Ceccarelli, E.A., 2014. *Recombinant protein expression in Escherichia coli: Advances and challenges*. *Front. Microbiol.* 5, 172. doi:10.3389/fmicb.2014.00172
- Rozkov, A., Avignone-Rossa, C.A., Ertl, P.F., Jones, P., O’Kennedy, R.D., Smith, J.J., Dale, J.W., Bushell, M.E., 2004. *Characterization of the metabolic burden on Escherichia coli DH1 cells imposed by the presence of a plasmid containing a gene therapy sequence*. *Biotechnol. Bioeng.* 88(7), 909–915. doi:10.1002/bit.20327
- Ruff, A.J., Arlt, M., van Ohlen, M., Kardashliev, T., Konarzycka-Bessler, M., Bocola, M., Dennig, A., Urlacher, V.B., Schwaneberg, U., 2016. *An engineered outer membrane pore enables an efficient oxygenation of aromatics and terpenes*. *J. Mol. Catal. B Enzym.* 134(B), 285–294. doi:10.1016/j.molcatb.2016.11.007
- Sazykin, I., Makarenko, M., Seliverstova, E., Rakin, A., Sazykina, M., 2019. *Cyclohexane, naphthalene, and diesel fuel increase oxidative stress, CYP153, sodA, and recA gene expression in Rhodococcus erythropolis*. *Microbiologyopen* 8(9), e00855. doi:10.1002/mbo3.855
- Scheller, U., Zimmer, T., Becher, D., Schauer, F., Schunck, W., 1998. *Oxygenation cascade in conversion of n-alkanes to α,ω -dioic acids catalyzed by cytochrome P450 52A3*. *J. Biol. Chem.* 273(49), 32528–32534. doi:10.1074/jbc.273.49.32528
- Scheps, D., Honda Malca, S., Richter, S.M., Marisch, K., Nestl, B.M., Hauer, B., 2013. *Synthesis of ω -hydroxy dodecanoic acid based on an engineered CYP153A fusion construct*. *Microb. Biotechnol.* 6(6), 694–707. doi:10.1111/1751-7915.12073
- Schewe, H., Kaup, B., Schrader, J., 2008. *Improvement of P450(BM-3) whole-cell biocatalysis by integrating heterologous cofactor regeneration combining glucose facilitator and dehydrogenase in E. coli*. *Appl. Microbiol. Biotechnol.* 78(1), 55–65. doi:10.1007/s00253-007-1277-1
- Schirmer, A., Rude, M.A., Li, X., Popova, E., del Cardayre, S.B., 2010. *Microbial biosynthesis of alkanes*. *Science* 329(5991), 559–562. doi:10.1126/science.1187936
- Schmid, A., Kollmer, A., Mathys, R.G., Witholt, B., 1998. *Developments toward large-scale bacterial bioprocesses in the presence of bulk amounts of organic solvents*. *Extremophiles* 2(3), 249–256. doi:10.1007/s007920050067
- Schrewe, M., Julsing, M.K., Bühler, B., Schmid, A., 2013a. *Whole-cell biocatalysis for selective and productive C–O functional group introduction and modification*. *Chem. Soc. Rev.* 42(15), 6346–6377. doi:10.1039/c3cs60011d
- Schrewe, M., Julsing, M.K., Lange, K., Czarnotta, E., Schmid, A., Bühler, B., 2014. *Reaction and catalyst engineering to exploit kinetically controlled whole-cell multistep biocatalysis for terminal FAME oxyfunctionalization*. *Biotechnol. Bioeng.* 111(9), 1820–1830. doi:10.1002/bit.25248
- Schrewe, M., Ladkau, N., Bühler, B., Schmid, A., 2013b. *Direct terminal alkylamino-functionalization via multistep biocatalysis in one recombinant whole-cell catalyst*. *Adv. Synth. Catal.* 355(9), 1693–1697. doi:10.1002/adsc.201200958

- Sellés Vidal, L., Kelly, C.L., Mordaka, P.M., Heap, J.T., 2018. *Review of NAD(P)H-dependent oxidoreductases: Properties, engineering and application*. *Biochim. Biophys. Acta Proteins Proteom.* 1866(2), 327–347. doi:10.1016/j.bbapap.2017.11.005
- Shteinman, A.A., 2015. *Shilov alkane platinum chemistry: 45 years*. *J. Organomet. Chem.* 793, 34–40. doi:10.1016/j.jorganchem.2015.03.020
- Shul'pin, G., 2016. *New trends in oxidative functionalization of carbon–hydrogen bonds: A review*. *Catalysts* 6(4), 50. doi:10.3390/catal6040050
- Shul'pin, G.B., Vinogradov, M.M., Shul'pina, L.S., 2018. *Oxidative functionalization of C-H compounds induced by the extremely efficient osmium catalysts (a review)*. *Catal. Sci. Technol.* 8(17), 4287–4313. doi:10.1039/c8cy00659h
- Song, J.W., Jeon, E.Y., Song, D.H., Jang, H.Y., Bornscheuer, U.T., Oh, D.K., Park, J.B., 2013. *Multistep enzymatic synthesis of long-chain α - ω -dicarboxylic and ω -hydroxycarboxylic acids from renewable fatty acids and plant oils*. *Angew. Chem. Int. Ed. Engl.* 52(9), 2534–2537. doi:10.1002/anie.201209187
- Soussan, L., Pen, N., Belleville, M.P., Marcano, J.S., Paolucci-Jeanjean, D., 2016. *Alkane biohydroxylation: Interests, constraints and future developments*. *J. Biotechnol.* 222, 117–142. doi:10.1016/j.jbiotec.2016.02.007
- Su, W., Xiao, W., Wang, Y., Liu, D., Zhou, X., Yuan, Y., 2015. *Alleviating redox imbalance enhances 7-dehydrocholesterol production in engineered *Saccharomyces cerevisiae**. *PLoS One* 10(6), e0130840. doi:10.1371/journal.pone.0130840
- Sudhamsu, J., Kabir, M., Airola, M. V., Patel, B.A., Yeh, S.Y., Rousseau, D.L., Crane, B.R., 2010. *Co-expression of ferrochelatase allows for complete heme incorporation into recombinant proteins produced in *E. coli**. *Protein Expr. Purif.* 73(1), 78–82. doi:10.1016/j.pep.2010.03.010
- Syed, K., Porollo, A., Lam, Y., Grimmett, P., Yadav, J., 2013. *CYP63A2, a catalytically versatile fungal P450 monooxygenase capable of oxidizing higher-molecular-weight polycyclic aromatic hydrocarbons, alkylphenols, and alkanes*. *Appl. Environ. Microbiol.* 79(8), 2692–2702. doi:10.1128/AEM.03767-12
- Tieves, F., Erenburg, I.N., Mahmoud, O., Urlacher, V.B., 2016. *Synthesis of chiral 2-alkanols from *n*-alkanes by a *P. putida* whole-cell biocatalyst*. *Biotechnol. Bioeng.* 113(9), 1845–1852. doi:10.1002/bit.25953
- Tufvesson, P., Lima-Ramos, J., Nordblad, M., Woodley, J.M., 2011. *Guidelines and cost analysis for catalyst production in biocatalytic processes*. *Org. Process Res. Dev.* 15(1), 266–274. doi:10.1021/op1002165
- Vallon, T., Glemser, M., Honda Malca, S., Scheps, D., Schmid, J., Siemann-Herzberg, M., Hauer, B., Takors, R., 2013. *Production of 1-octanol from *n*-octane by *Pseudomonas putida* KT2440*. *Chem. Ing. Tech. (Weinh)* 85(6), 841–848. doi:10.1002/cite.201200178
- van Beilen, J.B., Funhoff, E.G., 2007. *Alkane hydroxylases involved in microbial alkane degradation*. *Appl. Microbiol. Biotechnol.* 74(1), 13–21. doi:10.1007/s00253-006-0748-0
- van Beilen, J.B., Funhoff, E.G., 2005. *Expanding the alkane oxygenase toolbox: New enzymes and applications*. *Curr. Opin. Biotechnol.* 16(3), 308–314. doi:10.1016/j.copbio.2005.04.005
- van Beilen, J.B., Holtackers, R., Lüscher, D., Bauer, U., Witholt, B., Duetz, W.A., 2005. *Biocatalytic production of perillyl alcohol from limonene by using a novel *Mycobacterium* sp. cytochrome P450 alkane hydroxylase expressed in *Pseudomonas putida**. *Appl. Environ. Microbiol.* 71(4), 1737–1744. doi:10.1128/AEM.71.4.1737-1744.2005
- van Beilen, J.B., Panke, S., Lucchini, S., Franchini, A.G., Rothlis-berger, M., Witholt, B., 2001. *Analysis of *Pseudomonas putida* alkane degradation gene clusters and flanking insertion sequences: Evolution and regulation of the alk genes*. *Microbiology* 147(6), 1621–1630. doi:10.1099/00221287-147-6-1621

- van Beilen, J.B., Smits, T.H.M., Whyte, L.G., Schorcht, S., Röthlisberger, M., Plaggemeier, T., Engesser, K.H., Witholt, B., 2002. *Alkane hydroxylase homologues in Gram-positive strains*. Environ. Microbiol. 4(11), 676–682. doi:10.1046/j.1462-2920.2002.00355.x
- van Nuland, Y.M., Eggink, G., Weusthuis, R.A., 2016. *Application of AlkBGT and AlkL from Pseudomonas putida GPo1 for selective alkyl ester ω -oxyfunctionalization in Escherichia coli*. Appl. Environ. Microbiol. 82(13), 3801–3807. doi:10.1128/AEM.00822-16.
- Varnado, C.L., Goodwin, D.C., 2004. *System for the expression of recombinant hemoproteins in Escherichia coli*. Protein Expr. Purif. 35(1), 76–83. doi:10.1016/j.pep.2003.12.001
- Wachtmeister, J., Rother, D., 2016. *Recent advances in whole cell biocatalysis techniques bridging from investigative to industrial scale*. Curr. Opin. Biotechnol. 42, 169–177. doi:10.1016/j.copbio.2016.05.005
- Wang, M., Chen, B., Fang, Y., Tan, T., 2017. *Cofactor engineering for more efficient production of chemicals and biofuels*. Biotechnol. Adv. 35(8), 1032–1039. doi:10.1016/j.biotechadv.2017.09.008
- Wentzel, A., Ellingsen, T.E., Kotlar, H.K., Zotchev, S.B., Throne-Holst, M., 2007. *Bacterial metabolism of long-chain n-alkanes*. Appl. Microbiol. Biotechnol. 76(6), 1209–1221. doi:10.1007/s00253-007-1119-1
- Willrodt, C., Hoschek, A., Bühler, B., Schmid, A., Julius, M.K. 2016. *Decoupling production from growth by magnesium sulfate limitation boosts de novo limonene production*. Biotechnol. Bioeng. 113(6), 1305–1314. doi:10.1002/bit.25883
- Willrodt, C., Hoschek, A., Bühler, B., Schmid, A., Julius, M.K., 2015. *Coupling limonene formation and oxyfunctionalization by mixed-culture resting cell fermentation*. Biotechnol. Bioeng. 112(9), 1738–1750. doi:10.1002/bit.25592
- Woodley, J.M., 2013. *Protein engineering of enzymes for process applications*. Curr. Opin. Chem. Biol. 17(2), 310–316. doi:10.1016/j.cbpa.2013.03.017
- Wu, G., Yan, Q., Jones, J.A., Tang, Y.J., Fong, S.S., Koffas, M.A.G., 2016. *Metabolic burden: Cornerstones in synthetic biology and metabolic engineering applications*. Trends Biotechnol. 34(8), 652–664. doi:10.1016/j.tibtech.2016.02.010
- Zehentgruber, D., Drăgan, C.A., Bureik, M., Lütz, S., 2010. *Challenges of steroid biotransformation with human cytochrome P450 monooxygenase CYP21 using resting cells of recombinant Schizosaccharomyces pombe*. J. Biotechnol. 146(4), 179–185. doi:10.1016/j.jbiotec.2010.01.019
- Zhang, D., Yang, H., Wang, X., Qiu, Y., Tian, L., Qi, X., Qu, L.Q., 2020. *Cytochrome P450 family member CYP96B5 hydroxylates alkanes to primary alcohols and is involved in rice leaf cuticular wax synthesis*. New Phytol. 225(5), 2094–2107. doi:10.1111/nph.16267