

Biocatalysis of poorly water-soluble substrates

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Biocatalysis of poorly water-soluble substrates



Murray Peter Meissner PhD Thesis July 2018



Biocatalysis of poorly water-soluble substrates

PhD Thesis

Murray Peter Meissner

July 2018

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Abstract

Biocatalysis has grown in industrial application to the point where there are now hundreds of established enzyme-catalysed processes spanning various industries. However, many compounds of interest in such chemical syntheses are poorly water-soluble. If these compounds are to be used as substrates for enzyme-catalysed reactions they will form heterogeneous reaction mixtures because water is the predominant solvent for biocatalysis. With the introduction of multiple phases comes new challenges for experimental assessments and process performance. A chief challenge lies in identifying and addressing the extent of substrate mass transfer limitations into the aqueous phase where biocatalysis takes place.

This thesis aims to explore systematic methods for handling poorly water-soluble substrates in reaction evaluations, which are crucial for biocatalytic reaction design. A standardised method of scoping reactions and gauging their performance is developed and presented. The method involves comparing reaction trajectories performed under varying conditions (enzyme and substrate concentrations) and can distinguish process 'bottlenecks' of enzyme kinetics, enzyme stability or non-enzyme related limitations. The method was shown to enable the qualitative (and to an extent quantitative) identification of substrate mass transfer limitations through time-course measurements of substrate conversion.

Major advances in protein engineering techniques have facilitated the 'tailoring' of enzymes for specific process requirements (e.g. improved thermostability, solvent tolerance, substrate scope/affinity, and catalytic activity). However, the investment in research effort, time and costs for protein engineering are disproportionate to the number of realised industrial processes. Furthermore, this thesis suggests this is due to a disconnect in the earliest stages of reaction design and conceptualisation where the required process performance is typically not considered or understood. Therefore, process performance requirements are suggested as a series of metrics in this thesis such that they may be used to guide reaction assessments and for benchmarking. These metric requirements are based on the value-class of the product (i.e. process margins) and cost of biocatalyst formulation, and include: reaction conversion, reaction yield, yield on cosubstrate (where appropriate), selectivity, product concentration, productivity/space-time yield, specific productivity and biocatalyst yield. These metrics were shown to be appropriate and informative in two case studies.

These two cases studies involved biocatalysis of poorly water-soluble substrates and were chosen in order to demonstrate the application of the proposed reaction assessment and subsequent benchmarking with the process performance metrics.

The first case study, which is the enantioselective desymmetrisation of a diester by recombinant pig liver esterase, highlights the importance of quantifying mass transfer limitations when gauging process metrics (especially biocatalyst yield). The required process metrics for a high-value product ($100 \in kg^{-1}$) were all satisfied with the exception of biocatalyst yield. In order to increase the biocatalyst yield to the required range of 10-40 g g_{biocat}-1, the exceptional metrics of space-time yield ($20 \text{ g L}^{-1} \text{ h}^{-1}$) or product concentration (74 g L^{-1}) could be reduced by an order of magnitude and still satisfy target process performance metrics. The second case study features the biocatalytic Baeyer-Villiger oxidation of a macrocyclic ketone to its corresponding lactone, and resulted in an even more complex solid-liquid-gas reaction medium. Here, the necessity and usefulness of alternative, online, analytical methods involving oxygen mass balances in the gas-phase and activity assays by sacrificial sampling are demonstrated. The use of co-solvents to improve substrate mass transfer in this case is also presented. Due to the lower value-class of

macrocyclic lactones (5-20 \in kg⁻¹) more stringent process metrics are required when compared with the first case study. Oxygen transfer was found to limit the rate of reaction, and as a result all process performance metrics fell short by an order of magnitude. However, the rate of oxygen transfer can still be improved through scale-up, sparging pure oxygen instead of air, or operating the reactor under pressure, which may improve performance metrics sufficiently.

Based on the two case studies, a generic and pragmatic procedure for conceptualising biocatalytic reactions is proposed with a special focus towards those involving poorly water-soluble substrates. This comprehensive route for development includes the precise order and types of experiments required to design a reaction from initial considerations up to the point where later-stage process development tools and directed improvement strategies can be applied in addressing the challenges of poorly water-soluble substrates in industrial biocatalytic processes.

Dansk Resume

Anvendelsen af biokatalyse er vokset i industrien, til et punkt hvor der nu er hundredevis af etablerede processer, inden for adskillige forskellige industrier. Men de fleste af de stoffer, der er af interesse inden for kemisk syntese, er ikke vandopløselige. Hvis disse stoffer skal bruges som substrater i enzym-katalyse reaktioner, vil de danne heterogene reaktionsblandinger, fordi vand er det mest almindelige opløsningsmiddel til biokatalyse. Dannelsen af flere faser skaber nye problemer for den eksperimentelle udførelse og processens effektivitet. Et hovedproblem er at identificere og adressere begrænsningen af substraters masseoverførsel i de vandfaser, hvor biokatalysen finder sted.

Denne afhandling søger at afklare systematiske metoder til at håndtere ikke vandopløselige substrater på, hvilket er kritisk for biokatalytiske reaktionsdesign. En standardiseret metode til at vurdere reaktioner og justere deres effektivitet er blevet udviklet og præsenteret. Denne metode indebærer at sammenligne reaktionskurver opnået under varierende forhold (enzym og substrat koncentrationer) og kan skelne mellem flaskehalse forårsaget af enzym kinetik, enzym stabilitet, og begrænsninger der ikke er enzym-relaterede. Metoden har vist sig at være i stand til at identificere kvalitative (og i en hvis grad kvantitative) begrænsninger for substraters masseoverførsel via målinger af substratomdannelse over tid.

Massiv udvikling inden for protein engineering gør det nu muligt at "skræddersy" enzymer til specifikke proceskrav (som at forøge termostabiliteten, solvent tolerance, affiniteten over for substrater eller den katalytiske aktivitet). Men investeringen i tid, penge og forskningsaktivitet er disproportional i forhold til antallet af succesfulde industrielle processer. Denne afhandling foreslår, at dette højst sandsynligt skyldes, at de tidlige stadier inden for reaktionsdesign og konceptudvikling, hvor processens effektivitet ikke er i fokus eller helt forstået, ikke er koblet til processens slutmål. Derfor vil proceseffektivitet i denne afhandling være målt på en række kerne procesparametre, således at de kan bruges til at guide vurderingen af reaktioner, samt som sammenligningsgrundlag. Målene for disse procesparametre er basseret på hvilken værdi-klasse produkter er i (altså procesmargen) og omkostninger for biokatalytiske formuleringer, og inkluderer reaktionsomdannelse, reaktionsudbytte, udbytte af co-substrater, selektivitet, produktkoncentration, produktivitet/space-time yield, specifik produktivitet og biokatalytisk udbytte.

To casestudier med biokatalyse og dårligt vandopløselige substrater blev valgt for at demonstrere denne måde at vurdere og efterfølgende sammenligne processers præstationer på.

Det første studie, som er en rekombinant pig liver-esterases enantio-selektive afsymmetrisering af en diester, viser vigtigheden i at kvantificere massoverførsel når man vurderer procesparametre (særligt biokatalytisk udbytte). De nødvendige procesparametre for et produkt af høj værdi var alle tilfredsstillede, bortset fra det biokatalytiske udbytte. For at få det biokatalytiske udbytte op til de nødvendige 10-40 g g_{biokat}-1, kunne den høje produktivitet på 20 g L⁻¹ h⁻¹ eller den høje produktkoncentration på 74 g L⁻¹ blive reduceret med en tiendedel, og stadigvæk opfylde processens procesmål. Det andet casestudie er en biokatalytisk Baeyer-Villiger oxidation af en makrocyklisk keton til dens respektive lacton, og resulterede i et endnu mere kompleks reaktionsmedie med faststof, væske og gas. Her blev nødvendigheden af at bruge en alternativ, online, analytisk metode, der involverede iltoverførsel i gasfase og at ofre prøver i aktivitets-assays, vist. Yderligere blev effekten af at bruge co-solventer for at øge substraters masseoverførselsevne også studeret. På grund af makrocykliske lactoners lave værdiklasse, var det nødvendigt at opnå højere procesparamtre for denne reaktion, sammenlignet med den tidligere. Det vidste sig at iltoverførsel var en begrænsning for reaktionshastigheden, og derfor var alle procesparamtre en tiendedel for små. Tilførelsel af oxygen kan dog stadig forbedres enten ved opskalering, brusning af ren ilt i stedet luft, eller ved at køre reaktionen unden tryk. Alle tre kan føre til forbedringer i processens effektivitet.

Baseret på disse to studier er en general og pragmatisk procedure for at danne koncepter for biokatalytiske reaktioner blevet foreslået, med et specielt fokus på de reaktioner der involverer dårligt vandopløselige substrater. Denne omfattende metode til reaktionsdesign har den præcise rækkefølge og typer af eksperimenter der behøves for at designe en reaktion, fra de første overvejelser og op til et punkt, hvor procesudviklingsværktøjer og målrettede forbedringsstrategier kan bruges til at adressere de udfordringer der er for dårligt vandopløselige substrater inden for industrielle biokatalyserende processer.

Preface

This thesis serves as partial fulfilment of the requirements for a PhD degree in Chemical Engineering. The work here presented was supervised by Professor John M. Woodley, performed over the period of July 2015-2018, and was mostly conducted at the Process and Systems Engineering Centre (PROSYS) within the Department of Chemical and Biochemical Engineering at the Technical University of Denmark. The work towards one case study (in Chapter 4) was carried out on an external stay at the Institute of Chemistry, University of Rostock under the guidance of Dr. Jan von Langermann and collaboration with Enzymicals AG.

The research for this work was partly funded by the Technical University of Denmark as well as the European Union (EU) project ROBOX (grant agreement n° 635734) under EU's Horizon 2020 Programme Research and Innovation actions H2020-LEIT BIO-2014-1. The EU project has facilitated fruitful collaborations with various partners through which the materials and enzymes required for this research were supplied.

Kgs. Lyngby July 2018 Murray Peter Meissner

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The photograph on the cover of this thesis pictures a biocatalytic Baeyer-Villiger oxidation experiment performed in a laboratory-scale stirred tank reactor.

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I would like to express my gratitude to my supervisor, Professor John Woodley, for his guidance and mentorship over the course of this PhD. Thank you for believing in me and offering such an opportunity to gain this remarkable experience abroad. In particular I have always thoroughly enjoyed our thought-provoking discussions, and through these you have taught me the true value and challenges of biocatalysis in industrial processing as well as the importance of always seeking deeper understanding and 'asking the right questions.' These have shaped my outlook on research and will remain with me forever.

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Thanks must be expressed to past and present colleagues at DTU and in Rostock for providing an enjoyable and conducive working environment.

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"Joyful is the person who finds wisdom, the one who gains understanding." - Proverbs 3:13

List of Publications

Peer-reviewed papers

- Murray P. Meissner, Mathias Norblad, John M. Woodley. 2018. Online Measurement of Oxygen-Dependent Enzyme Reaction Kinetics. *ChemBioChem*, 19:106-113.
- Mathias Nordblad, Mafalda Dias Gomes,[†] <u>Murray P. Meissner</u>,[†] Hemalata Ramesh, John M. Woodley. 2018. Scoping Biocatalyst Performance using Reaction Trajectory Analysis. *Organic Process Research & Development*, (submitted). [†]These authors contributed equally.

Conference presentations and posters

- <u>Murray P. Meissner</u>, Gustav Rehn, John M. Woodley. 2015. The current state of biocatalytic Baeyer-Villiger oxidations. *New Reactions with Enzymes and Microorganisms*, Stuttgart (DE).
- <u>Murray P. Meissner</u>, Gustav Rehn, Mathias Nordblad, John M. Woodley. 2016. Considerations for integrated processes for biocatalytic Baeyer-Villiger oxidations. 8th International Congress on Biocatalysis (BIOCAT2016), Hamburg (DE).
- <u>Murray P. Meissner</u>, Gustav Rehn, Mathias Nordblad, John M. Woodley. 2016. Solid phase biocatalytic Baeyer-Villiger oxidations. 5th International Conference on Novel Enzymes, Groningen (NL).
- <u>Murray P. Meissner</u>, Mathias Nordblad, John M. Woodley. 2017. Oxygen mass balance for enzymatic reaction rate estimations. 5th International Conference on Biocatalysis in Non-Conventional Media (BNCM2017), Rostock (DE).
- <u>Murray P. Meissner</u>, John M. Woodley. 2017. Continuous biocatalytic reactors. *Organic Process Research and Development Conference*, Stockholm (SE).
- <u>Murray P. Meissner</u>, John M. Woodley. 2018. Kinetic evaluation of oxygen-dependent enzymes converting poorly water-soluble substrates. *Gordon Research Conference on Biocatalysis*, Biddeford, ME (USA).

Abbreviations

API	Active pharmaceutical ingredient
BVMO	Baeyer-Villiger monooxygenase
CDMO	Cyclododecanone monooxygenase
CDW	Cell dry weight
CFE	Cell-free extract
CWW	Cell wet weight
DOT	Dissolved oxygen tension
E. coli	Escherichia coli
EC	Enzyme Commission
ECS-PLE06	Recombinant pig liver esterase
FC	Gas flow controller
FM	Gas flow meter
GC	Gas chromatography
HPLC	High-performance liquid chromatography
ISPR	In situ product removal/recovery
OTR	Oxygen transfer rate
vvm	Volumes (of air) per volume of reaction medium per minute
$Y_{O2}M$	Gas-phase oxygen fraction meter

Nomenclature

Greek symb	<u>bols</u>	
η	Efficiency factor (for immobilised preparations)	[-]
v	Volumetric gas flow rate	[sL min ⁻¹]
Roman chai	racters	
а	Specific activity	[U mg _{biocat} ⁻¹]
<i>CO</i> 2	Dissolved oxygen concentration in the bulk medium	[mol L ⁻¹]
<i>C</i> [*] <i>02</i>	Dissolved oxygen concentration at saturation	[mol L ⁻¹]
Eo	Initial enzyme concentration	$[mg_{biocat} L^{-1}]$
k _{cat}	Turnover number	[min⁻¹]
k _d	First-order rate constant for deactivation	[S ⁻¹]
K _{ic}	Competitive inhibition constant	[mmol L ⁻¹]
K _{iu}	Uncompetitive inhibition constant	[mmol L ⁻¹]
k₋a	Overall volumetric mass transfer coefficient	[h ⁻¹]
Kм	Michaelis constant	[mmol L ⁻¹]
<i>п</i> 02	Molar flow rate of oxygen	[mol h⁻¹]
р	Product concentration	[mmol L ⁻¹] or [g L ⁻¹]
Р	Pressure	[atm]
R	Ideal gas constant	[L atm K ⁻¹ mol ⁻¹]
<i>R02</i>	Oxygen consumption rate	[mol L ⁻¹ h ⁻¹]
R _{glucose}	Glucose consumption rate	[mol L ⁻¹ h ⁻¹]
S ₀	Initial substrate concentration	[mmol L^{-1}] or [g L^{-1}]

STY	Space-time yield	[g L ⁻¹ h ⁻¹]
t	Time	[min] or [h]
Т	Temperature	[°C] or [K]
TTN	Total turnover number	[-]
V _{max}	Maximum reaction velocity at substrate saturation	[mmol L ⁻¹ min ⁻¹]
y 02	Gas-phase oxygen fraction	[-]

Definitions

Catalyst kinetics	Rate of catalysis according to the Michaelis-Menten expression (also including inhibition and enzyme stability).
gbiocat	Refers to grams of lyophilised cell-free extract in the context of this thesis. We postulate that this is indeed the preferred biocatalyst formulation for simpler process handling (e.g. storage stability and ease of dosing) in industrial processes.
Half-life (t½)	Time required for an enzyme to lose half of its specific activity. According to a first-order deactivation model: $t\frac{1}{2} = \ln(2)/k_d$.
Inhibition	The phenomenon where enzymes are inhibited through negative interactions with a medium component (e.g. substrate or product).
Reaction kinetics	Rate of reaction including catalyst kinetics, rate of (substrate) mass transfer, effects of mixing and environmental conditions (e.g. pH, temperature, presence of co-solvents).

Table of Contents

Abstract	•••	i
Dansk Resume		iii
Preface		v
Acknowledgements		vi
List of Publications		vii
Abbreviations		viii
Nomenclature		viii
Definitions		ix
Chapter 1. Introduction		1-1:16
Chapter 2. 'Bottleneck' identification in early-stage biocatalytic reaction assessments		2-1:12
Chapter 3. Setting process performance metric targets for the direction and evaluation of biocatalytic reactions		3-1:19
Chapter 4. Case Study I – Scoping the enantioselective desymmetrisation of a poorly water-soluble diester by recombinant pig liver esterase and improving the process to handle higher substrate loads		4-1:12
Chapter 5. Case Study II – Part A: Significance of analytical methods when characterising heterogeneous reactions		5-1:17
Chapter 6. Case Study II – Part B: Reaction scoping for the oxidation of macrocyclic ketones catalysed by cyclododecanone monooxygenase (CDMO)		6-1:18
Chapter 7. General discussion		7-1:11
Chapter 8. Conclusions	•••	8-1:2
Chapter 9. Future perspectives		9-1:3

References

Chapter 1. Introduction

1.1. Biocatalysis as an established branch of chemical synthesis

Catalysis is fundamental to industrial chemical processing. A catalyst is defined as something which "accelerates a chemical reaction without affecting the position of equilibrium" – Wilhelm Ostwald, 1895. As many as three quarters of all industrial processes require catalysts, with more than 90% of newly developed processes being catalytic (Hagen 2015). Catalysts vastly improve the atom efficiency of reactions as they are not stoichiometric reagents and may be reused (Sheldon 2000; Sheldon and Blaser 2003). Catalysts may be gases, liquids or solids and may be heterogeneous (i.e. forming an additional phase in the reaction medium) or homogeneous (i.e. soluble and well-dispersed in the reaction medium). It is also possible to immobilise homogeneous catalysts onto a solid particle, e.g. zeolites, to transform them into heterogeneous catalysts. Most steps of crude oil processing and the petrochemical industry are catalytic, as well as for the polymer industry, pharmaceuticals, dyes and pigments, herbicides and pesticides, organic intermediate syntheses, and environmental control (e.g. purifying automobile and power generation exhaust gas emissions). Indeed, it would be impossible to imagine the world as we know it without the influence of catalysts in our everyday life.

Most catalysts are reliant on a metal ion acting as an active centre. Whilst some metals such as gold, platinum, and silver are already scarce and correspondingly expensive, there is now an ever larger rarity of almost all catalytic metals (Figure 1.1). Inevitably, this will drive prices even higher and cause market instability for metals in the future. This is due to mining out the accessible reserves and not effectively recycling these metals (Dodson et al. 2012). Furthermore, there is only a growing demand for minerals, metals and semi-conductors from new 'renewable' energy industries (e.g. wind turbines, electric cars, or solar panels) and little focus towards recycling or reusing the already mined resources. Therefore, it should be expected that a shift in the traditional processing routes of chemical industry must occur in order to sustain the global demand for what have today become common commodities (e.g. plastics, pharmaceuticals, food, fuel, etc.).

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				140.9077	144.24	(145)	150.36	151.964	157.25	158.9253	158.9253	162.50	164.9303	167.26	168.9342	173.04	174.967
			Γ	90	91	92	93	94	95	96	97	98	99	100	101	102	103
		Actinid	es‡	Th	Pa	U	Np	Pu	Am	Cm	Bk	Cf	Es	Fm	Md	No	Lr
				232.0381	231.0289	238.0289	(237)	(244)	(243)	(247)	(247)	(251)	(252)	(257)	(258)	(259)	(262)

Figure 1.1. The remaining years until depletion of elements based on current extraction rates (reproduced from Hunt et al. (2015), with permission from the Royal Society of Chemistry).

Nature has however already solved this issue of metal scarcity for the most effective use in its own catalysis. Enzymes are biological catalysts that often incorporate metal active centres and allow almost all biological reactions to take place under, relatively speaking, mild conditions (e.g. 37 °C, pH 7.5, in an aqueous medium – although exceptions are prevalent depending on the biological environment). The metal ions are often bound to cofactors, substrates, nucleic acids or amino acid chains thereby forming active proteins, the unique structure of which gives them extraordinary scopes of specific activities. Enzymes are classed based on the type of reactions they catalyse (Table 1.1). Since the 1980's, recombinant DNA technology has allowed the expression of proteins through the fermentation of renewable sugars in highly productive, benign host organisms (Bornscheuer and Buchholz 2005). This has allowed a vast expansion of enzyme applications because virtually any enzyme can now be produced, even at large volumes, in a renewable manner by means of altering genes and overexpression in high copy number plasmids. Recombinant DNA technology has also spawned the field of protein engineering where specific structural alterations (or mutations) can be made through modifying an amino acid sequence. Changing the structure of an enzyme in a rational manner allows protein engineers to: improve the catalytic rate of an enzyme, better accept native substrates as well as new non-natural substrates, increase selectivity, improve the stability of an enzyme towards different reaction conditions (e.g. temperature, pH, organic solvents), and reduce substrate/product inhibition

(Strohmeier et al. 2011). Protein engineering itself has also grown as a field since its conception (Bornscheuer et al. 2012). Whilst structure-function understanding allows the rational improvements to enzymes by point mutations, there is also now the approach of directed evolution, which does not require a known crystal structure of a given protein (Figure 1.2) (Arnold 2001; Turner 2009; Reetz 2011). The simplest procedure of directed evolution is to take a wild-type enzyme, perform random mutagenesis, and then screen the resultant library for variants that have the desired characteristic. This process may then be repeated with these new variants to further strengthen the enzyme's capabilities. All these advances have now effectively led to an open field of customisable catalysts. In the future, these techniques will likely become even more sophisticated as computational tools and bioinformatics are better integrated to allow rationally designed synthetic enzymes (Lutz 2010).

EC Major Class Number	Enzyme Class	Chemical Functionalisation
1	Oxidoreductases	Oxidation/reduction
2	Transferases	Group transfer
3	Hydrolases	Hydrolysis
4	Lyases	Formation of double bonds through cleaving groups; Breaking double bonds through
5	Isomerases	Isomerisation
6	Ligases (synthetases)	Bonding large molecules, simultaneously cleaving smaller leaving group(s) through hydrolysis

Table 1.1. Major enzyme classes nomenclature. EC – Enzyme Commission (of the International Union of Biochemistry and Molecular Biology).



Figure 1.2. Procedure for protein engineering by rational design and directed evolution. Colours indicate mutations or corresponding structural 'hotspots'. Adapted from Strohmeier et al. (2011).

Therefore, due to their (1) highly selective and sought after activities, (2) renewable origin and stable cost price^{*}, as well as (3) the facility to tailor the structure of enzymes enhancing activity, scope (substrate affinity) and/or stability, enzymes – or biocatalysts – have since found their application in research and industry and now play an increasingly important role in synthetic chemistry.

Enzyme-based processes are employed in various industries to produce complex intermediates and final products in an efficient and sustainable way (Sheldon and Woodley 2018). This broadening of focus in the chemical industry towards encompassing biocatalysis took place mainly due to the impressive selectivity of enzymes (Hughes and Lewis 2018). Additionally, biocatalysts have become important for organic synthesis because their unique functionalisations might allow a reduced number of synthesis steps when compared to conventional routes (Nestl et al. 2014). All of these benefits lead to 'greener' processes through atom efficiency, reduced

^{*} The production of enzymes from sugars means their cost is directly linked to the sugar market. Therefore, their cost is more stable in comparison with other catalysts (especially considering future price volatility that might be caused by projections of depletion (Figure 1.1)).

waste and by-products, mild process conditions, and use of non-toxic solvents (Sheldon 2017; Sheldon and Woodley 2018). Indeed, biocatalysts have become an established option at a range of different scales, mainly for the synthesis of biologically active compounds. In order to illustrate this, a selection of examples of industrial biocatalysis are compiled in Table 1.2. More details can be found and further examples in each of the sources used in Table 1.2 – the review by Sutton et al. (2012) being particularly comprehensive and written from the perspective of fine chemicals and pharmaceutical industries.

Lastly, it is common for biocatalysts to take on a number of different forms in industrial applications (Table 1.2). Usually the cheapest, crudest acceptable form of a biocatalyst will be used due to economic constraints (Pollard and Woodley 2007). The simplified processing routes for the most common industrial biocatalyst formulations are shown in Figure 1.3. All formulations naturally begin with fermentation, which requires the input of fermentation medium and an inoculum of the microbial host strain. Whole-cells may be desired for more sensitive enzymes, internal co-factor recycling mechanisms, or for their low cost. However for most other cases, cellfree extracts (or isolated enzymes) are preferred in order to bypass potential mass transfer limitations of substrate/product across the cell membrane, make a biotransformation independent of cell viability, decrease oxygen demand, as well as for longer term storage and more convenient process handling (Duetz et al. 2001). Typically, cell-free extracts are further filtered after homogenisation to remove solid particulates, but also to concentrate the active enzyme in the formulation. Purified target proteins are almost never used in industrial settings because of additional costly chromatography separation steps. Both whole-cells and cell-free extracts can also be lyophilised into powders by freeze-drying for long-term storage. Lastly, although costly (Tufvesson et al. 2011b), enzymes can also be immobilised typically onto a carrier material for use in continuous flow reactors and for biocatalyst recycling (Hanefeld et al. 2009; Sheldon and van Pelt 2013).



Figure 1.3. Block flow diagram of the upstream production for different biocatalyst formulations. The dashed lines indicate optional steps (freeze-drying), and the dotted lines differentiate extracellular or intracellular enzyme expression. *Lyophilised cell-free extract is the preferred formulation for longer term storage of enzymes and easier process handling.

Table 1.2. Industrial biocata Choi et al. (2015), Grunwalc	lytic process examples (cc I (2015), and Hughes and I	mpiled from Schmid ei _ewis (2018). n.d. – not	t al. (2001), Straathoff et al. (; disclosed; tpa – tonnes per a	2002), Vandamme annum; CFE – cell	e et al. (2006), -free extract.	Sutton et al. (2012),
Substrate	Product	Reaction Type	Enzyme	Biocatalyst Formulation	Scale [tpa]	Manufacturer
Racemic alcohols Racemic amines	Enantiopure alcohols <i>R</i> -Amide; S-Amine	Resolution Resolution	Lipase Lipase	CFE CFE	1000 < 1000	BASF BASF
D, L-5-Monosubstituted hydantoins	4-Hydroxy- <i>D</i> - phenylglycine	Resolution	D-Hydantoinase	Whole-cells	1200	Kanegafuchi
Racemic amino-acid amides	Non-proteinogenic L- amino acids	Resolution	Amidase	CFE	< 1000	DSM
(<i>R</i> , <i>S</i>)-Piperazine-2- carboxylic acid	S-Piperazine-2- carboxylic acid	Resolution	Amidase	Whole-cells	< 1	Lonza
<i>D,L</i> -Amino-∆²-thiazoline- 4-carboxylic acid	L-Cysteine	Resolution	Pseudomonas enzymes	Whole-cells	5000	Ajinomoto
R,S-2-Chloropropianate	S-2-Chloropropionate	Resolution	Dehalogenase	n.d.	2000	Imperial Chemical Industries
Racemic mandelonitrile	R-Mandelic acid	Hydrolysis	Nitrilase	CFE	< 100	BASF
Acrylonitrile	Acrylamide	Hydrolysis	Nitrile hydratase	Immobilised enzymes	> 650 000	Mitsubishi Rayon / Numerous
Penicillin G/V	6-Aminopenicillanic acid	Hydrolysis	Penicillin acylase	Immobilised enzymes	35 000	GSK / DSM
6-Aminopenicillanic acid	Semisynthetic penicillins	Selective coupling	Acylase	CFE	< 1000	DSM
N-protected L-aspartic acid, D/L-phenylalinine methyl ester	Aspartame	Selective coupling	Thermolysine	Immobilised enzymes	14 000	DSM
Niacin	6-Hydroxynicotinic acid	Hydroxylation	Niacin hydroxylase	Whole-cells	20	Lonza
2-Cyanopyrazine	5-Hydroxypyrazine- carboxylic acid	Hydroxylation	Nitrilase/Hydroxylase	Whole-cells	<1	Lonza

1-7

		Formulation	Scale [tpa]	Manufacturer
Hydroxylation	Hydroxylase	Whole-cells	< 1	Lonza
Cascade hydroxylation	Pseudomonas enzymes	Whole-cells	< 1	Lonza
Oxidation	Baeyer-Villiger monooxygenase	CFE	n.d.	Codexis
Oxidation	Pseudomonas enzymes	Whole-cells	< 100	Lonza
Reduction	B-Ketoreductase	CFE	n.d.	Codexis/Arch PharmLabs Ltd.
Reduction	B-Ketoreductase	Whole-cells	2500	Lonza
Reduction	Alcohol dehydrogenase	CFE	n.d.	Pfizer
Transamination	Transaminase	Immobilised enzymes	n.d.	Merck/Codexis
C-C bond formation	Pyruvate decarboxylase	CFE	500	BASF
Conversion of achiral precursors	L-Aspartic acid ammonia lyase	CFE	400	DSM
Glycosyl transfer	Cyclodextrin glucano- transferase	CFE	$> 10 \times 10^{6}$	Numerous
Isomerisation	Glucose isomerase	Immobilised enzymes	> 10 × 10 ⁶	Numerous
	Oxidation Oxidation Reduction Reduction Transamination C-C bond formation Conversion of achiral precursors Glycosyl transfer Isomerisation	OxidationmonooxygenaseOxidationPseudomonas enzymesReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseReductionB-KetoreductaseConversion ofItansaminaseConversion ofL-Aspartic acid ammoniaachiral precursorsCyclodextrin glucano-Glycosyl transferCyclodextrin glucano-IsomerisationGlucose isomeraseIsomerisationGlucose isomerase	OxidationmonooxygenaseCFEOxidationPseudomonas enzymesWhole-cellsReductionB-KetoreductaseWhole-cellsReductionB-KetoreductaseWhole-cellsReductionB-KetoreductaseCFEReductionB-KetoreductaseCFEReductionB-KetoreductaseCFEReductionB-KetoreductaseCFEReductionB-KetoreductaseCFEReductionB-KetoreductaseCFEReductionB-KetoreductaseCFEReductionPyruvate decarboxylaseCFEConversion ofL-Aspartic acid ammoniaCFEConversion ofL-Aspartic acid ammoniaCFESchiral precursorsCyclodextrin glucano-CFEGlycosyl transferGlucose isomeraseImmobilisedIsomerisationGlucose isomeraseImmobilised	OxidationmonooxygenaseCFEn.d.OxidationPseudomonas enzymesWhole-cells<100

1-8

1.2. Early-stage process development and assessment

There are many examples of industrial biocatalysis, however the process development path for each case has never been straightforward and is often exceptional. However, what has been established through these cases are the general tools that can be used to effectively develop processes. The various tools can be classed into two categories: reaction engineering or biocatalyst engineering.

One important aspect of reaction engineering involves changing and/or optimising the reaction environment to enhance the performance of a biocatalyst. There are many process variables to balance (e.g. temperature, pH, type and fraction of co-solvent) that may sometimes conflict with the biocatalyst's optima. For example, high product concentrations might be required to ensure a cost-effective downstream separation. If the correspondingly high substrate load for the reaction inhibits the biocatalyst, then substrate feeding strategies must be considered. For the case of liquid substrates, this simply involves optimising the substrate feed rate. However, solid or gaseous substrates are more challenging to feed, and might need to be delivered to the aqueous enzyme reaction environment through a second phase (a process sometimes called *in situ* substrate supply (ISSS)) (Kim et al. 2007). In these cases, the theoretical feed rate of a substrate is dependent on its partition coefficient between each phase – this is covered in more detail in Section 1.3. High product concentrations might also instigate product inhibition. A means of addressing this is to integrate product separation with the reaction step, a process called *in situ* product removal (ISPR). Systems that are limited by thermodynamic equilibria can also be driven by ISPR through selectively removing a product (Tufvesson et al. 2014). There are many methods of ISPR mostly relying on exploiting unique physical properties of the product to ideally concentrate it out of the enzyme's immediate environment in the reaction medium (Lye and Woodley 1999; Woodley et al. 2008; van Hecke et al. 2014). Some of these methods include: adding water-immiscible organic co-solvents into which product will preferentially partition (Klibanov 1990), using more non-conventional solvents such as ionic liquids (van Rantwijk and Sheldon 2007), deep eutectic solvents (Smith et al. 2014) or supercritical CO₂ (Matsuda 2013), adding a solid phase to either absorb or adsorb the product (Vicenzi et al. 1997; Hilker et al. 2004; Fam and Daugulis 2012), physically transporting the product via a selective membrane (Carstensen et al. 2012), crystallising or precipitating the product from the liquid phase (Buque-Taboada et al. 2006), to name a few. A challenge to implementing ISPR actually lies in the multitude of different techniques. With little consensus or standardised methodologies for selecting and applying ISPR to any given system, the application of ISPR is often bound to *ad hoc* experimental approaches on a case-by-case basis. Another challenge of ISPR is that, as a mass transfer process, the rate of removal is directly related to the

concentration gradient between the reaction phase and 'ISPR phase'. Since the purpose of ISPR is to lower the effective concentration of product in the reaction phase, this concentration gradient or driving force will too be reduced, potentially leading to an inefficient and costly removal process. This trade-off must be carefully considered. A means of improving the rate of ISPR in such cases might be achieved through protein engineering an enzyme's tolerance for product inhibition by allowing higher product concentration limits.

Another aspect to reaction engineering is the collection of kinetic information to be used for process modelling and conceptual design (Ringborg and Woodley 2016). These process models are especially useful for the selection of reactor type and the process of scale-up in later stages of biocatalytic process development (Tufvesson et al. 2013).

On the other hand, protein engineering as discussed in Section 1.1 is still the most powerful way of improving a biocatalyst's performance (Woodley 2013). Another subsidiary branch of biocatalyst engineering is genetic engineering which encompasses improving enzyme expression or the host organism. Experience shows that more than 100-fold improvement to a biocatalytic process may be realised through protein engineering, whilst reaction engineering can only improve processes up to 10-fold, and genetic engineering a few fold. However, the vast benefits of protein engineering come at the greatest cost both in terms of research investment as well as time for development (Truppo 2017) when compared to reaction engineering approaches. This alludes to a necessary balance or prioritisation of the different biocatalytic process development strategies. For example, in the development of a biocatalytic process for the production of atorvastatin, ISPR was initially considered to mitigate product inhibition. Yet a 2500-fold improvement in enzyme activity was achieved instead through the protein engineering. After such improvement the limitation of product inhibition was surpassed and a successful process is now in operation (Ma et al. 2010). It is clear that competencies in protein engineering, fermentation and protein expression, as well as reaction engineering are all essential, and must be integrated, in order to succeed in biocatalytic process development. However, rarely are the two approaches of reaction engineering and biocatalyst engineering cohesive and directed towards a specific goal. Massive strides in one or the other has historically led to a breakthrough in process performance, which has then inspired further development until the biocatalytic reaction is found feasible for full-scale production.

In order to better succeed in biocatalyst implementation, a more systematic methodology and guidelines for process development must be established – especially regarding the early stages. The early stages of biocatalytic reaction conceptualisation and assessment are critical towards the speed of process development, which has been perceived to be too lengthy at present and stands as a major hindrance for industrial implementation (especially in the pharmaceutical industry) (Truppo 2017). Specifically, such a systematic methodology would entail first specifying the target reaction of interest and the required process performance. Secondly, the exact order and type of reaction characterisation experiments must be outlined in order to learn the primary limitations of a given biocatalyst as efficiently as possible. Thereafter the feasibility of implementing different tools for process development (Tufvesson et al. 2013) can be assessed in terms of their effectiveness towards the final targets, cost, and development speed. Only in this way can research efforts be streamlined from all sides affording a successful biocatalytic reaction at an industrial scale. Yet there remain further challenges during reaction assessments brought about by poorly-water soluble substrates. These challenges must also be considered, and their solutions integrated into this systematic methodology.

1.3. Prevalence of poorly water-soluble systems and common features

The majority of biocatalysis is performed with water as the preferred solvent (Sheldon and Woodley 2018). This is due to the understanding that most enzymes naturally occur in aqueous systems, and also because they require water in their immediate environment in order to be active (Zaks and Klibanov 1988; Rupley and Careri 1991; Bell et al. 1995). As the application of biocatalysis to complement conventional chemical and catalytic approaches in organic synthesis continues to expand, an ever increasing number of reactions involve poorly water-soluble substrates. Furthermore, at the required industrial concentrations necessary for cost-effective industrial implementation (Lima-Ramos et al. 2014), this frequently leads to heterogeneous reaction mixtures composed of multiple phases (Erbeldinger et al. 1998). The introduction of ISSS/ISPR can also create a multiphasic reaction medium. Indeed, of the selection of industrial examples of biocatalysis presented in Table 1.2, exactly half of these processes feature poorly water-soluble compounds (of less than 10 g L⁻¹) especially amongst the bulky molecules. These heterogeneous reactions can take the form of gas-liquid, liquid-liquid, or solid-liquid mixtures (or combinations thereof).

There are many challenges associated with multiple reaction phases. The first is a practical issue regarding experimental practice in that it is problematic to measure representative component concentrations when sampling a non-homogeneous medium. Unusual questions arise such as: how many samples are necessary, what is an appropriate sampling fraction (volume), how should samples be taken, or whether physical sampling of the medium itself is the best analytical method in such cases? Secondly, poor solubility can introduce mass transfer limitations because transport must occur between phases. Mass transfer is improved through convection in the medium, i.e. mixing. Better convection through mixing achieves a dispersion of second phase, e.g. smaller liquid droplets or bubble sizes, which in turn improves the specific interfacial area of this phase. However, this dependence on mixing also introduces

1-11

new limitations at different scales (physical reactor sizes) because mixing is a function of specific power input (Noorman 2016). Simply put, it is effortless to homogenise a smaller volume, usually leading to unrealistic power inputs per unit volume when compared to large volumes. This is why good dispersion is challenging at larger scales and why potentially misleading results may be found in the laboratory. Lastly, and most importantly, poor aqueous solubility sets an upper limit on the concentration of substrate available for reaction. The rate of catalysis is a function of dissolved substrate concentration and is generally described by Michaelis-Menten kinetics for enzymes. Therefore, there is a risk that, because these substrates are not accessible for catalysis, heterogeneous reactions may simply be too slow to be industrially useful. Nevertheless many cases, such as oxygen-dependent biocatalytic reactions, prove that reactions featuring poorly water-soluble substrates (where oxygen only has a solubility of 0.27 mM at 25 °C and 1 atm when in equilibrium with air) are indeed realisable. Oxidations can theoretically reach volumetric productivities in excess of 100 mmol L⁻¹ h⁻¹ at scale (Charles 1985), which corresponds to a reasonable 10 g L⁻¹ h⁻¹ for a product of average 100 g mol⁻¹ molecular weight (higher molecular weight products will exhibit higher productivities).

The most effective means to improving aqueous solubility (C_{aq}) is to use organic solvents. The selection and use of solvents in biocatalysis falls under reaction engineering, and is a fundamental component of industrial processes featuring poorly water-soluble substrates. Organic solvents fall under two categories: those which are themselves miscible in water and those which can be considered water-immiscible (although still remain very slightly soluble in water). In almost all circumstances, the solubility of poorly water-soluble substrates will be vastly higher in organic solvents (C_{aux}) based on the principle of 'like dissolves like'. Therefore for watermiscible solvents, the overall capacity for dissolved substrate ($C_{overall}$) is improved based on the fraction of solvent present. The mass balance for such systems is as follows:

$$V_{total} \cdot C_{overall} = V_{ag} \cdot C_{ag} + V_{aux} \cdot C_{aux}$$
(1.1)

An example to illustrate this principle is the production of the poorly water-soluble pesticide 2hydroxybiphenyl. In one case, solvents were investigated for the purpose of ISPR to mitigate its toxicity towards whole-cell biocatalysts (Held et al. 1999). As a result, the compound is well characterised in terms of its solubility in water (< 1 g L⁻¹) as well as many organic solvents (Table 1.3). Applying eq. 1.1 to varying co-solvent fractions for fully water-miscible solvents (methanol, acetone, acetonitrile) shows how the overall solubility of the entire reaction volume can be modified (Figure 1.4). Therefore for cases such as these, the addition of water-miscible solvents can vastly improve substrate availability in the liquid-phase, even in excess of 200 g L⁻¹. It is important to note that some compounds are so poorly water-soluble that enzymatic activity can only be observed in the presence of solvents. Contrarily, this improvement would be disadvantageous for systems where high substrate/product concentrations are inhibitory. However, the exact concentration can still be controlled through the volume fraction of co-solvent. High concentrations of co-solvents can also sometimes induce the inhibition or deactivation of enzymes (Owusu and Cowan 1989; Iyer and Ananthanarayan 2008). Therefore, enzyme stability and activity in the presence of co-solvent fractions must always be studied.

Medium	2-Hydroxybiphenyl Solubility [g L ⁻¹]	Source
Water	0.7	(Cavender and O'Donohue 2001)
Methanol	396	(Tomlin 2004)
Acetone	376	(Tomlin 2004)
Acetonitrile	418	(Tomlin 2004)
n-Octanol ⁺	436	(Tomlin 2004)
Toluene [†]	404	(Tomlin 2004)
n-Hexane ⁺	318	(Tomlin 2004)

Table 1.3. Saturation concentration of 2-hydroxybiphenyl in different solvents.



Figure 1.4. Overall liquid-phase saturation concentration of 2-hydroxybiphenyl at varying water-miscible co-solvent fractions. \bullet – methanol; \blacktriangle – acetone; \blacksquare – acetonitrile.

[†] Water-immiscible.

For the case of water immiscible solvents, a second organic phase will form during reaction. The saturation concentrations of n-octanol, toluene and n-hexane in water are 0.30, 0.53 and 9.5×10^{-3} g L⁻¹, respectively. Therefore, the aqueous phase solubility of compounds such as 2-hydroxybiphenyl are only slightly improved through use of these co-solvents (Table 1.4). However, the overall medium capacity for these compounds is increased to a great extent, and most importantly concentrated in smaller volumes of auxiliary phase. This allows easier and cheaper downstream product purification in later stages (Straathof 2003a). The partition of substrate/product between the aqueous and auxiliary phases can be exploited for ISSS and ISPR when inhibition plays a role (Straathof 2003b). In such cases, high substrate and product loadings can still be realised without the effects of inhibition.

Table 1.4. Maximum component fractions of 2-hydroxybiphenyl in the total reaction volume ($C_{overall}$), aqueous phase (C_{aq}), and auxiliary phase (C_{aux}) at various volume fractions of water-immiscible solvents.

	n	-Octanol			Toluene		r	n-Hexane	
Vol. Fraction Co- Solvent [% (v/v)]	C _{overall} [g L ⁻¹]	<i>С_{аq}</i> [g L ⁻¹]	C _{aux} [g L ⁻¹]	C _{overall} [g L ⁻¹]	<i>С_{аq}</i> [g L ⁻¹]	C _{aux} [g L ⁻¹]	C _{overall} [g L ⁻¹]	<i>С_{аq}</i> [g L ⁻¹]	C _{aux} [g L ⁻¹]
0	0.70	0.70	-	0.70	0.70	-	0.70	0.70	-
10	44.2	0.86	434	41.0	0.95	402	32.5	0.70	318
50	218	0.86	436	202	0.95	404	160	0.70	318
100	436	-	436	404	-	404	318	-	318

1.4. Thesis aims

Despite poorly water-soluble compounds being so prominent in industrial cases of biocatalysis, little focus in literature is given towards their challenges and systematic ways of handling them. Therefore, the overall aim of this thesis is to describe special considerations when handling poorly water-soluble substrates during early-stage biocatalytic reaction characterisations. A second aim of this thesis is to establish how to approach such early-stage development as there are no generic guidelines or methodologies for such. Together, these aims complement each other to give a comprehensive outlook on reaction engineering for poorly-water substrates in biocatalysis, which should satisfy an important knowledge gap for future biocatalytic process development.

1.5. Thesis structure

Towards these aims, the following are discussed in the appropriate chapter of this thesis, respectively. The results chapters are divided into two parts corresponding to each primary aim as stated above:

Early-stage reaction assessments

- An experimental procedure for carrying out reaction scoping is outlined in Chapter 2. The outcome is the determination of a primary process limitation or bottleneck as early as possible in the development cycle of biocatalysts with a minimum number of three experiments.
- In Chapter 3 the use of process performance metrics to guide development is described. Values for these metrics are suggested based on techno-economic process requirements of different product value-classes.

Experimental challenges associated with poorly-water soluble substrates during reaction characterisations

• The influences of mass transfer limitations on process metric evaluations when assessing heterogeneous reactions is addressed in Chapter 4. This was illustrated by means of a case study, which was the enantioselective desymmetrisation of a poorly water-soluble substrate using pig liver esterase. The optimal biocatalyst dose and space-time yield at small-scale was assessed without incurring mass transfer limitations (ascertained using the methodology described in Chapter 2). Perspectives on further process development were also suggested based on benchmarking process performance with metric targets from Chapter 3.

- Chapter 5 illustrates the analytical challenges in assessing conversions of poorly watersoluble substrates through a case study of a complex biocatalytic Baeyer-Villiger oxidation of macrocyclic ketones in a gas-solid-liquid reaction. The need and accuracy of alternative analytical techniques is discussed with comparison to conventional techniques of sampling liquid media and spectrophotometric activity assays.
- In Chapter 6 the analytical methods described in Chapter 5 are applied in combination with the methodology detailed in Chapter 2 to scope the biocatalytic Baeyer-Villiger oxidation of a macrocyclic ketone to its corresponding lactone, eventually elucidating the overall process limitation for this given reaction and focus for future process development and scale-up based on benchmarking with the process metric targets from Chapter 3.

The overall findings of this thesis are discussed in Chapter 7 and, based on these, a route for earlystage reaction conceptualisation is proposed. Even more importantly, this discussion also highlights a better way of handling poorly-water soluble substrates in future biocatalysis. Based on this, conclusions were drawn in Chapter 8, and future perspectives given in Chapter 9.

1.6. Scope

The scope of this thesis is such that only cases featuring poorly water-soluble substrates are considered. That being stated, the sections addressing early-stage reaction assessments in Chapters 2 and 3 are generic for any given biocatalytic reaction (i.e. not poorly water-soluble substrates alone).

Later process development steps and improvement strategies are not directly addressed in this thesis as they have been described elsewhere in literature and are beyond the scope of this thesis. However, the most important general aspects for implementing future biocatalytic conversions of poorly water-soluble substrates are discussed in line with the findings of the specific case studies in this thesis.

Literature is further reviewed throughout this thesis in each respective chapter where appropriate.

Chapter 2. 'Bottleneck' identification in early-stage biocatalytic reaction assessments

This chapter describes a novel methodology that is used as a framework for initial experimental characterisations of the enzyme-catalysed reactions in case studies described later in this thesis (Chapters 4-6). The method makes use of a minimum number of three experiments that vary only two factors (biocatalyst loading and initial substrate concentration) to determine a major process limitation for a given reaction system. Most important, in the context of reactions featuring poorly water-soluble substrates, is the ability of this method to qualitatively distinguish mass transfer limitations from any other limitations, such as enzyme stability or reaction rates and binding affinities (i.e. kinetics).

The following manuscript, which has been submitted for publication, has been reworked from this chapter:

Mathias Nordblad, Mafalda Dias Gomes,[†] <u>Murray P. Meissner</u>,[†] Hemalata Ramesh, John M. Woodley. 2018. Scoping Biocatalyst Performance using Reaction Trajectory Analysis. *Organic Process Research* & *Development*, (submitted). [†]These authors contributed equally.

2.1. Introduction

A significant challenge to biocatalytic process development is the complex nature of the design task in terms of finding a viable synthesis route, enzyme candidate with the desired specific activity, and compromise between optimal reaction conditions for a process and that of the enzyme (Woodley 2017). Essential to a successful process development is the need for a broad range of scientific and engineering disciplines to align. First, the specific synthetic route must be decided upon while at the same time identifying an appropriate enzymatic activity towards this functionalisation with the inputs of chemists and molecular biologists. Thereafter, the enzyme needs to be expressed in an appropriate host organism and prepared in a final formulation for use in a process by biotechnologists. Process engineers and chemists also need to envisage process constraints based on the economic value class of the product in question.

Certainly, the enzyme will need to be screened to assess its suitability for the target reaction (Lima-Ramos et al. 2014; Ringborg and Woodley 2016), a process herein referred to as "reaction scoping". This characterisation must be carried out as early as possible in order to feedback required improvements to the respective partners involved in early-stage process development. The efficacy of this characterisation could ultimately accelerate the rate of overall process development by establishing the primary limitations and relevant improvement strategies at an early-stage. Indeed, an improved speed of development has been highlighted as a requisite need to successful enzyme implementation, especially in the pharmaceutical industry (Truppo 2017). Thus, standardised tools and methods for designing, producing and evaluating enzymes should be considered highly valuable towards this end (Wohlgemuth 2007). However, to date there is little consensus as to how this characterisation is best carried out and most cases are established following an *ad hoc* approach.

Examples of experimental methods currently used to perform such characterisations are the measure of reaction progress after a certain time (single-point measurements) or 'initial rate measurements' (Cornish-Bowden 2012). In the context of characterising enzymes, these techniques can elucidate kinetic parameters or enzyme stability towards different reaction environments, although are laborious in terms of the number of experiments required (Gardossi et al. 2010). If extensive experimentation is followed using these methods, an indication of feasibility or optimisation of reaction conditions can be made. However, the major process performance limitation (or bottleneck) of the overall system cannot be inferred from such experiments.

This chapter details a systematic methodology that can be used to resolve exactly this problem. The relative simplicity of the methodology is key to its applicability in early-stage

process development. It follows that if the primary process limitation of an enzyme were to be made known at an early-stage, more directed process (Tufvesson et al. 2011a) and protein engineering (Woodley 2013) improvement strategies could be identified and implemented. The methodology relies on specific experiments that can offer a qualitative, and to some extent quantitative, description of the bottleneck/process limitations of a given enzyme reaction.

Similar to the works of Blackmond (2005; 2015) and Duggleby (1994; 2001), the methodology makes use of high frequency time-course conversion progressions of reactions to elucidate underlying reaction kinetics. The mechanism of such kinetics is then distinguished using normalised plots, similar to an earlier approach by Selwyn (1965). Building on Selwyn's work (which was a method to identify biocatalyst inactivation), the methodology presented in this chapter can identify limitations in (substrate) mass transfer, enzyme kinetics and stability.

The methodology that follows describes and discusses the relevance of graphical reaction trajectories, normalised axes and reaction conditions. Thereafter, the actual experiments and their order are presented. Lastly, the identified bottlenecks are discussed in relation to the experimental conditions. For the purposes of illustrating the method, simulations were made using empirically determined enzyme kinetic parameters of glucose oxidase, wherein expressions for oxygen mass transfer and enzyme inactivation models could also be included. The exact expressions used in the model fall outside the scope of this chapter, however are described in detail in Nordblad et al. (submitted). Further experimental validations of this method, which confirm the simulated responses, are also given as three separate case studies in Nordblad et al. (submitted). Notwithstanding, validations of the method also appear in the later chapters of this thesis wherein it was applied.

2.2. Methodology

2.2.1. Time-course reaction profiles and normalised axes

Central to the methodology are experimental time-course reaction progressions. Different kinetic phenomena (such as mass transfer or enzyme kinetics) are aggregated into each data point of a reaction trajectory, and over longer periods, the shape of this conversion profile will be dictated by the fundamental limiting phenomenon. In this way, a full time-course progression can reveal better understanding of kinetic limitations than single-point measurements or initial rate measurements can provide. The accuracy in determining the shape of a reaction trajectory is enhanced through higher frequency measurements, such as those which direct, online analytical techniques can provide (Meissner et al. 2018). As already mentioned, single-point measurements aggregate all effects of catalyst kinetics, mass transfer and loss of activity over time, and the extent of these effects are all dependent on changes in reaction composition (i.e. over time), and

therefore cannot be decoupled from each other. Further, whilst initial rate measurements can account for catalyst kinetics over brief periods, they cannot identify the other effects over the entire course of a reaction.

The methodology makes use of normalised axes in plots to distinguish time-dependent effects. A similar graphical approach was described by Selwyn (1965). For example, if the biocatalyst concentration is doubled whilst maintaining all other reaction conditions, then it should be expected that the reaction time will be exactly halved due to the increased rate of reaction. (Time-dependent) deviations from this expected behaviour would be difficult to visually inspect if the ordinary time vs. conversion plots of each data set were to be compared. However, if the time axis is normalised by the independent variable by multiplying it with biocatalyst concentration, then the resulting plots of (time · biocatalyst concentration) vs. conversion should directly overlap if there are no time-dependent effects (or no biocatalyst concentrationdependent activity effects). Conversely, if any other response is observed in such a normalised plot, then there exists a time-dependent limitation, which is in itself a function of the enzyme's response to the changing reaction composition. Similarly, an experiment performed with double the substrate concentration (with all other reaction conditions maintained) should take exactly double the time to reach full conversion. Normalising this response by dividing by the independent variable (i.e. substrate concentration) will then further elucidate the mechanism of such a limiting effect if no graphical overlap is observed. Such profiles are discussed and presented in Section 2.2.3.

2.2.2. Reaction conditions

In order to effectively learn how an enzyme will cope with certain reaction conditions in an industrial process, as much as possible, similar conditions must be used when performing these time-course evaluations. In this way, the experimental conditions should be relevant towards helping meet process targets for a given industrial application.

Industrial processes regularly require high substrate loadings, often in excess of 50 g L⁻¹, in order to realise cost-effective downstream separations. Choosing similarly high substrate concentrations in lab-scale experiments will allow the detection and quantification of the effects of substrate and/or product inhibition on an enzyme which may occur at these high concentrations.

In addition, the cost of enzymes matter on an industrial-scale. Therefore, the dose of biocatalyst at lab-scale should reflect this constraint. This, together with high substrate loadings, should also give indication to a more realistic reaction duration. If enzyme inactivation occurs during this reaction period then its effect on reaction progress will be made evident.

Finally, industrial processes frequently require the addition of solvents and other agents, such as antifoam, to enable smooth process operation. Solvents help solubilise poorly water-soluble substrates (refer to Chapter 1 and Chapter 6), can help remove inhibitory products *in situ*, and can also simplify downstream product isolation. Adding them at this (early) stage of process scoping immediately informs of an enzyme's robustness towards different solvents.

2.2.3. Order of experiments

The methodology relies on three key experiments, all of which follow the conversion (reaction progress) over time, and are presented as respective steps in Figure 2.1. Here, the conversion can be defined as either the fractional or percentage conversion of substrate to product, or the conversion of substrate to product relative to the thermodynamic equilibrium limit (if applicable). The first step (i.e. experiment) is used as a benchmark. This experiment establishes the general type of reaction behaviour: specifically, if the rate of reaction varies with time and/or conversion (termed "higher-order kinetics") or if it does not (zero-order kinetics). In the second experiment, the catalyst load is varied relative to the first experiment, and its impact on the reaction trajectory is assessed thereby identifying the main limitation of the system (in all of the example plots in Figure 2.1, the variable of interest is increased relative to the base case). More specifically, this experiment distinguishes between limitations related to the catalytic activity (through kinetics or stability) and limitations not related to the catalyst, such as mass transfer limitations. In the third experiment, the substrate concentration is varied, again, relative to the baseline experiment. This experiment only applies to systems limited by either enzyme kinetics or stability, and gives an indication of the underlying mechanism in either category.



Figure 2.1. Experimental order and the resulting reaction trajectory responses for different process performance limitations. Catalyst activity limited: k_{cat} – turnover number; kinetically limited: K_M – Michaelis constant, K_{ic} – competitive inhibition constant, K_{iu} – uncompetitive inhibition constant; enzyme stability limited: k_d – first-order rate constant for inactivation, TTN – total turnover number; mass transfer limited: k_La – overall volumetric mass transfer coefficient. Uncompetitive inhibition and competitive inhibition have been respectively simplified to typical scenarios of product inhibition and substrate inhibition.
Zero- vs. higher-order kinetics

The methodology starts with a single time-course experiment, meant to assess the general behaviour of the reaction under the chosen reaction conditions. In this analysis, reactions exhibiting a constant reaction rate throughout the course of reaction are classed as exhibiting zero-order kinetics. This type of behaviour implies that, within the conditions of the experiment, neither a change in substrate/product composition (through enzyme kinetics) nor reaction time (through inactivation) affect the course of reaction. Conversely, a reaction that exhibits a reaction rate that changes over the course of the reaction is classified as having higher-order kinetics. This behaviour also includes all reactions that fail to reach completion (relative to the reaction equilibrium). It is worth noting that it is common for reactions to show zero-order behaviour initially and transition into higher-order kinetics at higher conversions or after some time.

Impact of catalyst loading

For the second step of the methodology, the catalyst load is varied to separate the three main classes of limitation: catalyst kinetics, catalyst stability or non-enzyme related.

In a reaction controlled entirely by enzyme kinetics, the plots of reaction courses with different catalyst loads should superimpose when the reaction time is normalised for the catalyst load.

In a system limited by catalyst stability, the reaction rate should vary less with conversion at higher catalyst loads. This is because inactivation is a time-dependent process, and therefore less inactivation would have occurred in a reaction that proceeds more quickly.

Finally, in some cases the reaction rate will be disproportionately lower at higher catalyst loadings. This is because something other than intrinsic catalyst kinetics or stability is limiting the reaction: most typically, the rate of substrate mass transfer between phases or compartments in a reaction system. It is important to note that in such cases, any further analysis of the catalyst performance will be hindered by extensive mass transfer (non-catalyst related) limitations. If such an analysis is desired, reaction conditions must be modified to either improve mass transfer, or to reduce the need for high mass transfer by reducing the catalyst load (as indicated in Figure 2.1). The assessment should then be done from the beginning with a new baseline experiment again.

A reaction exhibiting zero-order behaviour is not significantly affected by catalyst inactivation during the course of the reaction and is saturated by the substrate throughout the reaction. Further improving such a reaction requires improving the maximum activity of the catalyst relative to the substrate.

Impact of substrate loading

In the third step of the methodology, the substrate concentration is varied in addition to the biocatalyst loading to distinguish between mechanisms for both kinetic and stability limitations. Changing the substrate concentration directly modifies the conditions for the catalyst, and consequently the entire reaction course will be different. Additionally, changing the substrate concentration also changes the duration of the reaction because more substrate typically takes longer to convert with a given amount of catalyst.

In a case where it has been determined that the reaction is primarily limited by catalyst kinetics, it will be of interest to determine the dominating kinetic limitation. For soluble enzyme catalysis in a homogeneous system exhibiting higher-order behaviour, the rate of reaction will depend solely on the enzyme kinetics. These will be linked to either a high saturation constant for the substrate (i.e. high K_M), or competitive or non-competitive inhibition by one or more of the reaction components. To distinguish between these effects, reaction data for different substrate loads should be compared in a plot of conversion against reaction time normalised by both enzyme and substrate load.

In this example, for a reaction that becomes slower at higher substrate conversions due to a high K_M for the substrate, the rate of conversion will become disproportionally faster at increased substrate concentrations. This is because the catalyst becomes more saturated at a given conversion with more substrate. On the other hand, if the kinetic limitation is due to competitive inhibition by the product, the combined effect of inhibition and increased substrate load results in an overlap of the reaction data in the normalised plot. Finally, non-competitive inhibition by the product results in a disproportionally lower rate of conversion at increased substrate concentrations (this effect can also be seen when one substrate acts as a competitive inhibitor for another in a two-substrate reaction). These effects are illustrated in Figure 2.1.

If it turns out that the reaction rate diminishes over the course of a reaction due to inactivation of the enzyme, it could be of interest to determine how this activity is lost. This work considers three mechanisms for inactivation: total turnover number (*TTN*) type-stability where the rate of inactivation is proportional to the rate of reaction, conversion-independent stability where the rate of inactivation is proportional to the concentration of remaining active enzyme (and linked to a parameter that is fixed in the experiment, such as temperature), and conversion-dependent stability where the rate of inactivation varies with the concentration of enzyme and the concentration of a reaction component (for example: product-induced inactivation) (Bommarius and Paye 2013).

For soluble enzyme catalysis in a homogeneous system, the performance of a reaction limited by product (or substrate) induced inactivation will become progressively worse at higher substrate loads in a plot of conversion vs time normalised by catalyst and substrate load. If the inactivation is instead linked to *TTN* or conversion-independent inactivation, the reaction curves for different substrate loads will superimpose in the normalised plots. These two inactivation mechanisms cannot be separated in this type of experiment if stability is the only limiting factor.

2.2.4. Dominating bottleneck regimes

It is important to realise that underlying kinetic mechanisms can be masked by the dominating limitation. For example, little or no useful information regarding the catalyst kinetics or stability can be gleaned from a substrate load experiment in a system that is limited primarily by mass transfer. It is therefore suggested that the main limitation is first determined using the catalyst load experiment. If it turns out that mass transfer is the main limitation, the reaction system should be modified to address and overcome this limitation, before further investigation of the catalyst properties are attempted (e.g. by changing the mixing conditions, scaling up the reaction (for improving gas-liquid mass transfer (Stocks 2013)), or by implementing a reactor design that improves mass transfer (Gasparini et al. 2012)). Likewise, significant activity loss over the course of the reaction affects the analysis of kinetics. Stability issues should therefore be addressed and solved before kinetics are studied in detail, after being quantified over longer reaction periods (i.e. at higher substrate or decreased enzyme loadings).

The impact of these two varied parameters on the different limitations is illustrated in Figure 2.2. For the purposes of the illustration, it has been assumed the catalyst kinetics are dominated by substrate binding to a certain point (up to the order of magnitude of K_M), beyond which the enzyme operates under fully saturated conditions until, above a certain concentration, the substrate or product cause inhibition. These two effects are independent of the enzyme concentration and thus run parallel to this axis. The catalytic rate is proportional to the enzyme load up to a point, beyond which substrate mass transfer can become a limiting factor. An increase in substrate load results in increased mass transfer, and consequently moves the limitation to catalyst load. Finally, the reaction can also be limited by catalyst stability. Since the reaction time scales with the catalyst load (linearly under zero-order kinetics), the amount of enzyme required to avoid stability limitations is proportional to the substrate load.



Biocatalyst Concentration

Figure 2.2. Illustration of the different types of process limitations and how they relate to substrate and biocatalyst load – A: Inhibition, B: Mass transfer, C: Kinetically limited, D: Stability. The optimal region of operation (OR) in the window where no limitations occur is circled, here substrate concentrations are maximised whilst using the least amount of biocatalyst.

In the given example, the system is limited by different factors under different conditions, i.e. combinations of substrate and catalyst load. It should be noted that not all of the indicated domains/limitations will necessarily be relevant for any given case. Figure 2.2 provides a blueprint of an expected regime that can guide the changes in enzyme or substrate loading which an experimenter should make in order to be able to evaluate these different bottlenecks.

After the limitations of a given reaction have been established, it could also be possible to define a similar looking "operating window" inside which a reaction can proceed without constraints. The optimal region of operation is shown on Figure 2.2, where substrate concentration is maximised with the least biocatalyst dose, assuming the rate of reaction is sufficient.

2.3. Discussion

In the context of process development, once the limiting factor for process performance has been identified the next logical step is to address that limitation. When the main limitation is enzyme kinetics or stability, there is potential for improvement in modifying the structure of the enzyme molecule through protein engineering. Such modifications can improve the affinity for the substrate (reducing K_M) or improve the stability of the protein structure. An alternative (or complementary) approach, in cases where the substrate or product have a negative impact on performance, is to control the concentration of these reaction components. This can be effected through controlled substrate supply (e.g. feeding) and *in-situ* product removal strategies (Ramesh

et al. 2016). In cases where mass transfer is limiting, the most direct solution is to consider the reaction and reactor design: how substrate is introduced and transported to the enzyme, the choice of any co-solvents and choice of reactor and configuration of mixing, if any. Finally, in some cases (for all categories of limitation) the formulation and purity of the catalyst should also be considered. The choice between applying whole-cell catalysis, crude or purified soluble enzyme solutions or an immobilized preparation can have a great impact on both the activity and the stability of the catalyst as a whole. On the other hand, the choice of formulation also comes with a cost that can affect the feasibility of the process (Tufvesson et al. 2011b).

The simulated reactions used to illustrate the methodology (Figure 2.1) are based on reaction kinetics for a one-way reaction that will run to completion, barring total loss of activity due to inactivation. However, the suggested methodology is fully applicable to equilibrium/thermodynamically limited reactions as well because the impact of catalyst load will be the same for an equilibrium reaction as for a non-reversible one. Further, varying the substrate load should not, for changes within an order of magnitude, drastically alter the equilibrium conversion in most cases. However, care should be taken when choosing substrate concentrations when studying two-substrate equilibrium reactions, because changing the concentration of only one of the substrates in such a case can change the expected conversion.

The methodology presented in part here is intended as an addition to established experiment-based evaluation methods that are used to support process development. Ultimately, the best tool for the task at hand should be used. A single end-point measurement can thus be sufficient to verify that the reaction achieves required performance (i.e. conversion in a given time) for some cases. On the other hand, if detailed information regarding the kinetic parameters of the catalyst is required (for example to evaluate the success of a specific modification to the protein), an initial rate experiment under carefully controlled conditions is likely the most pragmatic option. The methodology presented here is thus meant to provide an alternative, limited-effort approach in cases where more qualitative information is required; to provide a sense of the characteristics of the reaction system of interest under process conditions and to guide further experimentation and development work.

2.4. Conclusions

The methodology presented in this chapter presents a generic tool for assessing biocatalytic reactions. Distinct to these reactions is the prevalence of mass transfer limitations and potential instability of protein structures under process conditions. Both of these effects can be identified in a qualitative manner using this methodology. Furthermore, the mechanisms of limitation caused solely by enzyme kinetics (e.g. catalytic activity (k_{cat}), substrate affinity (K_M), or substrate/product inhibition) can be distinguished. The methodology is simple in terms of experimental effort and should be carried out at an early-stage of biocatalytic process development in order to better inform future directions for research investment.

Chapter 3. Setting process performance metric targets for the direction and evaluation of biocatalytic reactions

This goal of this chapter was to better define a systematic way of addressing biocatalytic process development. A crucial aspect to biocatalytic reaction development is the need for industrially-relevant reaction targets (i.e. synthesis route). An equally important facet to reaction development is a knowledge of the required process performance metrics in terms of reaction rate, product concentration, biocatalyst yield, conversion and selectivity, to name a few. These metrics place constraints and help direct biocatalytic reaction development, which can be especially useful in early-stages or for benchmarking. Therefore, these metrics are herein stipulated based on experience and calculation. A precise and simple methodology for reaction evaluation, which may be used to measure these metrics, is detailed in Chapter 2. A particular focus is placed on defining the value of metrics based on product market applications, estimating the biocatalyst yield through considering the cost of biocatalyst formulation, and perspectives on immobilisation to improve biocatalyst yields.

The following is intended for publication.

3.1. Introduction

When designing an enzyme-catalysed reaction from scratch, the first thing to be considered is what synthetic route or specific reaction is needed. This is likely to be inspired by a portfolio of known enzyme activities or the ease of expanding the substrate scope of a given enzyme to accommodate the reaction of interest. However, just as important is the understanding of what level of performance is required to meet the demands of the envisaged process. Such understanding will give indication to the direction in which a particular biocatalytic reaction must head in terms of, for example, eventual product concentration or rate of reaction. In this way, these process performance requirements help guide reaction design by establishing constraints during development. Towards this objective, a system of metrics are defined, which can be used as benchmarks when evaluating biocatalytic reactions. Targets for these metrics are proposed based on the value class of the product market (application). In this way, the process and reaction constraints are well-defined and based on techno-economic requirements. Different enzyme formulations will require different levels of reaction efficiency due to the associated cost contribution to the process margins. This is considered and addressed in this work.

A basic assumption made in this work is that an enzyme candidate is already available for assessment (in its most suitable formulation) towards a specific reaction. These targets are therefore less relevant for enzyme discovery, however might give indication to viable candidates. Further, because these targets pertain to reaction performance, downstream specifics are not considered – although the inherent requirements of a feasible downstream separation are incorporated into this assessment through the metric targets (e.g. need for higher product concentrations).

3.2. Process performance metrics

Biocatalytic process performance can be gauged using a standard set of process metrics (Table 3.1). These metrics may be benchmarked against industrial requirements, and the importance and value of each respective metric may differ depending on the value of the product and its intended market application. Using such generic process metrics, inspired by economic requirements, allows the avoidance of such detailed economic information (Lima-Ramos et al. 2014). This is beneficial when this information might be sensitive or more likely unavailable. The simple nature of these metrics allows them to be calculated in a 'back of the envelope' fashion. Furthermore, they can then be used as 'rules of thumb' when trying to assess the feasibility of a biocatalytic reaction step, without the need for more complex knowledge of the full process, environmental or economic performance.

Whilst there is no particular order as to how the metrics are presented in this work, for different types of reactions there may be a hierarchy of importance. For example, processes to produce pharmaceutical intermediates might value selectivity above all other metrics. On the other hand, manufacturing processes for low-value products such as polymers might require the highest possible reaction yield given the predominance of substrate costs for these processes.

Process Metric	Definition	Unit
Reaction conversion	$\frac{n_{substrate, initial} - n_{substrate}}{n_{substrate, initial}} \times 100\%$	%
Reaction yield	$\frac{n_{product} - n_{product, initial}}{n_{substrate, initial}} \times 100\%$	%
Yield on co-substrate	$\frac{n_{\text{product}} - n_{\text{product, initial}}}{n_{\text{co-substrate, initial}}} \times 100\%$	%
Selectivity	Reaction yield Reaction conversion	-
Product concentration	$\frac{m_{product}}{V_{reactor}}$	g L ⁻¹
Productivity (or space- time yield)	$\frac{m_{product}}{\tau \times V_{reactor}}$	g $L^{-1} h^{-1}$
Specific productivity	$\frac{m_{product}}{\tau \times m_{biocatalyst}}$	$g g_{\text{biocat}}^{-1} h^{-1}$
Biocatalyst yield	m _{product} m _{biocatalyst}	g g _{biocat} -1

Table 3.1. Biocata	lytic proces	s performance	metrics

Reaction conversion and yield

A fundamental requirement of an industrial reaction is to create value by making an adequate amount of product from a lower value substrate. The measure of how much product is produced from a certain amount of substrate is the reaction yield. In a single-product reaction with no byproducts or product degradation under the process conditions, the reaction yield will be equivalent to the reaction conversion. In some cases, the reaction yield is dictated by chemical equilibrium (thermodynamics). Reaction yield is especially critical for lower value, bulk volume products where process economics can largely be dictated by the cost of substrates and raw feedstocks. On the other hand, processes for high-value products might also require high reaction yields to avoid costly downstream separations of similar substrate and product molecules. Lastly, in some reactions the cost of co-substrates can be considerable. Therefore in these cases, it may be valuable to include a yield on co-substrate metric in order to take this into consideration.

Selectivity

Selectivity is an important measure when multiple products are formed in a reaction (e.g. asymmetric reactions). The selectivity is an exact indication of the amount of product of interest that has been in formed in lieu of all other products. This is often a key metric when emphasising the benefits of biocatalysts over chemocatalysts. Naturally, reactions with higher selectivity will be more atom efficient, produce less waste by-products and have simpler downstream separations (Sheldon and Woodley 2018).

Product concentration

The product concentration leaving the reactor is an important metric for downstream process design. Higher product concentrations allow smaller, more cost-effective separation steps, which is critical when considering downstream costs are often significant towards process margins. Importantly, high product concentrations can often have an inhibitory effect on some enzymes because they are vastly different to reaction component concentrations encountered in Nature. The product concentration should also only be measured based on the volume passing downstream from the reaction step, which is distinct to processes using *in situ* product removal technologies. Here, the product is ideally concentrated into a second phase (e.g. water-immiscible organic solvent or solid phase) and will be separated from the reaction medium before being passed on for downstream separation and purification. Therefore for these cases, only the concentration of product in this second phase influences the cost of the downstream process and will be relevant.

Productivity/space-time yield

The productivity (or space-time yield) of a reaction dictates the scale of reactor and process required to produce a target amount of product in a given time, and is based on the total reaction volume. The space-time yield is a function of the enzyme dose (see biocatalyst yield, below) and the intrinsic activity of that enzyme preparation, as is the case with chemocatalysis. It can therefore be increased by simply adding more biocatalyst, however this comes at the expense of space in the reactor (for the case of immobilised preparations) as well as cost of the enzyme. For this reason, a trade-off between reaction volume, biocatalyst yield and the minimum required activity must be found. The connection between space-time yield and biocatalyst yield is covered in more detail in the case study of Chapter 4.

Specific productivity and biocatalyst yield

On the other hand, the specific productivity has the basis of amount of enzyme added rather than a volumetric basis, as is the case for space-time yield. For the case of biocatalytic reactions, the biocatalyst yield is effectively the yield of product on biocatalyst (not to be confused with the yield of biocatalyst from a fermentation). It is a measure of how much product can be produced per amount of biocatalyst and should be measured over the entire period of a biocatalyst's life in a process. The biocatalyst yield informs of the cost contribution of a biocatalyst towards the process (this is elaborated upon in Section 3.4). Since biocatalyst formulations vary, the units of biocatalyst yield also differs and must be scaled by the amount of actual target protein that is present in the formulation. Generally speaking, the more active an enzyme, the better the biocatalytic yield so this might stand as a generic target for protein engineering. However, not all processes for different value products have the same reliance on enzyme costs. The price of biocatalyst for large volume (low-value) applications is usually nominal and relatively stable (although a high product turnover is still required), whilst the cost of enzymes for higher value product markets might be less defined. Biocatalyst yield may also be improved by simply producing more product in a given batch (adding more substrate), lowering the dose of biocatalyst (at the expense of space-time yield), or recycling the biocatalyst in subsequent batches. Recycling an enzyme often requires it to be available in an immobilised preparation. It is crucial that an immobilised enzyme preparation has sufficiently high intrinsic activity and stability to justify the costs of immobilisation and space reduction in the reactor volume. Generally, only the crudest acceptable form of a biocatalyst should be considered in order to minimise the cost of enzyme in a process.

3.3. Markets for biocatalysis applications and target process metrics

It is important to recognise that while biocatalysis offers all the valuable process traits shown in Figure 3.1, the primary reasons for biocatalysis implementation in different markets is different (Wohlgemuth 2010; Woodley 2017). Most likely, this is linked to the complexity of the chemical reaction itself, where biocatalysis has become instrumental in the highly-selective synthesis of optically pure molecules often containing multiple chiral centres (Sheldon and Woodley 2018). However, the argument for the implementation of biocatalysis due to other factors, especially in lower value product markets, remains a challenge. In particular, the high development costs of engineered biocatalysts makes them too expensive to improve for bulk volume products. Additionally, processes producing low-value products require high product titres, even in excess of 100 g L⁻¹, which almost always leads to some form of protein denaturation and/or inhibition because such concentrations far exceed those found in Nature.



Figure 3.1. Value of biocatalysis in different markets (adapted from Woodley (2017)). APIs – Active pharmaceutical ingredients.

Table 3.2 summarises target process metrics for different value processes, compiled through cumulated experience and industrial collaboration. Lower value products will have smaller process margins and therefore the biocatalyst itself must be more efficient in terms of both productivity and product concentration output. In essence, 100% conversion, reaction yield and selectivity are always desirable, however sometimes this may be unachievable due to e.g. chemical equilibrium. If high conversions are required then the value of K_M might become especially important because if it is too large reaction rates will become too slow at higher conversions. Furthermore, high-value products do not necessarily require a 100% conversion because process economics are less dependent on the price of feedstock for these cases. However, unreacted substrate can still be detrimental to downstream processes in these cases, especially when the substrate shares similar physical properties to the product (e.g. chiral resolutions).

	Low-value	Medium- value	High-value
Product Price [€ kg ⁻¹]	5	20	100
Product Titre [g L ⁻¹]	100	50	10
Productivity [g L ⁻¹ h ⁻¹]	20	10	2

Table 3.2. Process performance metric targets for different product value classes.

The metrics in Table 3.2 are dictated by process constraints. The product concentrations and space-time yields stated here are such that realistic process margins may be achieved for different

scales of production. Further, these metric targets are minimum thresholds that must all be satisfied in order to realise an effective biocatalytic process. Improving process metrics beyond these values will always be beneficial for the cost-effectiveness and environmental impact of a given process. However, depending on the application, there may be a hierarchy of importance for the different metric targets.

3.4. Cost contribution of biocatalysts: biocatalyst yield

3.4.1. Estimating target biocatalyst yields

Setting a target for biocatalyst yield is more complex, since it is not only a function of process constraints (the space-time yield will determine a required intrinsic activity), but also the intended market value-class and final formulation. Different biocatalyst formulations have different implications for processes. For example, isolated enzymes in (lyophilised) cell-free extracts can potentially be exposed to higher substrate/product concentrations or more harsh reaction environments because the issues of cell wall integrity and whole-cell viability are circumvented. Generally, if appropriate, the most crude form of a biocatalyst will always be selected above those which take more preparation (e.g. purified proteins or immobilised preparations) due to economic constraints (Woodley 2017). For this reason, only the following most typical biocatalyst formulations have been considered: fresh 'wet' whole-cells directly from a fermentation, dry whole-cells prepared through freeze-drying for longer term storage, cell-free extracts (liquid formulations), and lyophilised cell-free extracts. A set of guideline assumptions to convert between these different formulations must be assumed based on realistic biotechnological downstream processes (Table 3.3).

Table 3.3. Conversion factors for different biocatalyst formulations. DCW – dry cell weight (i.e. dry
whole-cells); CWW - cell wet weight (i.e. wet whole-cells); CFE - cell-free extract; "prot" - total protein
content; "biocat" – total soluble protein content (including target enzyme).

Conversion	Value and Units	Source
Dry cell weight from wet whole-cells	0.3 gdcw gcww ^{-1*}	Bratbak and Dundas (1984)
Cell-free extract from wet whole-cells	8.25 mL _{CFE} gdcw ⁻¹⁺	Harrison et al. (2015)
Cell-free extract density	1 g _{CFE} mL _{CFE} ⁻¹	Assumed
Total protein in dry cell weight	0.5 g _{prot} gdcw ⁻¹	Assumed
Total soluble protein (i.e. usable biocatalyst)	0.5 g _{biocat} g _{prot} ⁻¹	Assumed

The only realistic way to benchmark different biocatalyst yields for different formulations is to base it on an economic cost per standardised unit of activity. Central to this is the cost of producing these different formulations (Table 3.4). Previously, an in-depth economic analysis of a 10 m³ (with 75% working volume) fed-batch fermentation for the production of 50 g_{DCW} L⁻¹ was made by Tufvesson et al. (2011). Capital expenses and operating expenses, including raw materials and labour, were considered for a plant located in Western Europe and production was assumed to take place in 35 annual batches of 48 h. Under these assumptions, the baseline production cost of one kilogram of (wet) cells was found to be 67 €. The corresponding mass of wet whole-cells, cell-free extract and protein in a lyophilised form was calculated based on the conversion factors in Table 3.3. From this, an overall base case fermentation cost of producing the corresponding mass of wet whole-cells could be calculated. Producing dry whole-cells might be of interest for longer term storage, and was assumed to follow directly from the base case fermentation with a freeze-drying step. An estimate cost of freeze-drying processes is 25 kg⁻¹ of dried material (Snowman 1997), which was compounded with the cost of fermentation. Producing a cell-free extract would be achieved by homogenising wet whole-cells following fermentation and separating the unwanted solid fraction (e.g. cell debris) by centrifugation. The cost increase for these extra processing steps per kg of material was estimated to be 1.7-fold (Tufvesson et al. 2011). Lastly, a solid powder (lyophilisate) of this cell-free extract might be desired for long-term storage and use. Therefore, the same cost contribution of freeze-drying was compounded to the cost of producing cell-free extract in order to obtain an estimate for the cost per kg of lyophilised material. A block flow diagram showing the different processing routes for these biocatalyst formulations is given in Chapter 1.

^{*} Based on *Escherichia coli* bacterial cells. Different hosts contain less water content, e.g. *Bacillus subtilis* and *Pseudomonas putida* cells contain 51.5% and 48.4% dry matter, respectively (Bratbak and Dundas 1984). † Based on the homogenisation of *Escherichia coli* cells in a downstream process for human insulin production (Harrison et al. 2015).

It is important to consider that accurate process costs are typically sensitive for industrial producers and not usually published. Therefore, the estimates given in Table 3.4 must be treated only as rough estimates. Interestingly, the extra cost of producing a lyophilised cell-free extract is only approximately 4-fold more than wet-whole cells. Furthermore, the process of lyophilising preparations of whole-cells or cell-free extracts only adds a marginal relative cost difference. It is also important to consider and address that biocatalyst activity may decrease during the process of lyophilisation or drying.

Table 3.4. Cost estimates for different biocatalyst formulations. A basis of 50 g _{DCW} L ⁻¹ from a 10 m ³ fed-
batch fermentation (corresponding to 37.5 kg _{DCW}) was used in order to match a previous analysis made
by (Tufvesson et al. 2011).

	Basis	Cost	Cost per kg	Cost per kg _{cww} basis
Wet whole-cells	119 kg _{cww}	67 € kg _{cww} ⁻¹ × 119 kg _{cww} = 8002 €	67 € kg _{cww} -1‡	67 € kg _{Cww} -1
Dry whole-cells	37.5 kg _{DCW}	8002 € + (37.5 kg _{DCW} × 21 € kg ⁻¹) [§] = 8787 €	8787 € / 37.5 kg _{DCW} = 234 € kg_{DCW} ⁻¹	74 € kg _{Cww} -1
Cell-free extract	309 kg _{CFE}	114 € kg _{CFE} ⁻¹ × 309 kg _{CFE} = 35236 €	1.7 x 67 € kg _{CWW} ⁻¹ = 114 € kg _{CFE} ^{-1**}	295 € kg _{Cww} ⁻¹
Lyophilisate	9.4 kg _{biocat}	35236 € + (9.4 kg _{biocat} × 21 € kg ⁻¹) [§] = 35432 €	35432 € / 9.4 kg _{biocat} = 3779 € kg_{biocat} ⁻¹	297 € kg _{Cww} ⁻¹

The productivities and product concentrations for all value-classes given in Table 3.2 result in a theoretical process rate constant of 3.33×10^{-3} min⁻¹ (which correlates to an average biotransformation batch length of 5 h). In a system with no kinetic limitations, the effective k_{cat} of each biocatalyst formulation should therefore be equal to this process rate constant in order to meet the required product concentration in the given time-frame of 5 h. However, the effective dose of each biocatalyst (biocatalyst yield) must be scaled to accommodate the different product concentrations required by different value-classes as well as the appropriate conversions to extrapolate any formulation from a wet whole-cell fermentation (Table 3.3). Assuming a starting biocatalyst yield for each product value-class for any given formulation specifies the maximum

[‡] Based on the fed-batch fermentation of 50 g_{DCW} L⁻¹ in 10 m³ (Tufvesson et al. 2011).

[§] An estimated step cost of 25 \$ kg⁻¹ of dry material was made for generic freeze drying processes (Snowman 1997).

^{**} The relative cost of crude enzyme (cell-free extract) from a whole-cell preparation was calculated to be 1.7-fold higher on a cost per kg basis (Tufvesson et al. 2011).

allowable biocatalyst dose (= biocatalyst yield × product concentration) as well as the maximum allowable biocatalyst cost per kg (= biocatalyst yield × product value) for each formulation (Table 3.5).

A volumetric basis of 1000 L and average molecular weight of substrate and product of 100 g mol⁻¹ were assumed in order to calculate an overall activity or turnover required for each process to reach full conversion (where 1 unit of activity corresponds to 1 µmol min⁻¹). A specific activity could then be estimated for each formulation based on the biocatalyst concentration, which was dictated by the assumed biocatalyst yield, and necessary k_{cat} to reach full conversion. The required process activity and the specific activity of each formulation infers the exact dose of biocatalyst required for a 1000 L basis to reach full conversion (Table 3.5).

Once the biocatalyst concentration to reach the required turnover is known, the exact cost of this concentration can be calculated using the specific cost of producing each formulation (Table 3.4). Furthermore, the maximum allowable cost can also be calculated based on the assumed biocatalyst yield and product value. This will be the same for all value-class products because the biocatalyst yield is normalised by the cost of product. The ratio of the actual cost to maximum allowable cost allows the feasibility of biocatalyst yield targets to be assessed on a cost per unit of required activity basis.

Product value class	Low-value	Medium- value	High-value
Maximum allowable biocatalyst concent	tration (= Produ	$\left[\frac{g}{L} \cdot \frac{g_{bi}}{2}\right]$	ocat g
Wet whole-cells [g _{CWW} L ⁻¹]	1.69	3.37	3.37
Dry whole-cells [g _{DCW} L ⁻¹]	0.53	1.06	1.06
Cell-free extract [g _{CFE} L ⁻¹]	4.37	8.73	8.73
Lyophilisate [g _{biocat} L ⁻¹]	0.13	0.26	0.26
Maximum allowable specific biocatalyst cost	: (= Biocatalyst	yield × Produ	ct value)
Wet whole-cells [€ kg _{CWW} ⁻¹]	297	297	297
Dry whole-cells [€ kg _{DCW} -1]	945	945	945
Cell-free extract [€ kg _{CFE} -1]	115	115	115
Lyophilisate [€ kg _{biocat} -1]	3779	3779	3779
Required batch activity (turnover) [U] ^{††}	3.33 × 10 ⁶	1.67 × 10 ⁶	3.33 × 10 ⁵
$(-\frac{1}{MW} \times 1000 \text{ L} \times \text{Process rate constant})$	2.22×10^{-3}	2 22 4 4 0-3	2.22×40^{-3}
Required k _{cat} [min ⁻¹]	3.33×10^{-3}	3.33×10^{-3}	3.33×10^{-3}
Minimum required specific activity (turne	over) (= $\frac{\text{Prod}}{\text{MW} \times \text{Bid}}$	$\frac{\text{uct Conc.}}{\text{ocatalyst conc.}} \times \beta$	k _{cat})
Wet whole-cells [U g _{CWW} ⁻¹]	1.98×10^{3}	4.94×10^{2}	9.89×10^{1}
Dry whole-cells [U g _{DCW} ⁻¹]	6.30×10^{3}	1.57 × 10 ³	3.15×10^{2}
Cell-free extract [U g _{CFE} -1]	7.64×10^{2}	1.91×10^{2}	3.82×10^{1}
Lyophilisate [U g _{biocat} -1]	2.52×10^{4}	6.30×10^{3}	1.26 × 10 ³
Biocatalyst dose for 1000 L (= $\frac{\text{Req. batch activity}}{\text{Minimum and activity}}$)			
Wet whole-cells [kg _{cww}]	1.7	3.4	3.4
Dry whole-cells [kg _{DCw}]	0.5	1.1	1.1
Cell-free extract [kg _{CFE}]	4.4	8.7	8.7
Lyophilisate [kg _{biocat}]	0.1	0.3	0.3
Actual biocatalyst co	ost per batch		
(= Biocatalyst dose × Cost p	er kg of produc	ction)	
Wet whole-cells [€]	113	226	226
Dry whole-cells [€]	124	248	248
Cell-free extract [€]	497	994	994
Lyophilisate [€]	500	1000	1000
Maximum allowable biocatalyst cost			
(= Biocatalyst dose × Max. allowab	le specific bioc	atalyst cost)	
Wet whole-cells [€]	500	1000	1000
Dry whole-cells [€]	500	1000	1000
Cell-free extract [€]	500	1000	1000
Lyophilisate [€]	500	1000	1000

Table 3.5. Costs and activity model for different biocatalyst formulations. A volumetric basis of 1000 L and average molecular weight (MW) of substrate and product of 100 g mol⁻¹ were assumed.

⁺⁺ 1 unit of activity (U) = 1 μ mol min⁻¹

For the case of Table 3.5, target biocatalyst yields were the ultimate objective function and were calculated such that a break-even in enzyme cost margins would be reached for the most expensive formulation (lyophilised cell-free extract). Whole-cells are more than 4-fold cheaper to produce than lyophilisates, and thus might help relax a biocatalyst yield target if this formulation is appropriate for the biotransformation. Therefore, Table 3.6 summarises the upper and lower boundaries of target biocatalyst yields required to meet enzyme cost margins for wet whole-cells and lyophilised cell-free extracts, respectively.

Product value class	Low-value	Medium-value	High-value
	(5 € kg⁻¹)	(20 € kg⁻¹)	(100 € kg⁻¹)
Wet whole-cell yield [g g _{CWW} ⁻¹]	15 - 60	3 - 15	0.7 - 3
Dry whole-cell yield [g g _{DCW} ⁻¹]	40 - 190	10 - 50	2 - 10
Cell-free extract yield [g g _{CFE} ⁻¹]	5 - 20	1 - 6	0.3 - 1
Lyophilisate yield [g g _{biocat} ⁻¹]	170 - 760	40 - 190	10 - 40

Table 3.6. Target biocatalyst yield metrics for different formulations. The lower and upper values use the basis of break-even enzyme cost margins for wet whole-cells and lyophilisates, respectively.

The yield targets in Table 3.6 are heavily dependent on the underlying assumptions used in the 'back of the envelope' calculations preceding them. Therefore, they have been rounded off to better reflect their significant figures. Further, they only represent the biocatalyst yield required for one single batch, in which both the target productivity and product titre are reached. Regardless, although slightly lower, the ranges given in Table 3.6 reflect those previously estimated by Tufvesson et al. (2011). The biocatalyst yield target increases with increasing formulation purity (in terms of water reduction) because less biocatalyst is required to achieve the same reaction turnover, but also because these formulations are more expensive to produce. While some of the yields may appear quite high for many enzyme cases, there are exceptional examples of industrial biocatalytic processes featuring biocatalyst yields of up to 2000 g g_{immob}⁻¹ (Katchalski-Katzir and Kraemer 2000), 5000 g g_{immob}⁻¹ (Jensen and Rugh 1987) and >7000 g $g_{DCW^{-1}}$ (Kobayashi et al. 1992) for the continuous production of 6-aminopenicillanic acid, high fructose corn syrup and acrylamide, respectively. A key to realising such yields could lie in the ability to reuse enzymes (further discussed in Section 3.4.3), the use of continuous processing or in the trade-off between biocatalyst concentration and space-time yield, which is a major discussion topic of Chapter 4.

3.4.2. Influence of poor substrate affinity and enzyme stability

An enzyme limited by poor substrate affinity or stability will impact the required biocatalyst yield because more enzyme will be required to achieve the same baseline reaction activity. In order to assess this, the same cost-basis calculation methodology in Section 3.4.1 was followed. However, the following Michaelis-Menten expression was used to simulate conversion progress over time whilst incorporating expressions for both enzyme stability and substrate affinity:

$$\frac{dp}{dt} = aE_0e^{-k_d\cdot t}\frac{\left(S_0 - p(t)\right)}{K_m + \left(S_0 - p(t)\right)}$$

Where: p is the product concentration as a function of time, a is the specific activity of a biocatalyst (linear function of k_{cat}), E_0 and S_0 are the respective initial enzyme and substrate concentrations, k_d is the first-order inactivation rate constant, and K_M is the Michaelis-Menten constant (measure of substrate affinity).

In this way, kinetic parameters could be varied and their influence on specific activity observed. If the enzyme were to be limited by one of these parameters, the magnitude of required improvement to the specific activity (i.e. k_{cat}) of the enzyme could be estimated. This would thereafter feed into the cost analysis as an increased demand on enzyme loading (and therefore biocatalyst yield target) to reach the same required process turnover, ultimately affecting the cost margins. This could then be benchmarked with the base case scenario in Section 3.4.1, where no limitations were present.

If an enzyme were to have poor affinity for its substrate, the reaction velocity would drop prematurely while substrate is consumed during the reaction. The example case studied here assumes a K_M of 25 mM, which corresponds to a substrate concentration of 2.5 g L⁻¹ for a substrate of 100 g mol⁻¹ molecular weight. Interestingly for cases such as these, only the cost of enzyme in high-value product classes suffers. This is because the starting substrate concentration of 10 g L⁻¹ for this value class is already close to the K_M limit, whereas the other value class product concentrations are orders of magnitude larger (Table 3.2). This might be especially crucial when extremely high-value products are considered as they may only be present in minute concentrations in processes. Compared to a system with no limit for substrate affinity ($K_M << S_0$), high-value class products will require a 2.3-fold higher biocatalyst yield to compensate for the inefficient catalyst caused by K_m limitations. Whilst medium-value and low-value products only require 1.3- and 1.1-fold higher biocatalyst yields. Retrospectively, protein engineering substrate affinity would have the greatest impact on higher value products, where the biocatalyst could effectively be made more than 2-fold more efficient. These results are shown graphically in Figure 3.2.



Figure 3.2. Effect of substrate affinity on biocatalyst yield for different valueclass products.

On the other hand, protein instability will affect enzymes for all value class products equally because (in this model) it is only a function of reaction time. Naturally, if the enzyme is denatured by other factors, e.g. high substrate/product concentrations, then this result would not translate. For a case where the enzyme exhibits poor stability ($t\frac{1}{2} = 10$ h), the biocatalyst yield target would need to be increased 1.2-fold in order to counter the extra cost of activity required to push the reaction to completion. Likewise, an enzyme exhibiting more severe instability ($t\frac{1}{2} = 2.5$ h) will need 1.8-fold higher biocatalyst yield targets (Figure 3.3). This again highlights the magnitude of cost reduction that might be achieved through protein engineering enzyme stability. It ought to be noted that, while in this single-use batch reaction model enzymes with a half-life of more than 10 h will have little beneficial effect on reducing biocatalyst costs, this is indeed a prerequisite for biocatalysts that are to be recycled for subsequent uses. In such cases, a half-life as long as possible (e.g. days) can be instrumental to improving biocatalyst yields in processes. It also ought to be mentioned that biocatalytic processes rarely suffer from stability limitations if only single batches are required.



Figure 3.3. Effect of enzyme stability on biocatalyst yield.

Protein engineering the increased activity of an enzyme will always benefit a process where the cost of enzyme is significant because theoretically less biocatalyst will then be required to perform the same turnover at the same space-time yield (i.e. increasing biocatalyst yield). Improving the expression of target protein in the final biocatalyst formulation will also have a dramatic effect on the specific activity, and this could be overlooked in enzyme development. Therefore, if the enzyme performs adequately in terms of stability and productivity, and further improvement can still be made towards its intrinsic activity, then this should remain a generic target for protein engineering.

3.4.3. Cost-benefit of immobilisation

A means of increasing the yield of a biocatalyst can also be to potentially recycle that same biocatalyst for further reaction batches. Every batch completed with the same biocatalyst effectively doubles its yield (Figure 3.4). The most common route to realising biocatalyst recycling is to immobilise a cell-free extract into a solid particle (Hanefeld et al. 2009). In essence, this concentrates the activity of a given biocatalyst to a small fraction of the reaction volume, simplifying the separation of catalyst from the medium. There are a multitude of available methods to achieve this such as: binding an enzyme to a carrier structure (using either physical, ionic or covalent interactions), encapsulating the enzyme in a microstructure with a polymer matrix, or cross-linking enzyme aggregates/crystals (Sheldon and van Pelt 2013). However, immobilisation comes at a cost, not only in terms of economy, but also activity.



Figure 3.4. Effect of number of recycles on biocatalyst yield assuming a single batch yield of 3 g g_{biocat}^{-1} and no activity loss upon recycle.

First, based solely on the cost of producing immobilised biocatalysts, Tufvesson et al. (2011) estimated that immobilised biocatalysts cost 3.6-fold more than cell-free extracts on a price per kg basis. This includes the cost of support material as well as the processing capital expenses and operating expenses.

Secondly, there is an overall penalty on the intrinsic activity of immobilised biocatalysts when compared to their 'free' homogeneous counterparts. Immobilisation supports can only contain a certain loading of protein to begin with. A typical loading could be approximately 30 g_{biocat} kg_{immob}⁻¹ where usually 1:1 volumes of cell-free extract are immobilised (Guisan 2006). An immobilised biocatalyst can thus potentially hold approximately the same specific activity of a cell-free extract, however often some activity is lost during the immobilisation process itself. Furthermore, once immobilised, enzymes can lose conformational flexibility and therefore might have reduced activity. Any losses in activity during reaction due to environmental conditions (e.g. breakage caused by shear stresses), upon recycle, or during storage must also be taken into account.

The most crucial drawback to immobilising enzymes however is the introduction of potential mass transfer limitations, as is the same for most heterogeneous catalysts (Moo-Young and Kobayashi 1972). Most of the immobilised activity is concentrated within the catalyst particle and access to substrate can only proceed via the slow process of substrate diffusion. The diffusion rate itself is a function of the support structure (e.g. particle size, pore channel diameter, porosity, etc.). This is also a drawback when an enzyme experiences product inhibition because the product will take longer to diffuse from the enzyme's immediate environment within the catalyst particle. The reduced reaction rate due to mass transfer limitations can be quantitatively measured through a mathematical expression called the "effectiveness factor" (η) (Liese and Hilterhaus 2013). The effectiveness factor is the ratio of the hypothetical maximum catalytic rate of a "free" enzyme (measured at the surface of the immobilised particle) and the rate of diffusion within the particle. A model output of this effectiveness factor as a function of immobilised particle diameter and enzyme loading on the support, based on real experimental kinetic parameters, is shown in Figure 3.5. A reaction rate reduction of 20-80% ($\eta = 0.2-0.8$) is therefore common, especially in larger immobilised particles or with higher enzyme loadings on the carrier material.



Figure 3.5. Effectiveness factor as a function of spherical particle radius for immobilised biocatalysts containing varying enzyme loadings (shown next to the curves as mg mL_{carrier}⁻¹). The specific activity of the enzyme was 100 U mg_{biocat}⁻¹ (left) and 1000 U mg_{biocat}⁻¹ (right); the substrate concentration at the surface was assumed to be 10-fold that of the immobilised enzyme K_M ; diffusivity of the substrate within the porous structure = 4×10^{-4} cm² min⁻¹ (parameters and diffusion models for immobilised enzyme catalysis obtained from Regan et al. (1974)).

As enzymes are made more active, for example through protein engineering or improved expression, the efficiency factor is made worse. This is because diffusional limitations (which are independent of enzyme activity) far outweigh the speed of reaction at the surface of a particle. Figure 3.5 illustrates this effect where efficiency factor profiles within the same particle for an enzyme of 100 U mg_{blocat}-1 are compared to an enzyme engineered to have a specific activity an order of magnitude higher, which is not uncommon. Therefore, improving the activity of enzymes through protein engineering (as is common practice) has a detrimental impact on implementing immobilised biocatalysts in processes. Table 3.7 shows the particle size reduction required to maintain an efficiency factor of 0.8 between these two cases. At higher enzyme loadings, it is possible that the size of particulates would therefore need to be so small that their separation from a medium might be challenging, effectively defeating the objective of immobilising enzymes in the first place. Furthermore, such small particle sizes may not even be feasible to prepare.

Enzyme Loading	100 U mg ⁻¹	1000 U mg ⁻¹	Volume of Carrier for 5000 U L ⁻¹ $[mL_{carrier} L^{-1}]$
on Carrier	specific activity	specific activity	
200 mg mL _{carrier} ⁻¹	1.2 μm	0.4 μm	47
50 mg mL _{carrier} ⁻¹	2.5 μm	0.8 μm	89
10 mg mL _{carrier} ⁻¹	5.3 μm	1.7 μm	211
5 mg mL _{carrier} ⁻¹	7.6 μm	2.4 μm	294
1 mg mL _{carrier} ⁻¹	16.9 μm	5.3 μm	660

Table 3.7. Immobilised particle radii required to maintain $\eta = 0.8$ for different loadings of enzymes for a specific activity of 100 U mg⁻¹ and 1000 U mg⁻¹. Porosity and minimum carrier diameter assumed to be 0.3 and 250 µm, respectively.

All these limitations towards activity will impose a need for increased dose of immobilised biocatalyst compared to other biocatalyst formulations. However, there is an upper limit to this concentration of immobilised particles in a reactor based on the physical space they occupy within the reaction volume as well as the increased cost of using such amounts of immobilised preparations.

Despite decades of research into enzyme immobilisation, there remains no clear generic method that is best suited for industrial reactions. This might be due to the complexity of some immobilisation mechanisms, cost, or even a general lack of vision for their suitability in an industrial biotransformation. Most studies lack comparative data between free enzyme forms and immobilised forms during biotransformations to see their relative performance. Process metrics, as described in this chapter, are rarely stated in these studies. Furthermore, the reaction cycles boasted in these studies are not necessarily with industrially relevant conditions (e.g. product concentrations) or reaction timeframes. That being said, there are a number of commercial biocatalytic processes relying on immobilised enzymes to allow continuous flow reactions (Rao et al. 2009). Therefore, with the right focus, enzyme immobilisation might yet find even wider application.

3.5. Conclusions

In conclusion, process performance metric targets are essential to help guide biocatalytic process development. Understanding the end-use market of the product of interest will either relax or impose more stringent process performance requirements based on the economic value of the product. The value of product coupled with the cost of producing different enzyme formulations and their respective specific activity infers the target biocatalyst yield to realise a feasible process.

Using the metric targets as early as possible during reaction characterisation and evaluation stages allows a better understanding of process constraints and the reaction

environment. It also allows the benchmarking of reaction performance to check whether process improvement strategies will be necessary, and what these might entail. The feasibility of implementing these different improvement strategies can then be assessed at the earliest possible stage of process development. Thereby, leading to either continuing process development in a timely and resource efficient manner or redefining the reaction targets to better suit the achievable process performance.

Chapter 4. Case Study I – Scoping the enantioselective desymmetrisation of a poorly water-soluble diester by recombinant pig liver esterase and improving the process to handle higher substrate loads

In this case study, the methodology relying on reaction trajectory analysis presented in Chapter 2 was used to identify mass transfer limitations for a given system. This reaction was the enantioselective conversion of a poorly water-soluble diester (solubility < 60 mM in water) to a monoester. The substrate formed a second liquid phase. Previously, this reaction had been wellcharacterised in terms of pH and temperature optima and some aspects of process performance. However, the precise influence of substrate mass transfer and poorly water-soluble nature of the substrate were not considered. Therefore, this work had this as a special focus. With the constraints of mass transfer on space-time yield considered, it was possible to evaluate and improve biocatalyst yield through increasing substrate concentrations. Eventually the complete conversion of approximately 75 g L⁻¹ substrate was achieved in 3.65 h yielding an adequate productivity of 20 g $L^{-1} h^{-1}$ with a biocatalyst yield of 4.36 g g_{biocat} -1. This work highlights the importance of scale-up during reaction characterisations, simplicity of applying the methodology from Chapter 2, and future directions for reaction improvement to address product inhibition and substrate supply. An important outcome from this chapter is to use benchmarking (with target process metrics as detailed in Chapter 3) to help guide future development by balancing overperforming metrics (such as space-time yield in this case), ultimately satisfying all process performance requirements for industrial application.

The following is intended for publication.

4.1. Introduction

Biocatalysis has become an established branch of chemistry in its own right owing to the excellent stereo-, regio- and enantioselectivity of biocatalysts, importantly under mild reaction conditions (Straathof et al. 2002; Sheldon and Pereira 2017). Consequently, biocatalysts can realise more sustainable manufacturing processes, satisfying 10 of the 12 principles of green chemistry: waste prevention and high atom efficiency (avoiding the formation of by-products) through their high selectivity, less hazardous syntheses, suited towards safer solvents (most often carried out in water), low energy demand due to their operation under mild process conditions, origin from renewable sugar feedstocks through fermentation, avoidance of derivatization, their nature as catalysts and not as stoichiometric reagents, the ability to apply real-time process analytics to prevent pollution, and most importantly, inherently safer processes (Sheldon and Woodley 2018). These traits also infer improved process economics. Additionally, biocatalysts have a unique feature in that they may be improved by protein engineering, offering an additional degree of freedom towards process implementation (Woodley 2013). Such improvements are gained through tailoring the amino acid sequences of enzymes to enhance stability, activity or selectivity (Strohmeier et al. 2011). Indeed, numerous biocatalytic processes have been successfully commercialised to date (Schmid et al. 2001; Sutton et al. 2012; Choi et al. 2015; Hughes and Lewis 2018), however standardised methods of process development are lacking (Tufvesson et al. 2013). One means of gauging reaction performance during development is to use process metrics inspired by techno-economic requirements for a given product value-class (Lima-Ramos et al. 2014). A systematic method for scoping reactions and estimating such process metrics was detailed in Chapter 2. In this work, this procedure was applied to a reaction featuring a poorly water-soluble substrate. The system of interest is the enantioselective desymmetrisation of dimethyl cyclohex-4-ene-cis-1,2-dicarboxylate (1) to (1S,2R)-1-(methoxycarbonyl)cyclohex-4ene-2-carboxylic acid (2a) catalysed by recombinant pig liver esterase (ECS-PLE06; Scheme 4.1) (Wohlgemuth 2011; Süss et al. 2014). The diester substrate is poorly water-soluble (approximately 60 mM in water at 25 °C for structurally similar dimethyl 1,4cyclohexanedicarboxylate) and forms a second liquid phase. The monoester product of this reaction is of value as a pharmaceutical intermediate towards the synthesis of biologically active molecules (Scheme 4.1) (Boland et al. 1985), as well as the antibiotic plantencin (Yun et al. 2008), active pharmaceutical ingredients in treatments towards HIV (Andreini et al. 2011) and cytomegalovirus (Herpes) (Compton et al. 2006).



Scheme 4.1. Desymmetrisation of diester **1** to (1S,2R)-monoester **2a** catalysed by recombinant pig liver esterase (ECS-PLE06) (adapted from Süss et al. (2014)).

Previously, this reaction and enzyme strains were characterised in terms of pH and temperature optima as well as reaction performance (Süss et al. 2014; Süss et al. 2015; Hinze et al. 2016). A simple method of pH control was implemented that made use of inexpensive sodium bicarbonate breaking down in the presence of the carboxylic acid product **2a**, thereafter forming benign CO₂ and passing from the reaction medium. This was found to be more effective than conventional titration of base to control the pH. Eventually this promising reaction showed adequate performance to convert 200 mM substrate with an enantiomeric excess of > 99.5% (Süss et al. 2014). However, this eventual reaction performance arose from a series of improvised experiments and the bottleneck towards large-scale production has yet to be determined. Furthermore, the precise influence and nature of poorly water-soluble substrate was not directly addressed. Therefore, the aim of this work was to perform a bottleneck identification relying on time-course progression analyses to determine the major process limitation, according to the procedure detailed in Chapter 2. Using time-course measurements in this way it is possible to distinguish the dominating bottleneck for prospective performance of ECS-PLE06 under given conditions: enzyme kinetics (e.g. poor substrate affinity), enzyme stability or, more likely, substrate mass transfer. With this limitation in mind, the reaction was able to be further scaledup and its suitability for process implementation was assessed.

4.2. Materials and Methods

Reagents

All reagents were of analytical grade and supplied by Sigma-Aldrich (St. Louis, MO). Dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate (**1**) and lyophilized recombinant pig liver esterase (ECS-PLE06; EC 3.1.1.1) were kindly supplied by Enzymicals AG (Greifswald, Germany).

Activity assay

The activity of pig liver esterase was quantified using the same assay detailed by Süss et al. (2014) through spectrophotometric measurements of the degradation of *p*-nitrophenyl acetate (*p*NPA) to *p*-nitrophenolate. Reaction mixtures contained 850 µL of phosphate buffer (pH 7.5, 50 mM), 50 µL of enzyme stock solution and 100 µL of *p*NPA in DMSO (10 mM). Initial rate measurements were taken at 400 nm ($\varepsilon_{30 \, ^{\circ}C} = 17759 \, \text{L} \, \text{mol}^{-1} \, \text{cm}^{-1}$) over 100 s at 30 °C. One unit (U) of esterase activity was defined as the generation of 1 µmol of *p*-nitrophenolate per minute under the standard activity assay conditions. Measurements were made using a UV-vis spectrophotometer (Specord 50, Analytik Jena AG, Jena, Germany). Reference samples contained dH₂O and negative controls were performed with the absence of enzyme.

Small-scale apparatus

Small-scale reactions were performed in a 100 mL round bottom flask using a magnetic stirrer for mixing and an external water bath for temperature control at 40 °C. 40 mL of saturated NaHCO₃ buffer (pH 8.15) was used to control the pH. ECS-PLE06 (\geq 0.3 U mg⁻¹) was dosed to this medium, and the reaction was started by the addition of 0.6 g of **1** (80 mM). Samples (500 µL) were taken periodically and concentrations of substrate and product were determined using the procedure detailed in the analytical methods section. Reaction progress could be indirectly followed by use of a CO₂ bubble counter fixed to the top of the apparatus.

Fed-batch 250 mL stirred tank reactor

Fed-batch experiments were performed in a 300 mL glass jacketed stirred tank reactor (STR). 42 g of NaHCO₃ (corresponding to a 2 M final concentration) was added with 1500 U of ECS-PLE06 (6 U mL⁻¹ final concentration) to 190 mL dH₂O. Temperature was maintained at 40 °C by use of an external thermostat and mixing ensured by a pitched 4-blade turbine with overhead stirring motor at 300 rpm. pH was measured by an InLab Semi-Micro electrolyte sensor (Mettler Toledo, Colombus, OH) and was found to be 8.15 ± 0.38 (mean \pm SD) over all experiments using the sodium hydrogen carbonate buffered system. 60 mL of substrate **1** (corresponding to a final concentration of 1.2 M) was fed by a R99-E syringe pump (Razel Scientific Instruments, Fairfax, VT) over the first 10 hours to make up a final reaction volume of 250 mL. Triplicate samples $(500 \ \mu L)$ were taken periodically over 24 h and concentrations of substrate and product were determined using the procedure detailed in the analytical methods section.

Analytical methods

Concentrations of **1** and **2a** were quantified by GC-FID (TRACE 1310 Series, Thermo Fisher Scientific, Waltham, MA). Triplicate samples of 500 µL were taken from reaction media and acidified with 500 µL of concentrated HCl (1:1 volumes) to render **2a** insoluble in the aqueous environment and to denature residual protein activity. Samples were then diluted and extracted with methyl tert-butyl ether (MTBE) containing n-decane (25 mM) as an internal standard through vortex on the highest setting and phase separation by centrifugation. 1 µL of the organic phase fraction was injected into a TR-1701 column (30 m x 0.25 mm x 0.25 µm; Thermo Fisher Scientific, Waltham, MA) where the oven temperature was ramped from 130 °C to 200 °C at a rate of 5 °C min⁻¹ and thereafter held at an isotherm of 200 °C for 1 min. Injector and detector temperatures were maintained at 250 °C throughout. The carrier gas flow rate was 1.2 mL min⁻¹ with a split ratio of 35:1. Experimental substrate and product concentrations were determined using a standard curve generated by subjecting samples of known concentrations to the same acidification/solvent extraction procedure.

4.3. Results and Discussion

Understanding the influence of poorly water-soluble substrate on this system first required a series of time-course experiments with varied enzyme concentrations to identify potential mass transfer limitations. With this limitation on reaction rate considered, process performance metrics (as defined in Chapter 3) were assessed. Procedures (e.g. scale-up and fed-batch operation) were then followed with the goal of maximising product concentration. The best reaction performance was then benchmarked with target process performance metrics based on the high-value of the product and improvement strategies discussed.

The first step in this process was elucidating the major reaction limitation by varying enzyme loadings at a fixed, moderate substrate dose. Figure 4.1a shows these time-course progressions of reaction with incremental enzyme doses at a substrate concentration of 80 mM. Experiments were performed in a stirred round bottom flask (40 mL). Normalising the time axis by the varied parameter (enzyme concentration) revealed that the reaction becomes substrate mass transfer limited at enzyme doses greater than 6 U mL⁻¹ because the reaction trajectories diverge (Figure 4.1b). Interestingly, the product was found to have a beneficial effect by better dispersing the substrate in the reaction medium since it acts as an emulsifier. Methanol was also formed as a by-product, which likely also helps improve substrate solubility in the medium. The slight improvement in reaction rate due to these factors can be observed after initial product formation, especially at the lowest enzyme doses.



Figure 4.1. (a) Conversion as a function of time for the ECS-PLE06 catalysed reaction of 80 mM diester **1** to monoester **2a** using 40 mL saturated NaHCO₃ as a buffer (pH 8.15) at 40 °C (left). (b) Data replotted with the x-axis normalised by enzyme concentration to reveal the major process limitation (right). ECS-PLE06 concentrations, $[E_0]$, were varied as follows: • - 1.54 U mL⁻¹, \blacksquare - 2.99 U mL⁻¹, + - 4.50 U mL⁻¹, * - 5.94 U mL⁻¹, • - 8.72 U mL⁻¹, \blacktriangle - 11.37 U mL⁻¹.

According to Figure 4.2, the upper limit of productivity due to substrate mass transfer limitations was 21 g L⁻¹ h⁻¹. Clearly, the expected linearly proportional response of space-time yield to enzyme concentration breaks down after this point, indicating a non-enzyme related limitation. On the other hand, biocatalyst yield appears to improve at lower enzyme concentrations because the same amount of substrate is still converted, although the reactions take longer. Another way of improving the biocatalyst yield metric is to increase the substrate loading thereby converting more substrate with the same amount of enzyme. In this manner, a better reflection of biocatalyst yield is obtained because, in reality, industrial processes should operate with the highest possible space-time yield in order to afford cost-effective processes (shorter batches and smaller reaction volumes). However, the biocatalyst yield should still be evaluated under conditions where the reaction is not kinetically limited. This is especially important to consider in cases such as this, which are limited by liquid-liquid mass transfer.



Figure 4.2. Space-time yield (STY; •) and biocatalyst yield (\blacktriangle) as a function of enzyme activity dose with a fixed substrate concentration of 80 mM in all experiments. The dashed line indicates linearity through the origin.

Therefore, the substrate concentration was increased 5-fold (400 mM) and an enzyme dose of 6 U mL^{-1} was maintained because mass transfer limitations occurred at higher enzyme concentrations. Full conversion and no kinetic limitations were observed at this higher substrate concentration, which was confirmed with the overlap of trajectories on a normalised time axis (Figure 4.3a). The resultant improvement in biocatalyst yield from 0.89 to 4.36 g g_{biocat}⁻¹ at the same maximum space-time yield is shown in Figure 4.3b.



Figure 4.3. (a) Reaction with 80 mM substrate (\bullet) and 400 mM substrate (\blacktriangle) in 40 mL saturated NaHCO₃ buffer (pH 8.15) at 40 °C and with an enzyme dose of 6 U mL⁻¹ on a normalised plot (left). (b) Space-time yield (STY; blue \bullet) and biocatalyst yield (red \blacktriangle) as a function of enzyme activity dose with 80 mM substrate; data in yellow are those obtained with 400 mM substrate (right).

In order to investigate the potential of further increasing substrate concentration, it was necessary to also increase the scale of reaction to a stirred tank reactor (250 mL). At this scale, better control and measurement of the reaction environment was made possible through defined stirring, temperature and pH control. Substrate concentrations greater than 500 mM caused the enzyme to agglomerate and denature. Therefore, three 400 mM batch doses of **1** were supplied at 3 h intervals in a fed-batch manner to mimic the previous reaction profiles observed at smaller scale. However, full conversion was not reached even after 24 h (data not shown). In order to rule out potential substrate inhibition caused by too large single substrate doses, a constant feed rate of substrate at 5.81 g min⁻¹ was instead supplied to the reactor by use of a syringe pump. This would hypothetically result in a calculated 1.19 M product and a STY of 22.0 g L⁻¹ h⁻¹ in 9 h of reaction time if the reaction was not limited, as would be expected based on previous results. During fed-batch reaction, product formation ceased after 7 h after which substrate accumulated at a rate equal to that of the pump feed rate (Figure 4.4a). However, the mass balance of substrate to product was closed throughout indicating that indeed the reaction had stopped after 400 mM

inhibition or product induced deactivation could be the cause. In order to confirm this, the endpoint reaction medium was dosed with a fresh 6 U mL⁻¹ of ECS-PLE06 and left to incubate at 40 °C for 48 h. Conversion was only marginally improved by approximately 10% (data not shown) indicating that product inhibition or product induced deactivation was the most likely cause of activity loss.



Figure 4.4. (a) Fed-batch conversion of 1.2 M diester 1 (•) to monoester 2a (\blacktriangle) in a 250 mL (end volume) stirred tank reactor. pH was maintained with saturated NaHCO₃ buffer (pH 8.15) at 40 °C and included an enzyme dose of 6 U mL⁻¹ (above). (b) Mass balance corresponding to the sum of substrate and product in the reaction medium (•) and the actual fed-batch rate of substrate supply to the reactor by a syringe pump indicated with a dashed line (below). Error bars reflect 95Cl (n = 3).

Indeed, this was was confirmed after the stability of ECS-PLE06 was evaluated with respect to temperature (40 °C), high buffer strength solutions (saturated NaHCO₃), and by-product methanol concentrations of up to 5% (v/v) (corresponding to a high molar concentration of ~1.3 M; Figure 4.5). ECS-PLE06 showed a negligible activity loss after 24 h in the presence of all environmental factors. Furthermore, the enzyme gained activity whilst being incubated in NaHCO₃ buffer and methanol, which might point towards an affinity of ECS-PLE06 for strongly buffered environments containing methanol.



Figure 4.5. Stability of ECS-PLE06 as a function of time at room temperature in 50 mM phosphate buffer at pH 7.5 (control, solid bars), at 40 °C in 50 mM phosphate buffer (pH 7.5; diagonal striped bars), at 40 °C in saturated NaHCO₃ buffer (1.14 M, pH 8.15; opaque bars), and with 5% (v/v) methanol (~1.3 M) at 40 °C in saturated NaHCO₃ buffer (horizontal striped bars). Error bars reflect 95Cl (n = 4).

In an attempt to address product inhibition/deactivation a simple *in situ* product removal (ISPR) technique using reactive crystallisation was attempted. Here, the charged product anion in solution would potentially precipitate from solution when bound to a metal cation. An ideal 'self-healing' system could utilise a metal bicarbonate salt to both buffer the pH during reaction as well as simultaneously precipitate product and simplify downstream isolation steps without negatively affecting enzyme performance. Unfortunately all trials with the prospective metals Ca²⁺, La³⁺, Mn²⁺ and Ce³⁺ failed to crystallise the product from solution in the presence of Tris-HCl buffer under the reaction pH of 7-8. For this specific case, two-liquid phase approaches would not be feasible because the substrate, being hydrophobic, would partition more strongly than product into an organic phase. Instead, further focus could be made towards more expensive ISPR methods such as selective anion exchange resins (Bechtold et al. 2006) or charged membranes (Strathmann 2010). Another possibility could be to remove the product in an external loop through pH swing (precipitation) or cooling crystallisation, although this would require a means to retain the biocatalyst from the harsh conditions in the external loop (Buque-Taboada et al. 2004; Buque-Taboada et al. 2006).

Nevertheless, in this work improved performance was achieved with final process metrics summarised in Table 4.1. The product of interest in this work is an intermediate towards an active pharmaceutical ingredient (API)/biologically active molecule. Based on the market application, this product can therefore be classed as high-value and the corresponding process metric targets from Chapter 3 could be used as benchmarks. The reaction performance far exceeded almost all the target metrics with the exception of biocatalyst yield (and specific productivity). However, a reaction must satisfy all required metrics in order to be viable for industrial applications. The simplest route of improving biocatalyst yield is to reduce biocatalyst loading at the expense of productivity, which was satisfactory by an order of magnitude. The enzyme was found to be robust and retained activity for well over 24 h so could be suited to extended batch lengths. Another more complex method to improve biocatalyst yield without compromising other metrics would be to address product inhibition allowing a higher product turnover for this particular reaction. Different techniques of in situ product removal (ISPR) were studied and discussed previously. The biocatalyst yield could also be improved by further optimising the expression of ECS-PLE06 in the lyophilised preparation, which would increase the specific activity of the biocatalyst affording smaller doses to achieve the same productivity. Allin-all based on the process metrics, the reaction shows great promise for full-scale industrial implementation despite the mass transfer of poorly water-soluble substrate restricting the spacetime yield.

Process Metric	High-value Class Targets (100 € kg⁻¹)	Actual Values
Reaction conversion [%]	(100)	100
Product concentration [g L ⁻¹]	10	74
Space-time yield [g L ⁻¹ h ⁻¹]	2	20.2
Specific productivity [g g _{biocat} ⁻¹ h ⁻¹]	1.7 - 7.6	1.20
Biocatalyst yield [g g _{biocat} ⁻¹]	10 - 40	4.36

Table 4.1. Benchmarking best process performance metrics for the ECS-PLE06catalysed desymmetrisation of **1** to **2a**.
4.4. Conclusions and future perspectives

This work points towards a promising biocatalytic reaction based on this performance of ECS-PLE06. Scale-up of up to 400 mM batches of product can proceed with an expected biocatalyst yield and productivity of 4.36 g g_{biocat}⁻¹ and 20.2 g L⁻¹ h⁻¹, respectively. Beyond this relatively high concentration limit (\sim 75 g L⁻¹), product inhibition will prevent further conversion of substrate. Biocatalyst yield can be improved to fall into the required window of $10-40 \text{ g}_{\text{biocat}^{-1}}$ (for highvalue products) by simply dosing with less enzyme, although the batch length will be proportionally extended. Alternatively, biocatalyst yield could be improved through increasing the specific activity of the lyophilised cell-free extract by further optimising the expression of ECS-PLE06. If higher product concentrations are required then alternative ISPR techniques must be considered. Furthermore, substrate must be dosed in a fed-batch manner to avoid enzyme agglomeration and activity loss. Through the application of a standardised methodology relying on time-course progressions it was concluded that the reaction was limited by mass transfer. This was due to the low solubility of substrate in the water-based medium. ECS-PLE06 is a robust enzyme that is highly thermostable and tolerant towards solvents (Hinze et al. 2016), therefore mass transfer limits could still potentially be reduced by increasing temperature or adding watermiscible co-solvents to this system to help improve substrate solubility in the aqueous reaction medium. This specific system also highlights the elegance of 'self-healing' systems where pH can easily be maintained by the reaction medium itself through an oversaturated benign carbonate buffer reacting with product cation species.

Chapter 5. Case Study II – Part A: Significance of analytical methods when characterising heterogeneous reactions

This chapter forms the first of two detailing the assessment of an enzyme-catalysed system featuring a (gas)-solid-liquid Baeyer-Villiger oxidation. The inclusion of a solid-phase through poorly water-soluble macrocyclic ketones and lactones presented the highest levels of complexity in terms of experimental evaluation. The analytical challenges brought about by the presence of solid substrate with regards to sampling a heterogeneous medium and performing activity assays are described in this chapter, and in two parts.

First, significant sample-to-sample variation of the solid-liquid reaction medium prevented more conventional direct liquid-phase sampling. Therefore, an online method of monitoring reaction progress using oxygen mass balance in the gas-phase of the reactor outlet was developed. The method was successfully validated and demonstrated by using two model reactions: firstly, the oxidation of glucose by glucose oxidase and, secondly, the Baeyer-Villiger oxidation of macrocyclic ketones to lactones. Initial reaction rate constants and time-course progressions calculated from the oxygen mass balance were validated against conventional online methods of dissolved oxygen tension and pH titration measurements. A feasible operating window and the sensitivity to dynamic changes of reaction rates was established by controlling oxygen transfer through the operating parameters of the reactor. Such kinetic data forms the basis for reaction characterisation, from which bottlenecks may be made evident and directed improvement strategies can be identified and implemented.

Secondly, the presence of a solid-phase hindered conventional spectrophotometric activity assays. Consequently, an alternative activity assay was developed that made use of direct sacrificial sampling.

What follows in Chapter 6 is a more detailed description of the Baeyer-Villiger oxidation reaction and the application of each analytical method.

5.1. Part I: Gas-phase measurements of oxygen mass balance to follow reaction kinetics

This chapter has formed the basis of the following published peer-reviewed journal article: <u>Murray P. Meissner</u>, Mathias Norblad, John M. Woodley. 2018. Online Measurement of Oxygen-Dependent Enzyme Reaction Kinetics. *ChemBioChem*, 19:106-113.

5.1.1. Introduction

Conventional wisdom holds that the characterisation of enzyme-catalysed reactions is best performed by time-course experiments as close to industrially relevant conditions as possible. However, this is rarely the case. Perhaps this is because process performance metrics have been poorly defined and applied during reaction evaluations (Chapter 3). Chapter 2 describes how the resulting reaction progress curves, under a range of defined conditions, may reveal performance limitations (or bottlenecks), such as enzyme stability, substrate/product inhibition or mass transfer (Blackmond 2005; Blackmond 2015). Central to this characterisation is the necessity for accurate analytics.

Broadening the application of biocatalysis to a wider range of industrially useful substrates will require focus on how to deal with organic solvents and poorly water-soluble substrates (Schmid et al. 2001). Indeed, industrially relevant substrate/product loadings in excess of 50 g L⁻¹ will most often exceed solubility limits, or induce inhibition, frequently necessitating the need for additional water-immiscible solvent phases (Lu et al. 2004; Süss et al. 2014; Reimer et al. 2017; Sheldon 2017; Uthoff et al. 2017). This situation is increasingly common and brings the significant challenge of sampling heterogeneous mixtures, in such a way as to give representative and useful measurements (Domínguez de María and Hollmann 2015).

Oxidation reactions form an important class of reactions in organic chemistry (Faber 2011; Turner 2011). The continuous supply (and conversion) of oxygen during these reactions provides an alternative method for measuring reaction rate that is not dependent on sampling the reaction medium. Based on this premise, it was decided to use this method for kinetic characterisation. Hence, the objective of the work in this chapter is to document a relatively simple methodology to characterise oxygen-dependent reactions with poorly water-soluble components and/or two liquid-phases by eliminating the need for direct sampling of a multiphasic reaction medium. The method is based on using the oxygen mass balance measured by online gas-phase analysis. The primary advantage of this method is that it is insensitive to sampling heterogeneity (due to non-ideal mixing of multi-phasic mixtures) because it measures solely in the gas-phase. Additionally, the method is generic for any oxygen-dependent reaction; this means it can cope with different substrates, and the development of a new analysis method

for each new compound can be avoided. Furthermore, as an online method, it may also be used for process control because it yields real-time kinetic predictions. Inspired by similar applications of this approach in the fields of wastewater treatment (Kappeler and Gujer 1992), solid-state fermentation (Gumbira-Sa'id et al. 1993; Mitchell et al. 2000) and in more conventional fermentations by means of measuring respiratory quotients (Villadsen 2015), the application of this method to enzymatic reaction kinetic measurements is novel. Of particular interest is that the oxygen consumption rates (equating to productivities) of enzyme-catalysed reactions in an industrial setting are up to two orders of magnitude higher than those in the previous examples, which increases accuracy.

To illustrate and validate the methodology, first the well-known, stable enzyme glucose oxidase (EC 1.1.3.4; GOx) was used to catalyse the oxidation of D-glucose to D-glucono-1,5-lactone, which thereafter spontaneously hydrolysed to D-gluconic acid (Scheme 5.1). It should be noted that the effective 1:2 stoichiometry for oxygen:glucose conversion is due to the extra peroxide degradation loop catalysed by catalase (EC 1.11.1.6; Cat), which is unique to oxidase-based reactions.



Scheme 5.1. Reaction scheme catalysed by glucose oxidase (GOx) and catalase (Cat), which is used to decompose hydrogen peroxide, a harmful reaction intermediate. **1**: D-glucose; **2**: D-glucono-1,5-lactone; **3**: D-gluconic acid.

The accuracy of the gas-phase analytical equipment with respect to differing productivities (achieved by adjusting oxygen transfer rates (OTRs) and enzyme loading) was estimated by comparison with glucose analysis in liquid-phase samples by HPLC and, in this way, feasible operating windows for the method were found. In addition, estimating initial rates and time-course substrate consumptions by gas-phase measurements were also compared with other, more conventional online methods, such as dissolved oxygen tension (DOT) and pH titration measurements. The inherent challenges of applying these more conventional approaches are also discussed. Lastly, the method is also applied to a more complex, heterogeneous reaction involving co-solvent, which is catalysed by a Baeyer-Villiger monooxygenase (BVMO). This reaction was the oxidation of macrocyclic ketone **4** to lactone **5** (Scheme 5.2) by cyclododecanone monooxygenase (EC 1.14.13.x; CDMO) from *Rhodococcus ruber* SC1 (Schumacher and Fakoussa 1999; Kostichka et al. 2001). Cofactor regeneration was facilitated by glucose dehydrogenase (GDH; EC 1.1.1.47),

which catalysed the analogous reaction of GOx in Scheme 5.1, however without the need for molecular oxygen. The BVMO-catalysed reaction demonstrated not only the usefulness of such an online method for following reaction progress if standard liquid-phase sampling is made problematic, but also the generality of applying gas-phase mass balances to any system requiring oxygen. An explanation of the theory and nomenclature used in the application of this method follows.



Scheme 5.2. Oxidation of macrocyclic ketones to lactones catalysed by cyclododecanone monooxygenase from *Rhodococcus ruber* SC1 (CDMO). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) cofactor was recycled by using glucose dehydrogenase (GDH), which accepted D-glucose (1) as a co-substrate and produced D-glucono-1,5-lactone (2). 4: cyclopentadecanone; 5: cyclopentadecanolide.

5.1.2. Theory

A black box approach over the gas-phase during biocatalytic oxidation was applied (Figure 5.1).



Figure 5.1. Black box schematic of reaction apparatus. FC = gas flow controller; FM = gas flow meter; $y_{02}M$ = gas-phase oxygen fraction meter (reproduced from Meissner et al. (2018), with permission from John Wiley and Sons).

The number of moles of oxygen in the gas outlet stream, $\dot{n}_{O2,out}$ [mol h⁻¹], can be calculated by applying the ideal gas law to the total volumetric gas flow rate, v_{out} [sL h⁻¹], and oxygen fraction in the gas-phase, $y_{O2,out}$ [-], which are measured variables:

$$\dot{n}_{O_2,out} = \frac{P \cdot v_{out}}{RT} \times y_{O_2,out}$$
(5.1)

The number of moles of oxygen flowing into the system is calculated during a steady-state where no reaction is taking place because the following simplification holds:

$$\dot{n}_{O_2,in} = \dot{n}_{O_2,out}$$
 (5.2)

During reaction, the oxygen consumption rate, R_{02} [mol L⁻¹ h⁻¹], can then be found by mass balance, and the glucose consumption rate from reaction stoichiometry for glucose oxidase^{*} (Scheme 5.1):

$$R_{O_2} = \frac{dC_{O_2}}{dt} = \frac{\dot{n}_{O_2,in} - \dot{n}_{O_2,out}}{V} = \frac{1}{2}R_{glucose}$$
(5.3)

The initial rate of reaction is found in a similar manner from an initial portion of data when the reaction velocity can be considered linear. Replacing $\dot{n}_{O2,out}$ with an averaged oxygen flow rate, $\dot{n}_{O2,avg}$, observed during this period of steady-state reaction, allows the calculation of the initial rate. The 95% confidence intervals were determined using the total sample size of data that were averaged to calculate each initial rate (n > 100 in almost all instances).

The off-gas mass balance can also be combined with classic two-film theory to yield the following overall expression, where $k_L a$ [h⁻¹] is the overall volumetric mass transfer coefficient, and C_{02}^* and C_{02} [mol L⁻¹] are the dissolved oxygen (DO) concentrations at saturation and in the bulk medium, respectively:

$$\frac{\dot{n}_{O_2,in} - \dot{n}_{O_2,out}}{V} = \frac{dC_{O_2}}{dt} = k_L a \left(C_{O_2}^* - C_{O_2} \right)$$
(5.4)

5.1.3. Materials and Methods

Reagents

All reagents were of analytical grade and supplied by Sigma-Aldrich (Buchs, Switzerland). HiQ® Synthetic Air 5.0 was supplied by AGA A/S (Copenhagen, Denmark). Lyophilised catalase from bovine liver ($\geq 2000 \text{ U mg}^{-1}$; EC 1.11.1.6) and superoxide dismutase (SOD) from bovine erythrocytes ($\geq 3000 \text{ U mg}^{-1}$; EC 1.15.1.1) were purchased from Sigma-Aldrich. Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4) was provided by DuPont Industrial Biosciences (Wageningen, The Netherlands) in the form of lyophilised cell free extract. Cyclododecanone monooxygenase from *Rhodococcus ruber* SC1 (EC 1.14.13.x) was provided by InnoSyn BV (Geleen, The Netherlands) in the form of cell free extract. Lyophilised glucose dehydrogenase (EC 1.1.1.47) for

^{*} For CDMO $R_{02} = R_{glucose}$ due to reaction stoichiometry.

NADPH cofactor regeneration was provided as GDH280 by evoxx technologies GmbH (Monheim am Rhein, Germany).

GOx batch reaction conditions

Experiments were performed in a sealed 250 mL bioreactor with a working volume of 150 mL (MiniBio with my-Control software from Applikon Biotechnology (Delft, The Netherlands)). The bioreactor included stirring (500-1000 rpm), aeration through a sintered frit (0.5-2.0 aeration volumes per reactor volume per minute (vvm)), pH and temperature control capabilities. The operating temperature was maintained at 25 °C and pH at 7.5 by potassium phosphate buffer (200 mM). pH-stat experiments by titration of NaOH (1 M) were performed in the absence of buffer. Reaction kinetics were determined through complete oxidation of glucose (200 mM) by the addition of, unless otherwise stated, GOx (200 mg L⁻¹) and catalase (10 000 U L⁻¹) to ensure total H_2O_2 removal. Samples of 1 mL were periodically taken for analysis by HPLC. Dissolved oxygen in the liquid-phase was measured by a Solvent-Resistant Oxygen Probe (PyroScience GmbH, Aachen, Germany). To improve measurement accuracy, the gas-phase at the reactor outlet was dried by use of a condenser and sequential drying tube filled with silica drying beads before being passed through a Smart-Trak® 50 Series Digital Mass Flow Meter (Sierra Instruments, Inc., Monterey CA, USA) and BlueInOne CO_2/O_2 Gas Analyser (BlueSens gas analyser GmbH, Herten, Germany).

BVMO batch reaction conditions

The oxidation of macrocyclic ketones to lactones catalysed by CDMO were also performed in the same bioreactor used for GOx reactions (working volume of 150 mL). Reaction temperature was controlled at 30 °C and pH at 7.5 by NaOH (6 M) addition and potassium phosphate buffer (100 mM). D-Glucose (200 mM) and GDH280 (25 U mL⁻¹) were used for cofactor regeneration. NADP⁺ (2.5 mM), FAD (0.1 mM), catalase (150 U mL⁻¹), and SOD (20 U mL⁻¹) were for their stabilising effect on BVMOs (Goncalves et al. 2017). Antifoam 204 (0.5% (v/v)) was added to prevent foam formation. Cyclopentadecanone substrate (100 mM) was added to the reaction medium dissolved in methanol co-solvent (20% (v/v)). Lastly, the reaction was initiated by the addition of CDMO cell free extract (5% (v/v); protein concentration and specific BVMO activity not available).

GC analysis

End-point analyses of the BVMO experiment reaction medium could only be carried out after reaction completion due to the formation of a well-dispersed emulsion. 5 replicate samples of 1 mL were drawn from the reactor and substrate/product extraction was facilitated by 1:1 volumes of ethyl acetate containing naphthalene (1.5 g L⁻¹) as an internal standard. After vigorous mixing by vortex for 30 s and separation of organic from aqueous components by centrifugation, 300 µL

of the organic fraction was analysed by Clarus 500 GC-FID (Perkin Elmer). 1 μL of sample was injected into an Elite-5 column (Perkin Elmer) where the oven temperature was ramped from 150 °C to 200 °C at 4 °C per minute and thereafter at a rate of 40 °C per minute to a final isotherm at 300 °C for 1 minute. 0.60 mL min⁻¹ helium with a split ratio of 10:1 was used as carrier gas. Injector and detector temperatures were maintained at 280 °C throughout. Experimental substrate and product concentrations were determined using a standard curve generated by subjecting samples of known concentrations to the same solvent extraction procedure.

HPLC analysis

Glucose analyses were carried out by HPLC following the identical procedure detailed by Toftgaard Pedersen et al. (2017).

Solubility measurements

The solubility of the macrocyclic ketone and lactone, cyclopentadecanone and cyclopentadecanolide, was measured in triplicate by accurately weighing an amount of each into a 1 L volumetric flask containing dH₂O. The solution was mixed by orbital shaking and incubated at 30 °C for 3 days to allow for equilibration. The mixtures were then filtered, dried, and weighed again. The difference in mass from beginning to end was taken to be the amount dissolved in water (solubility).

5.1.4. Results and Discussion

Oxygen mass balance method validation

As a brief validation that time-course reaction progression and initial rate estimations made by oxygen mass balance in the gas-phase correlate to standard HPLC analyses, an example of data output from gas-phase measurements is shown in Figure 5.2. The initial oxygen transfer rate calculated by the difference $(\dot{n}_{O2,in} - \dot{n}_{O2,avg})/V$ was found to be 33.42 ± 0.54 mmol L⁻¹ h⁻¹ (mean ± 95CI). The predicted $R_{glucose}$ was therefore, from reaction stoichiometry, 66.84 ± 1.08 mmol L⁻¹ h⁻¹, which was within a 98% agreement with the rate measured by HPLC (68.19 ± 2.05 mmol L⁻¹ h⁻¹). Indeed, the closeness of prediction over the full time-course using gas-phase analysis can be seen in Figure 5.3.



Figure 5.2. Measured total volumetric flow rate of gas in the outlet stream (green) and oxygen fraction in the off-gas (blue) as a function of time. The number of moles of oxygen (red) were calculated through the ideal gas law and the grey dashed line indicates reaction initiation by the addition of 200 mg L⁻¹ GOx. pH was maintained at 7.5 by titration of 1 M NaOH. Steady-state periods that were taken as references for initial rate calculations are indicated. Agitation rate = 1000 rpm; aeration rate = 1.0 vvm (reproduced from Meissner et al. (2018), with permission from John Wiley and Sons).



Figure 5.3. Predictions of dynamic glucose concentrations during a time-course experiment using the off-gas mass balance method (yellow), dissolved oxygen measurements (blue) and pH titration method (green) compared with measurements by HPLC (black circles). The grey dashed line indicates reaction initiation by the addition of 200 mg L⁻¹ GOx. The reaction was performed in the absence of buffer with the titration of 1 M NaOH for pH control. Agitation rate = 1000 rpm; aeration rate = 1.0 vvm (reproduced from Meissner et al. (2018), with permission from John Wiley and Sons).

Method accuracy at different OTRs

Next, the validity of the off-gas oxygen method was tested at a range of reaction rates in order to ascertain a feasible operating window under which it might be applied. A possible strategy to fix the reaction rate was to ensure the reaction was purely limited by oxygen transfer kinetics and not enzyme kinetics. In order to do so, each experiment was dosed with an excess of GOx. The reaction performance could therefore be controlled by the OTR by adjusting physical reactor parameters: stirrer speed and aeration rate, independent of enzyme activity. In all instances, except under the highest OTR (98.89 \pm 1.44 mmol L⁻¹ h⁻¹), 200 mg L⁻¹ GOx was sufficient to reach this oxygen limited reaction state, which was confirmed by observing the same kinetics in a duplicate experiment under the same conditions with double the enzyme dose (data not shown).

The upper limit of this operating window was dictated by the maximum allowable reactor settings. Increasing scale or changing the reactor will therefore redefine the window under which this method may be applied.

Clearly the accuracy of the method varies with the ability to match oxygen supply and consumption in the reaction. If either the reaction rate or oxygen transfer rate are too low, then an inadequate response from the gas sensors may lead to inaccurate kinetic predictions. On this basis it was reasoned that a useful operating range for the method must be investigated. The range of OTRs and results are summarised in Table 5.1. Narrow 95% confidence intervals (< 3% on average) point towards a high precision of the method. This may be attributed to the large sample size of data that was used to calculate initial rates, an inherent benefit of using such online measurements with a quick data collection capacity.

Table 5.1. Summary of initial reaction rates obtained under various oxygen transfer rate settings (R_x = mean ± 95CI). Accuracy reflects the closeness of gas-phase mass balance prediction with that of HPLC analysis.

Agitation Rate	Aeration Rate	Measured <i>R₀₂</i> [mmol L ⁻¹ h ⁻¹]	Predicted <i>R_{glucose}</i> [mmol L ⁻¹ h ⁻¹]	HPLC <i>R_{glucose}</i> [mmol L ⁻¹ h ⁻¹]	Accuracy [%]
500 rpm (<i>P/V</i> = 0.2 kW m ⁻³)	0.5 vvm 1.0 vvm 2.0 vvm	15.12 ± 1.07 23.20 ± 1.08 40.76 ± 0.91	30.25 ± 2.14 46.39 ± 2.17 81.52 ± 1.82	36.37 ± 0.32 47.23 ± 0.15 65.12 ± 0.53	83.2 98.2 74.8
1000 rpm (<i>P/V</i> = 1.6 kW m ⁻³)	0.5 vvm 1.0 vvm 2.0 vvm 2.0 vvm ⁺	50.93 ± 0.45 55.56 ± 0.62 59.31 ± 1.27 98.89 ± 1.44	101.87 ± 0.91 111.12 ± 1.23 118.61 ± 2.54 197.79 ± 2.88	83.09 ± 2.02 113.99 ± 2.77 116.00 ± 1.00 173.71 ± 3.02	77.4 97.5 97.8 86.1

Figure 5.4 illustrates the accuracy of rate estimations based on the online method relative to those obtained through HPLC analyses in a parity plot. For the sake of generality, initial reaction rates have been presented as space-time yields (STYs) in g L⁻¹ h⁻¹. The two methods show a high degree of linear correlation ($R^2 = 0.9556$). The method appears to have a slight bias towards overestimating the reaction rate; however, most of the data points for the two methods fall within 85% of each other.

[†] Double GOx dose = 400 mg L^{-1} .



Figure 5.4. Parity plot of space-time yields (STYs; productivities) measured by HPLC vs. those predicted by oxygen mass balance. Data were taken from all experiments and were adjusted to represent more typical reactions where the stoichiometric coefficient of oxygen to product is equal. Dashed lines indicate 85% similarity; dotted line indicates unity (reproduced from Meissner et al. (2018), with permission from John Wiley and Sons).

Sensitivity to dynamic changes

The response of the off-gas method towards dynamic rate changes was observed by starting a reaction with 30 mg L⁻¹ GOx and then doubling the reaction rate after some time by dosing the reaction with a further 30 mg L⁻¹ GOx (Table 5.2). Oxygen supply was adequate at a volumetric mass transfer coefficient (k_La) of 85 h⁻¹ owing to the fact that no rate limitations were observed. Although a slightly higher reaction rate was predicted by the gas-phase mass balance method when compared to that obtained by HPLC analyses, the increase in rate after doubling the enzyme loading was exactly as expected at 1.95-fold the previous value. Therefore, it was concluded that the method could accurately predict dynamic rate changes caused by the corresponding enzyme addition. It follows that the method could therefore also cope with dynamic rate variations encountered in other oxidation reactions. Generally it was found that the sensors had a response time of less than 5 s to disturbances in steady-states, although this is dependent on the gas holdup of the reactor.

GOx Dose	Predicted <i>R_{glucose}</i> [mmol L ⁻¹ h ⁻¹]	Predicted <i>R_{glucose}</i> Increase	HPLC <i>R_{glucose}</i> [mmol L ⁻¹ h ⁻¹]	HPLC <i>R_{glucose}</i> Increase
30 mg L ⁻¹ 60 mg L ⁻¹	29.91 58.18	1.95x	23.81 49.27	2.07x

Table 5.2. Initial reaction rates as a function of dynamic enzyme dose from oxygen mass balance and HPLC analysis.

Comparison with online methods

Lastly, the off-gas method was put into context with two other online methods that can be used to monitor reaction kinetics: DOT measurements and alkali titration for pH maintenance.

Predicting rates by dissolved oxygen measurements requires an estimation of the k_La of the system under the conditions that are to be run in a time-course experiment. In this instance, the oxygen transfer characteristics of the 150 mL reactor used were estimated using the dynamic gassing-out method (de Figueiredo and Calderbank 1979) through dissolved oxygen measurements under standard conditions: in water at 25 °C and 1 atm. The k_La as a function of stirring speed and aeration rate is shown in Figure 5.5.



Figure 5.5. Average $k_L a$ as a function of stirring speed and aeration rate in 150 mL water at 25 °C and 1 atm (SD < 1% in all instances; n = 5; reproduced from Meissner et al. (2018), with permission from John Wiley and Sons).

Measurements of the DOT over time during an experiment (Figure 5.6) converted through Henry's law to oxygen concentrations in the liquid medium allow the OTR to be calculated at each instance of the experiment. The prediction of glucose consumption follows from the calculated OTR and reaction stoichiometry (Figure 5.3).



Figure 5.6. Time-course reaction performed in the absence of buffer solution with 1 M NaOH as base titrant. The grey line indicates reaction initiation by the addition of 200 mg L⁻¹ GOx. Dissolved oxygen tension (DOT) is shown in blue and the net alkali pump volume is shown in green. Agitation rate = 1000 rpm; aeration rate = 1.0 vvm (reproduced from Meissner et al. (2018), with permission from John Wiley and Sons).

The number of moles of oxygen or substrate consumed during such a reaction should also correlate with the number of moles of NaOH base titrated to maintain the pH in an unbuffered system since the resultant reaction product is an acid. The number of moles of base added can be calculated from the measured alkali pump volume and known concentration of the base solution (Figure 5.6).

The glucose consumption kinetics predicted by each method and those established by HPLC analyses are presented in Figure 5.3 and initial rates compared in Table 5.3. Each method was able to follow the reaction to an extent, although with varying degrees of accuracy. In terms of both initial rate and predicted kinetics over the full time-course of the experiment, the gasphase mass balance method was most comparable to HPLC analysis, which was considered to be the benchmark being the standard analytical method for this reaction. A weakness of predictions by dissolved oxygen measurements is that an accurate measurement of k_La under actual reaction conditions is essential, as opposed to a k_La estimated under standard conventions. k_La is dependent on medium properties such as the presence of salts, sugars or the enzyme itself (Pulido-Mayoral and Galindo 2004), which could be a reason for an inaccurate k_La estimate found under conventional standard conditions using the dynamic gassing out method. Using oversaturated enzyme reactions (i.e. reactions performed under OTR limited conditions) to measure k_La was postulated to be superior to conventional methods such as the dynamic gassing out method because it inherently incorporates a more realistic medium composition, required by the enzymatic reaction itself (Ortiz-Ochoa et al. 2005; Garcia-Ochoa and Gomez 2009). In the case of this experiment, the k_La during reaction is closer to 96.7 h⁻¹ rather than the estimated 84.7 h⁻¹, which is why the predicted glucose consumption kinetics were slower than those obtained by HPLC analyses. The accuracy of the pH titration method for kinetic predictions was most likely reduced due to the difficulty in making an accurate measurement of the peristaltic pump flow rate. Furthermore, any residual buffer salts in solution could cause a deviation of alkaline addition from that which is specifically required to neutralise the acidic product formation. Clearly a method relying on pH titration is less generic because not all reactions feature acids or bases. In any case, the method of gas-phase kinetic prediction does not suffer from these drawbacks and can be applied more generally to any oxidation reaction without the need for liquid-phase measurements.

Method	Predicted <i>R_{glucose}</i>	HPLC <i>R_{glucose}</i>	Accuracy
	[mmol L ⁻¹ h ⁻¹]	[mmol L ⁻¹ h ⁻¹]	[%]
Off-gas Measurement	66.84 ± 1.08	68.19 ± 2.05	98.0
DO Measurement	58.41 ± 0.10		85.7
pH Titration	51.81 ± 0.12		76.0

Table 5.3. Comparison of initial rates predicted by three online methods for oxidation kinetic predictions (R_x = mean ± 95CI). Accuracy reflects the closeness of each method's prediction with that of HPLC analysis.

Method application to BVMO catalysed reactions

To demonstrate the broader applicability of gas-phase mass balances, a further reaction catalysed by CDMO was tested featuring a poorly water-soluble substrate and product (25.4 ± 4.7 and 24.2 ± 5.7 mg L⁻¹ (mean \pm SD, n = 3), respectively), and methanol co-solvent phase to enhance substrate solubility. Due to the low solubility of both substrate and product, the reaction medium was heterogeneous, appearing turbid. Reaction monitoring by direct, liquid-phase sampling turned out to be unfeasible due to inconsistencies caused by large sample-to-sample variation. However, this oxygen-dependent reaction could be monitored using the online off-gas method previously validated using the glucose oxidase system. An example of experimental output is shown in Figure 5.7.



Figure 5.7. Reaction kinetics of the oxidation of cyclopentadecanone to cyclopentadecanolide catalysed by CDMO. The grey line indicates reaction initiation by the addition of 5% (v/v) CDMO cell free extract. The fraction of oxygen in the outlet gas stream ($y_{O2,out}$) is shown in blue and the predicted concentration of lactone product made by oxygen mass balance is shown in green (reproduced from Meissner et al. (2018), with permission from John Wiley and Sons).

At this enzyme loading, more than 25 g L⁻¹ product was produced in 15 h resulting in a space-time yield of 1.92 g L⁻¹ h⁻¹. An end-point analysis of the reaction medium by gas chromatography (GC-FID) confirmed the final conversion to be 92%.

The oxygen consumption trend by off-gas analysis shows two distinct reaction rate periods separated by a step change at 2.5 h. This behaviour was consistent with previous experiments and was distinct for this BVMO reaction, having not been observed in reactions with GOx. We hypothesise that this may be caused by loss of methanol co-solvent by gas stripping. There may exist a critical surface tension or methanol concentration under which poorly-soluble ketone or oxygen availability becomes limiting. It is also possible that the first reaction rate period reflects the unhindered rate of CDMO converting all dissolved substrate in the medium containing co-solvent, followed by the next reaction rate region wherein the mass transfer of cyclopentadecanone into the medium then dominates (this scenario would likely be solvent independent). A different cause for reaction rate decrease may be due to product inhibition after a limiting amount of product has formed during reaction. However, this is less likely to be the case considering the sharp step change nature of rate response.

Regardless, such an experiment highlights the strength of such an online method to discover kinetic effects in real-time, which is difficult to achieve through discrete sampling and analysis. Furthermore, these results satisfy the scope of demonstrating the more general application of gas-phase mass balances to other reaction systems. This application is further demonstrated in Chapter 6.

Effects of scale, limitations and advantages

One challenge with the gas-phase method is the measurable net difference in molar oxygen flow between reactor inlet and outlet. Assuming considerable reaction rate, the net difference will depend on both k_La and gas-phase holdup, both of which increase with scale (Stocks 2013). Since k_La values are up to 5-fold higher at larger scales, so too can OTRs be expected to be higher and therefore overall reaction rates are vastly improved from smaller-scales if oxygen mass transfer has been found to be limiting. Therefore, it is expected that method accuracy should improve with scale due to more oxygen being consumed in reaction. The online method could also prove advantageous in large-scale applications for monitoring and control of (bio)oxidation reactions where manual sampling of the reactor could be challenging due to e.g. overpressure, sterility, heterogeneity, safety, manpower or analytics. However, larger gas holdups expected at industrial scales might also affect the dynamic sensitivity of the method (i.e. the ability to detect sudden changes). Because of the reliance of the method on a net difference in molar oxygen in the gas-phase, it is also doubtful that this method will be as accurate at space-time yields lower than those encountered in this study (< 1 g L⁻¹ h⁻¹), which might be prevalent at smaller scales.

If a reactor overpressure or increased temperature is required, deviations from standard conditions (1 atm, 25 °C) will be seen. Such effects from these cases could be accounted for by adjusting the assumptions behind calculating molar quantities using volumetric flow measurements and the ideal gas law (see Section 5.1.2 for more details).

The online methodology followed in this work could potentially be automated to a degree by generating an appropriate software; doing so would enable an increased throughput for experiments.

With respect to the generality of the method, only the oxygen consumption/liberation from a net reaction is measured and not from individual half-reactions as is the case of (oxygen) uncoupling from product formation (e.g. with P450 monooxygenases) or when multiple reaction products are formed. Therefore, the black box approach to estimating kinetics from oxygen mass balances will make it difficult to distinguish uncoupling or to follow enantioselective reactions. In such instances, direct substrate/product analyses would remain the benchmark for performing kinetic characterisations. However, this method should find useful application with systems involving challenging substrates where liquid-phase sampling is made impractical (e.g. heterogeneous reactions or enzyme-catalysed surface modifications of solids (Fischer-Colbrie et al. 2006)) as exemplified with the BVMO case described in the previous section.

5.1.5. Conclusions

This study proves that the method of gas-phase oxygen mass balance is able to adequately predict bio-oxidation kinetics. In order to apply the method, an operating window of space-time yields under which it may be applied must be established. In this study, this window was found to be between 1-15 g L⁻¹ h⁻¹, which was inherently linked to the oxygen transfer capabilities of the lab-

5-15

scale apparatus used in this study. Therefore, the generic concept of using oxygen mass balance to determine kinetics holds, and this could find application in the studies of heterogeneous reactions, as well as for industrial measurement and control of oxidation processes.

5.2. Part II: A substitute activity assay method independent of spectrophotometric measurements

The Michaelis-Menten kinetics for the oxidation of cyclopentadecanone by CDMO was determined using a standard spectrophotometric assay (Figure 5.8). V_{max} was found to be 2.56 U mL_{CFE}⁻¹ and K_m was found to be 24 μ M. Therefore, at a substrate saturation of 113 ± 21 μ M (Meissner et al. 2018) the reaction should always proceed at V_{max} .



Figure 5.8. Enzyme activity as a function of substrate concentration. The Michaelis-Menten kinetic prediction is represented by the dotted line. Assays were determined spectrophotometrically by the consumption of NADPH causing an absorbance decrease at 340 nm. Reactions were carried out at 30 °C in 1 mL cuvettes with 50 mM Bis-Tris-propane buffer at pH 9.0, 0.3 mM NADPH, and 5% (v/v) CDMO CFE. Substrate was dissolved in 5% (v/v) n-propanol as co-solvent.

However, considerable variance was encountered whilst assessing the activity of CDMO using this conventional spectrophotometric assay (Figure 5.8). This could be due to insoluble substrate particles or background protein activity from the cell-free extract interfering with the spectrophotometric measurement. Therefore, an alternative activity assay was devised making use of direct substrate and product analyses by GC following solvent extraction by ethyl acetate from sacrificial reaction samples of 1 mL. This method presented more accurate activity measurements, especially under dilute conditions ([S] < 250 μ M). It is also important to note that

no activity was measurable without the addition of co-solvent. An example of one such activity measurement performed under dilute conditions over a period of one hour is shown in Figure 5.9. Importantly, the rate of substrate consumption and product formation are equivalent over the first half the experiment indicating that the mass balance in measurement closes. The apparent decreased product formation rate thereafter could potentially be due to product degradation at pH 7.5 through lactone ring hydrolysis. The exact methods and analytical protocols as well as the application of this method to evaluate enzyme stability are detailed further in Chapter 6.



Figure 5.9. Cyclopentadecanone consumption, cyclopentadecanolide formation and product yield as a function of reaction time. Measurements made by GC analyses of an extract from a sacrificial reaction sample (1 mL). Reactions carried out at 28 °C in 50 mM Tris/HCl buffer pH 7.5, 0.3 mM NADPH, 0.1% (v/v) CDMO CFE, and 0.25 mM substrate dissolved in n-propanol (5% (v/v)) as well as 25 U mL⁻¹ glucose dehydrogenase and 0.5 M glucose for cofactor regeneration. Error bars reflect 95Cl (n = 3).

Chapter 6. Case Study II – Part B: Reaction scoping for the oxidation of macrocyclic ketones catalysed by cyclododecanone monooxygenase (CDMO)

In this chapter, the analytical methods described in Chapter 5 were applied to assess the multiphasic Baeyer-Villiger oxidation of macrocyclic ketones to lactones catalysed by cyclododecanone monooxygenase (CDMO). First, enzyme-catalysed Baeyer-Villiger oxidations and the means to address poorly water-soluble solid substrates are briefly reviewed. Experimental evaluations followed to study the effect of different types of co-solvents on the activity as well as stability of CDMO. Based on this, an ideal co-solvent was selected and an optimal temperature and co-solvent fraction was determined using a Design of Experiments approach by making use of time-course reaction profiles (measured using the online oxygen mass balance analytical method detailed in Chapter 5). Finally, the primary process limitation was assessed using the methodology detailed in Chapter 2 and the route for further process development discussed after benchmarking with the metric targets from Chapter 3.

The structure of the chapter follows that of a manuscript as it is ultimately intended for publication. As such there is some repetition with the previous chapter. Although minimal, it allows the chapter to stand-alone as a potential publication.

6.1. Introduction

Oxidations in the chemical industry are a particularly challenging class of reactions that are limited by the necessary use of harmful oxidants, unwanted side reactions, and poor selectivity (Kroutil et al. 2004; Faber 2011; Turner 2011). Oxygen-dependent biocatalysts offer solutions to many of these limitations, and come in many varieties each offering their own specialised activities: oxidases, mono- and dioxygenases, dehydrogenases, and peroxidases, to name a few (Hollmann et al. 2011). The most important traits of oxygen-dependent enzymes towards synthetic application lie in their facility to use molecular oxygen and their outstanding selectivity.

Baeyer-Villiger monooxygenases (BVMOs) are oxidoreductases that act on their substrate and are bound with a co-factor, which is usually a flavin moiety (Mascotti et al. 2015; Romero et al. 2018). Whilst Baeyer-Villiger oxidation has been understood for over a century, their successful implementation to date has been moderate (ten Brink et al. 2004; Sutton et al. 2012). Enzymatic Baeyer-Villiger oxidation was first recognized and reported in 1948, in the biocatalytic degradation of steroids (Turfitt 1948). Following this, subsequent studies showed that Baeyer-Villiger oxidations facilitated by BVMOs are abundant in fungi and other prokaryotic organisms, as reported by Fried and co-workers and Peterson and co-workers in 1953 (Fried et al. 1953; Peterson et al. 1953). Over the years, several BVMOs have been made available in recombinant forms, which greatly increased the diversity of substrates upon which BVMOs can act through protein engineering. Furthermore, recombinant DNA technology has allowed the expression of BVMOs in benign host organisms which has enhanced their potential for industrial application. BVMOs catalyse the transformation of three major types of substrates: cyclic ketones, aryl ketones and linear ketones (Iwaki et al. 2002; Kamerbeek et al. 2003; Kyte et al. 2004; Mihovilovic et al. 2005a; Rehdorf et al. 2007) to their respective lactones. Extensive reviews of Baeyer-Villiger oxidations using several types and forms of BVMOs along with their applications in synthetic chemistry have been published by Mihovilovic and co-workers (2005b) as well as Alphand and Wohlgemuth (2010).

Lactones are an interesting product class and derive from either the oxidation of cyclic ketones (Walton and Stewart 2002; Mihovilovic et al. 2002; Carboni-Oerlemans et al. 2006; Rioz-Martínez et al. 2009; Kotlewska et al. 2011; Fink et al. 2013; Kara et al. 2013) or the esterification of hydroxy acids (Antczak et al. 1991; Sharma and Chattopadhyay 1999; Efe et al. 2008; Götz et al. 2013). They find numerous applications as, sometimes specialty, monomers for polymer synthesis (Knani et al. 1993; Moore et al. 2005; Lange et al. 2007; Kobayashi 2009; Heise and Palmans 2010), intermediates in flavour and fragrance synthesis (Serra et al. 2005; Fink et al. 2011), benign solvents (Alonso et al. 2013) or even as fuel additives (Horváth et al. 2008; Bond

et al. 2010). Therefore, lactones in these target markets can be classified as low- to medium-value products.

Despite their unique chemistry, BVMOs have only found application in one large-scale process (Alphand et al. 2003), where even gram-scale preparative reports are rare (Schulz et al. 2005). This process makes use of recombinant whole-cell *Escherichia coli* expressing cyclohexanone monooxygenase from Acinetobacter calcoaceticus (Doig et al. 2001) to oxidise the model substrate (-)-(1S,5R)-bicyclo[3.2.0]hetp-2-en-6-one to an asymmetric pair of lactones. The bottleneck of this process was the inhibitory and/or toxic nature of both substrate and products on the whole-cell biocatalyst (Kim et al. 2007). Overcoming substrate inhibition was attempted by making use of a substrate feeding strategy, although the problem of product inhibition remained (Doig et al. 2002). A better approach was to make use of an adsorbent resin that could facilitate in situ substrate supply (ISSS) and in situ product removal (ISPR) simultaneously, aptly named as an *in situ* substrate feeding and product removal (SFPR) strategy (Hilker et al. 2004a,b). In this case, the product has a stronger partition to the resin allowing substrate to be released into the reaction medium and, at the same time, to maintain product under inhibitory concentration limits. The high oxygen demand of both oxidation and whole-cell metabolism as well as resin and whole-cell viability were satisfied using a 1-litre bubble column with a sintered glass sparger (Hilker et al. 2006). Eventually, 20-25 g of combined product was isolated from the resin after a reaction window of about 20 h leading to a space-time yield of $1.2 \text{ g L}^{-1} \text{ h}^{-1}$. This corresponds to a reaction yield of up to 80% with each lactone exhibiting excellent enantiomeric excess (ee > 99%) (Hilker et al. 2005; Hilker et al. 2008).

To date, most reported Baeyer-Villiger oxidations of cyclic ketones have pertained to five or six membered ring ketones with little focus towards macrocyclic ketones (Iwaki et al. 2006; Fürst et al. 2017; Delgove et al. 2018b). A limitation in the enzymatic oxidation of macrocyclic ketones is their poor solubility in an aqueous reaction medium. Macrocyclic ketones, being highly hydrophobic, are therefore not as readily available for enzymatic reactions as compared to smaller ring size ketones and other more water-soluble substrates (Delgove et al. 2018a). Unlocking this branch of oxidations using biocatalysis would therefore require a successful solution in dealing with the low substrate capacity for these reactions (Schmid et al. 2001). Furthermore, industrially-relevant substrate/product loadings of more than 50 g L⁻¹ would in many cases also lead to a heterogeneous reaction mixture as well as potential substrate and/or product inhibition (Lu et al. 2004; Süss et al. 2014; Reimer et al. 2017; Sheldon 2017). A low substrate solubility can result in a poor driving force for diffusive mass transfer, which can hamper the productivity (or rate) or such processes. Hence, achieving productivity targets in such cases frequently requires an additional agent to aid in solubilising the substrate. A common means to achieve enhanced substrate miscibility in an aqueous reaction mixture is to use organic co-solvents.

There are a few considerations which should be properly investigated before choosing a co-solvent for a biocatalytic reaction. The presence of a specific solvent may have an effect on an enzyme's molecular structure, which could have implications towards its activity or stability during reaction. The relative activity of most enzymes is highest when placed in aqueous environments because extensive hydrogen bonding and dipolar interactions with the polypeptide chains of the enzyme drastically decrease their conformational mobility, thereby stabilising the enzyme (Stepankova et al. 2013). Hence, altering the environment surrounding the enzyme by introducing co-solvents may expedite enzyme denaturation or alter the active site of an enzyme (Owusu and Cowan 1989; Iyer and Ananthanarayan 2008).

Additionally, the ability of different solvents to improve substrate solubility should also be considered. This is a cumulative function of several properties of the solvent in question, such as: dielectric constant, hydrogen bonding, polarity index, hydrophobicity, inertness towards the substrate and product and the partition coefficient (P_{0/W}). The natural logarithm of the partition coefficient (Log P_{0/W}) is a measure of the hydrophobicity or hydrophilicity of a solvent. Laane and co-workers (1987) was one of the first to analyse the relationship between the activity of lipase towards catalysing the transesterification of tributyrin and heptanol and the Log P_{0/W} constants of different solvents used in the transformation. A sigmoidal function was found, and it has been concluded that for very low values of $Log P_{0/W} < 1$ (corresponding to highly water-miscible solvents), biocatalytic activity was low or insignificant. Organic co-solvents with Log P_{0/W} less than 2 caused distortion of the geometry of water molecules at the enzyme surface, which led to inactivation and a lower activity of lipase. For Log $P_{0/W}$ values between 2 and 4, the activity can be variable depending on the solvent, while for Log $P_{0/W}$ greater than 4 (corresponding to waterimmiscible solvents), the biocatalyst showed high and constant activity. Choosing a solvent having higher Log P_{0/W} values (greater than 3), typically leads to a biphasic system consisting of an organic solvent phase and an aqueous phase containing the enzyme. Whilst the enzyme present in the aqueous phase may be unaffected, if the substrate is also hydrophobic with high $Log P_{0/W}$, it will partition preferentially into the organic fraction. The formation of a biphasic system may therefore introduce substrate mass transfer limitations and interfacial adsorption of an enzyme protein (Straathof 2003). This may not be prevalent in cases with lipases because they have the unique ability to work at organic-aqueous interfaces. A co-solvent which provides a balance between the loss of enzyme activity or stability and increased substrate solubility should therefore be selected to maximize the productivity or rate of biocatalysis.

However, when substrate or product inhibition limits the rate of biocatalytic reaction, the purposeful addition of a second liquid phase (by means of organic solvent) can also be an important *in situ* substrate supply (ISSS) and *in situ* product removal (ISPR) technique (Freeman et al. 1993; Woodley et al. 2008). Inhibition is mitigated through substrate/product partitioning into the organic phase, avoiding accumulation in the aqueous reaction phase, and thereby enhancing productivity. The same effect may also be achieved using solid phase adsorbents/absorbents, which can be more benign towards enzyme structures than organic co-solvents (Guo et al. 2010; Schmölzer et al. 2012; Hilker et al. 2004a).

Alternative routes of utilising poorly water-soluble substrates in biocatalytic reactions have focused on the development and application of supercritical carbon dioxide and ionic liquids (van Rantwijk and Sheldon 2007; Jessop 2011; Claus et al. 2018).

Apart from using co-solvents, surface active agents such as surfactants may also aid substrate mass transfer through the formation of micro-emulsions and micellar structures. These emulsions feature vastly higher interfacial areas than traditional two-liquid phase reaction media.

The aim of this study was to demonstrate a BVMO-based process oxidising the poorly water-soluble bulky cyclic ketone, cyclopentadecanone, to its corresponding lactone. The final process was successfully scaled-up to 100 L with similar productivities not reported since the first reported large-scale BVMO-based process (Alphand et al. 2003; Hilker et al. 2008). To this end, cyclododecanone monooxygenase (CDMO; EC 1.14.13.x) from *Rhodococcus ruber* SC1 was selected for its activity towards larger-ring size cyclic ketones and stability (Schumacher and Fakoussa 1999; Kostichka et al. 2001). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) cofactor was recycled using glucose dehydrogenase (GDH; EC 1.1.1.47) by converting the co-substrate, D-glucose, to D-glucono-1,5-lactone. D-Glucono-1,5-lactone then spontaneously hydrolysed to gluconic acid in the presence of water. The overall reaction is shown in Scheme 6.1.



Scheme 6.1. BVMO-catalysed oxidation of poorly water-soluble cyclopentadecanone (1) to cyclopentadecanolide (2) by CDMO from Rhodococcus ruber SC1. NADPH cofactor was recycled using GDH, which converts glucose to glucono-1,5-lactone (thereafter spontaneously hydrolysing to gluconic acid).

First, the substrate and product physical properties in an aqueous medium were studied. A crystallisation experiment was performed to verify if similarly hydrophobic product would crystallise after formation on the surface of substrate particles, which could potentially further hinder substrate mass transfer.

Following this, four solvents were selected for their potential to help improve substrate solubility. Ranging in water-miscibility (Log $P_{0/W}$), their influence during an activity assay was established. The activity assay was based on sacrificial sampling and total extraction of 1 mL aliquots for analysis by gas chromatography (GC). This was done to ensure accuracy when dealing with the poorly water-soluble compounds that otherwise caused disturbances in a standard spectrophotometric assay.

The stability of CDMO towards these co-solvents was then evaluated by exposing the enzyme to each solvent over a period of 12 h (assumed to be a reasonable window of operation for a batch process). Relative activity was measured every 4 h with reference to the enzyme's initial activity by using the sacrificial sampling activity assay. Additionally, CDMO stability towards gas-liquid interfaces as well as oxygen in solution were measured by sparging an enzyme stock solution with nitrogen gas and synthetic air, respectively.

Showing a robustness towards solvents even at high concentrations, CDMO was then tested under trial process conditions. A method employing oxygen mass balances in the gas-phase of a reactor was previously demonstrated and validated in order to overcome sampling challenges brought about by heterogeneity caused by poorly water-soluble substrates (Meissner et al. 2018). This method was used to follow reaction time-course progressions, and the end-point conversion measured by GC analysis. A full factorial experimental design with mid-point replicates was used to find operating optima for co-solvent fraction in the medium and reaction temperature. Also, the influence of stabilisers, shown to have a beneficial effect by Goncalves and co-workers (2017), was tested during reaction.

At this stage the process performance was ready to be benchmarked at lab-scale in order to identify the major bottleneck towards reaction kinetics. The utility of the methodology detailed in Chapter 2 was assessed and used to identify and confirm that substrate mass transfer was in fact limiting. In light of this, future outlooks on process development are discussed.

6.2. Materials and Methods

Reagents

All reagents were of analytical grade and supplied by Sigma-Aldrich. CDMO from *Rhodococcus ruber* SC1 (EC 1.14.13.x) was supplied in the form of cell-free extract by Innosyn BV (Geleen, The Netherlands; specific protein content or BVMO activity not available). GDH (EC 1.1.1.47) for NADPH regeneration was supplied as lyophilised GDH280 powder by evoxx technologies GmbH (Monheim am Rhein, Germany).

Sacrificial sampling activity assays

A bulk reaction mixture for activity assays was prepared in a 25 mL volumetric flask and comprised of: Tris/HCl buffer at pH 7.5 (50 mM), D-glucose (2.5 mM in buffer) and GDH for cofactor regeneration (25 U mL⁻¹), NADPH (0.15 mM in buffer), cyclopentadecanone (0.25 mM) in 5% (v/v) n-propanol (unless otherwise stated), and CDMO cell-free extract. 1 mL aliquots of this mixture was then pipetted into separate 15 mL test tubes and placed into an orbital shaker at 30 °C and 150 rpm. The large headspace and shaking ensured oxygen transfer by diffusion was enhanced to not become limiting in activity measurements. Enzyme was dosed such that the full time-course of reactions would last for at least 45 min to allow enough time between sampling for stopping the reaction by extraction. At 5 min intervals individual samples were taken by extracting the full contents of a test tube in a 1:1 volume of ethyl acetate containing internal standard. Substrate and product concentrations in the organic fraction were then analysed by GC. Specific activity was calculated from the linear portion of substrate depletion data.

Small-scale batch reaction conditions

Lab-scale experiments were performed in a sealed 250 mL MiniBio stirred tank reactor from Applikon Biotechnology (Delft, The Netherlands). The reaction medium, which had a working volume of 150 mL, comprised of: D-glucose (200 mM) and GDH (25 U mL⁻¹) for cofactor regeneration, NADP⁺ (0.5-2.5 mM), potassium phosphate buffer at pH 7.5 (100 mM), cyclopentadecanone substrate (50-100 mM) dissolved in methanol (5-20% (v/v)), and finally CDMO cell-free extract (5% (v/v)) to initiate the reaction. Experiments to assess the influence of stabilising agents also included flavin adenine dinucleotide (FAD; 0.1 mM), catalase (150 U mL⁻¹) and superoxide dismutase (20 U mL⁻¹) at optimal doses found by Goncalves and co-workers (2017). pH control was maintained by the titration of NaOH (6 M) and reaction temperature controlled with a heating jacket. The stirring speed was set to 1000 rpm, which corresponded to a power input of 1.5 kW m⁻³, and oxygen supply was achieved by sparging the medium with air at 1.0 volumes per reactor volume per minute (vvm). Reaction kinetics were calculated through gas-phase mass balance (Meissner et al. 2018). The volumetric flow rate of gas leaving the reactor was measured using a Smart-Trak 50 Series digital mass flow meter (Sierra Instruments, Inc.,

Monterey CA, USA) and oxygen fraction with a BlueInOne CO_2/O_2 gas analyser (BlueSens GmbH, Herten, Germany). The off-gas was dried before measurement by being passed through a condenser and sequential drying tube filled with silica drying beads.

Design of Experiments (DoE)

Models for the full-factorial experimental design were developed in MATLAB Release 2016b using the Interactive response surface modelling toolbox "rstool", The Mathworks, Inc. (Natwick MA, USA).

GC analysis

End-point analyses of substrate and product concentrations (conversions) in reaction media were made by a Clarus 500 GC-FID (PerkinElmer) equipped with an Elite-5 column (PerkinElmer) following solvent extraction with 1:1 (v/v) of ethyl acetate containing naphthalene (1.5 g L^{-1}) as internal standard. The detailed procedure and temperature profile is outlined by Meissner and co-workers (2018).

6.3. Results and Discussion

6.3.1. Substrate and product physical properties

Understanding the substrate and product physical properties in water was an important step in realising their use in a biocatalytic reaction. Substrate and product solubility in water was determined to be, 25.4 ± 4.7 and 24.2 ± 5.7 mg L⁻¹ (mean \pm SD, n = 3), respectively (Meissner et al. 2018).

Being very poorly soluble in water, product would precipitate from solution upon formation. It was plausible that because the substrate and product were of similar molecular structure and physical properties, crystallisation of product may occur on the surface of substrate particles in solution. This newly formed product layer could then prevent substrate from diffusing into solution, and mass transfer would be limited or even eventually prevented. To confirm whether or not this phenomenon was taking place, product was supersaturated in water at high temperature (60 °C). The solution was then slowly cooled to 4 °C forcing the product to precipitate out of solution. At the same time, a substrate particle was added to the water to act as a seed crystal. It was observed, and further confirmed by GC, that completely separate product formation occurred even in the presence of substrate particles (Figure 6.1). Therefore, substrate mass transfer would not be limited by product formation during reaction.



Figure 6.1. Photograph of separate crystals formed from crystallising product (highlighted in blue) in the presence of an externally added substrate crystal (circled in red).

6.3.2. Effect of co-solvents on activity

When assessing the activity of CDMO towards cyclopentadecanone, considerable variance was found when performing a conventional assay relying on the absorbance decrease of NADPH at 340 nm. This could be due to insoluble substrate particles or background protein activity from the cell-free extract interfering with the spectrophotometric measurement. To avoid this possibility, direct substrate and product analyses by GC following solvent extraction by ethyl acetate from sacrificial reaction samples of 1 mL was employed. This method generated more accurate activity measurements, especially under dilute conditions ([S] < 250 μ M) and is described in more detail in Chapter 5. It is also important to note that no activity was measurable without the addition of co-solvent.

Four co-solvents were selected to solubilise cyclopentadecanone and ranged in Log $P_{0/W}$ such that this effect may be observable on the activity of CDMO (Figure 6.2). These four solvents were also selected due to their availability in chemical processing as conventional solvents. The volume fraction of co-solvent was kept to 5% (v/v) such that all substrate could be solubilised, however deleterious effects of co-solvent on e.g. enzyme stability would be limited (although this was later quantified). The co-solvents were as follows: cyclohexane (Log $P_{0/W} = 3.2$), methanol (Log $P_{0/W} = -0.76$), n-propanol (Log $P_{0/W} = 0.28$), and acetonitrile (Log $P_{0/W} = -0.33$). These solvents are common in industrial processing and vary in water solubility as well as reported enzyme compatibility.



Figure 6.2. Substrate (cyclopentadecanone) concentration profiles during sacrificial sampling activity assays solubilised in 5% (v/v) cyclohexane (squares), methanol (triangles), n-propanol (diamonds), and acetonitrile (circles). Dotted lines indicate the linear portion of activity taken to be initial rates.

Clearly, the substrate partitions too strongly into an organic phase consisting of water-immiscible solvent, such as cyclohexane, to permit enzymatic activity under these dilute conditions. Instead, either the substrate loading in the organic-phase needs to be vastly increased, or water-miscible solvents with lower Log $P_{0/W}$ are more appropriate for solubilising substrate. However, this could come at the trade-off of enzymatic stability over longer periods of time. Therefore, this was investigated in the following section.

6.3.3. Effect of temperature, co-solvents, gas-liquid interfaces and oxygen on

stability

The stability of CDMO was assessed by exposing a stock solution of enzyme to an environmental stress, which would be encountered during reaction at process-scale. To isolate these effects, only one factor was changed at a time to allow the independent study of stability. In each circumstance a negative control was also performed, which simply involved leaving the enzyme stock solution at room temperature without any other medium enhancement. Because most industrial batch processes are running in a window of 12 h or less (one working day), stability was only assessed over this period. Stability was measured by the loss in enzymatic activity from beginning to end by relative activity, periodically every 4 h.

First, the effect of temperature on CDMO was studied because at higher temperatures reaction rates could be elevated and mass transfer improved, however this may come at the expense of enzyme stability. Indeed, the relative activity of CDMO at 40 °C showed an increase to 129% above that observed at 30 °C. However, all activity was lost after 4 h at 40 °C, whilst the relative activity of enzyme kept at 30 °C remained stable over the full time-course of an experiment (data not shown).

The effect of co-solvents at high concentration (20% (v/v)) was then studied (Figure 6.3). As expected, the solvent with the highest Log $P_{0/W}$, cyclohexane, was favourable towards the enzyme. However, it was previously shown to limit substrate availability due to its own water-immiscibility, and was therefore not considered appropriate for process applications. Interestingly, CDMO showed good stability in the presence of most of the other lower Log $P_{0/W}$ solvents as well. No measurable activity was seen when the enzyme was exposed to acetonitrile at 20% (v/v), rather denatured protein precipitates were observed. n-Propanol caused a loss of approximately 60% activity after 12 h, whilst methanol had no effect on activity loss. This result demonstrates not only the robustness of CDMO towards organic solvents, but also that conventional understanding regarding the relation between Log $P_{0/W}$ and enzyme stability may not always apply. Instead, enzyme tolerance towards different solvents should always be tested on a case-by-case basis.



Figure 6.3. CDMO stability as a function of exposure to 20% (v/v) co-solvents. Solid bars = negative control; diagonal striped bars = methanol; horizontal striped bars = cyclohexane; opaque bars = n-propanol. Enzyme exposed to acetonitrile showed no activity throughout. Error bars reflect 95Cl (n = 3).

Oxygen supply is conventionally achieved by sparging and dispersing gas bubbles into the liquid medium in large-scale processes. In some instances, the presence of gas-liquid interfaces has been shown to have a destabilising effect on enzymes (Ganesh et al. 2000; Patil et al. 2000; Bommarius and Karau 2005; Bhagia et al. 2018). In others, stability is further compromised due to dissolved oxygen or peroxide in solution oxidising sensitive amino acids in the structure of an enzyme (e.g. cysteine residues) and causing inactivation (Stauffer and Etson 1969; Scott Willett and Copley 1996; Slavica et al. 2005). Naturally these effects should always be studied on all oxygen-dependent enzymes to ensure their process viability. A negligible deactivating effect of gas-liquid interfaces on CDMO was observed when sparging a stock solution with nitrogen gas (Figure 6.4). However, oxygen concentration appeared to have a deleterious effect on enzyme activity wherein approximately 50% residual activity remained after 12 h. If it were possible to identify and remove non-critical, oxygen sensitive amino acids in the structure of CDMO by protein engineering, an improved stability towards aeration may be realised.



Figure 6.4. CDMO stability as a function of gas-liquid interfaces (bubbling with N_2 gas; diagonal striped bars) and oxygen concentration (bubbling with air; horizontal striped bars). Solid bars reflect the negative control. Error bars reflect 95Cl (n = 3).

6.3.4. Scoping initial reaction conditions

Now that the effects of stability were better understood, the reaction could be worked up to a labscale reactor to allow the collection of time-course process performance data as well as better temperature, pH and dissolved oxygen control.

Methanol at high concentration (20% (v/v)) showed no deleterious effect on enzyme performance and whilst elevated temperatures of 40 °C prevented enzyme activity after 4 h, it remained to be seen if instead there was some optima towards process performance at a lower methanol concentration or temperature between 30-40 °C. To investigate this window, a fullfactorial experimental design approach was employed (Box et al. 2005). The design of experiments (DoE) relied on gathering process performance data at the boundary conditions to build a model, which was thereafter validated using three replicates at the mid-point of these experimental conditions (Table 6.1). Process performance was gauged on the final product concentration and conversion, which were derived from full time-course progression experiments with 50 mM substrate and 5% (v/v) CDMO cell-free extract. This resulted in experimental windows of up to 12 h, which allowed the effects of enzyme stability under these different process conditions to surface. Product formation over time was measured and predicted using oxygen mass balances in the gas-phase of the reactor (Meissner et al. 2018), and final conversions were confirmed using GC analysis. Higher concentrations of substrate hindered the stirring capabilities of the small-scale apparatus due to the increased presence of solid particles in the medium.

Variable Name	Property	'+'	4	Zero
X1	Methanol fraction [% (v/v)]	20	5	12.5
X2	Temperature [°C]	40	30	35

Table 6.1. Design of Experiments (DoE) for testing CDMO performance towards methanol co-solvent fraction and reaction temperature. '+' and '-' refer to boundary conditions and 'Zero' conditions refer to mid-point replicates.

The resulting model was found to be valid at the 95% confidence interval because experimental error collected at the mid-point of the DoE overlapped model prediction variance. The experimental results are shown in Table 6.2 and the model output predictions in Figure 6.5. The results simply confirm that process performance is only achieved at low temperature (30 °C) and high methanol fraction in the medium (20% (v/v)), and not at some local maxima in between. Under these conditions, the highest overall product concentration and conversion was observed in both experiments and by model prediction.

Variable X1	Variable <i>X2</i>	Product Concentration [g L ⁻¹]	Conversion [%]
-	-	13.3	45.7
+	-	13.4	92.6
-	+	6.60	62.0
+	+	2.37	9.58
Zero	Zero	10.2	87.6
Zero	Zero	12.0	90.6
Zero	Zero	7.03	90.2

Table 6.2. Experimental results for DoE.



Figure 6.5. DoE model output for final product concentration (left) as well as conversion (right) as a function of temperature and co-solvent (methanol) fraction.

Additionally, a simple experiment to test the influence of stabilising agents, which had been reported to have a beneficial effect on BVMO stability (Goncalves et al. 2017), was performed. These stabilisers included superoxide dismutase and catalase for dealing with residual oxygen radicals and hydrogen peroxide as well as an excess of cofactors: 0.1 mM flavin adenine dinucleotide (FAD) and 2.5 mM NADPH. Most important of these was the large NADPH requirement since this would be significantly expensive at large-scales. Two experiments were performed: one including all the stabilisers, and one without and at a much lower concentration NADPH (0.5 mM; Figure 6.6). The overlay of conversion profiles for each instance revealed that there was no observable difference from experimental variance in process performance to be gained by adding stabilisers. This may result from a number of reasons, however the most prominent of these is that CDMO itself is already a very robust enzyme (as shown by its resistance to large fractions of water-miscible polar co-solvents). Furthermore, the original benefit of adding stabilisers might be more pronounced when dealing with purified BVMOs and not cell-free extracts. This is because cell-free extracts may already contain a number of similar active proteins and cofactors that help in stabilising the BVMO. Therefore, it is important that the formulation of biocatalyst is considered during stability studies, especially when purified enzymes are involved.



Figure 6.6. Conversion profile of 5% (v/v) CDMO converting 50 mM cyclopentadecanone in 20% (v/v) methanol with stabilising agents (including 2.5 mM NADPH; green) and without (0.5 mM NADPH; blue).

6.3.5. Bottleneck identification

With this characterisation work forming a basis of understanding for how best to handle CDMO and poorly water-soluble cyclopentadecanone in reaction, the major process limitation, or bottleneck, for this reaction was elucidated. Time-course experiments are a powerful means to achieve this (Blackmond 2005; Blackmond 2015). Chapter 2 presents a methodology with which the bottleneck towards process implementation may be identified. A reference experiment is compared with another wherein the catalyst concentration is doubled (Figure 6.7a). If the reaction rate also doubles, then there is no kinetic limitation. This can be seen graphically as an overlay of the two data sets in a Selwyn plot (1965).



Figure 6.7. (a) Time-course conversion profiles of CDMO converting 50 mM cyclopentadecanone in 20% (v/v) methanol (left). (b) Selwyn plot of the same data sets (right). The reference experiment contained a catalyst loading of 5% (v/v) CDMO cell-free extract (blue) and the second experiment contained double the enzyme concentration (10% (v/v) CDMO cell-free extract; green).

The two data sets do not overlap in a Selwyn plot (Figure 6.7b). Furthermore, doubling the catalyst loading did nothing to improve the rate of reaction. These phenomena confirm substrate mass transfer as the limiting bottleneck for kinetics. This could either point towards cyclopentadecanone mass transfer or oxygen transfer as being limiting.

Oxygen mass transfer is strongly dependent on the physical apparatus used for reaction and can be influenced by power input, operating with pure oxygen instead of air, type of aeration, and even scale (Stocks 2013). It was hypothesised therefore that oxygen mass transfer was more likely to be the limiting factor at this small-scale because the power input was limited to reflect that of large-scale processes (approximately 1.5 kW m⁻³). The resulting effect was that the capacity for oxygen supply was reduced, and this could be reflected in the data.

6.3.6. Final metrics, benchmarking and future outlook

Eventually the substrate concentration was also doubled to 100 mM, although the reaction proceeded at the same rate owing to the bottleneck of oxygen supply at this scale. The final process metrics are given in Table 6.3. A lactone product could find application as a monomer for polymer synthesis, flavour and fragrance intermediate, benign solvent or fuel additive and is therefore classed as a low- to medium-value product. In this case the achieved process performance falls short in all metrics due to the demanding process requirements of low- to medium-value product classes. Converting approximately 4-fold more substrate to meet the target product concentration metric should not be too problematic due to the robust nature of the BVMO, however the rate of reaction was deficient by an order of magnitude. Space-time yield was found to be limited by the rate of oxygen transfer, which is especially critical for low-value products. The simplest strategy to improve oxygen transfer in this case would be to scale-up the reaction system. Indeed, the volumetric mass transfer coefficient $(k_L a)$ can also be improved by an order of magnitude through scale-up (Stocks 2013). The rate of oxygen transfer (and therefore reaction under oxygen limited circumstances) can therefore be improved approximately 5-fold due to mixing at scale (Noorman 2016). Other strategies for improving the rate of oxygen transfer could include using pure oxygen instead of air, operating the reactor under pressure, or alternative oxygen supply methods (such as membrane aeration). However considering the lowvalue of the product, these alternatives might prove more costly and thus less appropriate. Nevertheless even after addressing the space-time yield and product concentration requirements in these ways, the biocatalyst yield will still be insufficient. The only option for improvement in this case would be to look at increasing the specific activity of the cell-free extract through either protein engineering or further optimising the expression of active CDMO contained within. Therefore, this case shows the value of benchmarking to direct future research efforts at an earlystage in the development cycle.

Process Metric	Low-value Class Targets (5 € kg⁻¹)	Medium-value Class Targets	Actual Values
		(20 € kg ⁻¹)	
Reaction conversion [%]	(100)	(100)	92.6
Product concentration [g L ⁻¹]	100	50	22.3
Space-time yield [g L ⁻¹ h ⁻¹]	20	10	1.58
Specific productivity [g mL _{CFE} ⁻¹ h ⁻¹]	1.0 - 4.6	0.3 - 1.1	0.03
Biocatalyst yield [g mL _{CFE} ⁻¹]	5 – 20	1-6	0.45

Table 6.3. Benchmarking process performance metrics for the CDMO-catalysed oxidation of cyclopentadecanone.

The difference in potential success between this case and that of Chapter 4 is linked to the endproduct value class. Previously, ECS-PLE06 had relatively little trouble with reaching target process performance metrics for a high-value product (e.g. APIs), especially when an asymmetric reaction was involved. On the other hand, low- and medium-value products usually require more development effort as is showcased here. Perhaps the limited number of cases of BVMO implementation in industry is due to a lack of scope towards high-value products, which would otherwise balance the already challenging demands of a complex (e.g. gas-liquid-solid) oxidation reaction.

6.4. Conclusions

The enzyme-catalysed oxidation of macrocyclic ketones was only made possible through the application of a water-miscible solvent. Furthermore, CDMO presents a robust BVMO that is stable even in the presence of high solvent (methanol) concentrations. This case exemplifies the usefulness and necessity of alternative analytical techniques when trying to perform biocatalysis involving poorly water-soluble substrates. Such techniques were applied to characterise the reaction, and oxygen mass transfer was found to be the limiting bottleneck. Therefore, it is recommended that the most pragmatic way of furthering the process development of this reaction would be to increase the scale of reaction because OTRs are significantly improved at scale due to higher gas hold-ups. This was indeed observed in subsequent scale-up studies at a scale of 100 L.
Chapter 7. General discussion

In hindsight of the work carried out in previous chapters, the following chapter is a general discussion of the most relevant considerations for the biocatalysis of poorly water-soluble substrates. The discussion starts with a suggested route of assessment for early-stage biocatalytic reactions. This protocol includes the order and types of experiments that, if followed, will elucidate a comprehensive understanding of the given reaction's performance in a structured and efficient manner. The outcome of such a protocol may be used for: benchmarking with target process performance metrics (Chapter 3), conceiving the most feasible improvement strategies as well as subsequent process development and scale-up. Particularly relevant for poorly water-soluble substrates is the influence of scale on reaction performance, which is reflected during reaction assessments performed within this protocol. Therefore, this is also discussed. Finally, the use of non-aqueous biocatalysis is presented as a means not only to nullify this influence of scale, but ultimately the other challenges of implementing poorly water-soluble substrates encountered in this thesis.

7.1. A protocol for early-stage process conceptualisation

Despite several hundred biocatalytic processes being implemented to date (and sometimes outperforming their more conventional chemocatalytic counterparts (Ma et al. 2010; Savile et al. 2010)), the number of viable processes is disproportionate to the research effort that has been undertaken on improving biocatalysts across various fields, such as protein engineering (Strohmeier et al. 2011). Indeed, significant advances have led to well-established methods of engineering enzymes (Reetz 2011; Bommarius et al. 2011; Bornscheuer et al. 2012), however there are very few cases where these methods have been used to match enzymes for specific process applications. There is also often little focus on attaining an adequate expression of engineered enzymes, which has implications for achieving a necessary specific activity (and biocatalyst yield) of a biocatalyst formulation in a process setting. Further, a disconnect in process development often occurs in the early-stages, where standardised methods of evaluating a biocatalytic reaction are lacking and the envisaged process itself may be poorly understood (Tufvesson et al. 2013). For all these reasons it is therefore inevitable that the development of a biocatalyst is all too often carried out blindly without the final process in mind, leading to failure or irrelevance towards industrial implementation.

The analyses carried out in the earlier case studies of this thesis (Chapters 4-6) were central to proposing a standardised protocol. Based on what was observed, established and learnt

in those case studies, a proposed flow for conceptualising this initial phase of reaction scoping was developed and is shown in Figure 7.1.



Figure 7.1. Flow of early-stage biocatalytic process development.

Creating a viable biocatalytic process from scratch requires a systematic development that should always hold industrial process performance targets as well as reaction targets (or synthesis route) at the forefront. Here, "scratch" means that a product of interest and a suitable enzyme candidate towards its synthesis have so far been identified. In such a scheme, the nature of both targets will ideally identify and feed process constraints to the next stage of reaction evaluation, and then proceed to influence each subsequent step in the process development cycle. The fundamental target that must first be decided is the precise synthesis reaction to be performed by an enzyme. While this sounds trivial, the reaction target needs to be decided through collaboration of retrosynthetic chemists for their expertise in organic pathway synthesis, molecular biologists for their expertise in known enzyme activities and potential for expanding substrate scopes, as well as chemical engineers. All should have the final product goal or subsequent synthesis steps towards that goal in mind. The intended product market will determine its value-class and therefore the necessary process performance to meet economically viable process margins (i.e. profit from a given process based on the values of product, substrate and reaction yield). One way of establishing the required process performance is to set additional targets for process metrics based on the product value-class (Chapter 3).

The target process metrics give an indication of feasible enzyme doses, the required speed of reaction (intrinsic activity) and required product concentration from the reaction step. However, under these process constraints, some knowledge of the reaction must first be established. Such an initial reaction characterisation should include investigating the substrate and product physical properties under reaction conditions (especially relevant for poorly watersoluble substrates). This includes investigating potential substrate and product degradation under ranges of reaction conditions (e.g. pH, temperature, or presence of co-solvents) as well as the analytical procedures required to measure their component fractions during a reaction (Chapter 5). In addition, at this stage the enzyme's tolerance to specific reaction conditions must also be assessed to establish pH and temperature optima. These optima need to strike a balance between enzyme activity, enzyme stability as well as substrate/product degradation (if applicable). The Michaelis-Menten kinetics of an enzyme in question should be assessed at this stage in order to understand the maximum possible activity and affinity towards the substrate (Ringborg and Woodley 2016). It is important that the formulation of this enzyme is also taken into account – a purified protein will exhibit different activities and stability than a cell-free extract or whole-cell preparation - because protein expression and isolation steps become relevant. The selection of this formulation should be based on economic viability, ease of use in an industrial process (e.g. long-term viability during storage at preferably room temperature), and suitability for required process performance. Lastly, the combination of all these factors should lead to an understanding of the reaction kinetics, where all these effects (e.g. enzyme stability, substrate mass transfer, enzyme kinetics, thermodynamics) are compounded. The final experimental output from this stage to be used in further reaction development is a standardised time-course conversion profile under realistic reaction conditions.

The immediate goal of this early-stage development methodology is to identify a major limitation/bottleneck to process performance. A full reaction characterisation and/or optimisation is superfluous in terms of both time and experimental effort. Thus, in Chapter 2 a simple methodology relying on the graphical analysis of time-course progressions under different reaction conditions (enzyme and substrate loading) was described to demonstrate the identification of such a process bottleneck in an efficient and simple manner. The outcome suggests whether the reaction is limited by intrinsic activity (as is the case of zero-order reaction kinetics), enzyme stability, enzyme kinetics, or substrate mass transfer. The exact mechanism of enzyme stability or kinetic limitation (e.g. substrate mass transfer is found to be limiting, it will dominate all other kinetic effects and reaction performance will be hindered unless resolved through reaction engineering (Chapter 1). Furthermore, Chapter 5 highlights some experimental challenges and their solutions when trying to scope heterogeneous reactions using this methodology.

If process performance falls short of the metric targets after benchmarking, the contributing bottleneck would have been identified following the methodology described in Chapter 2, and a directed improvement strategy formulated. The key implication of knowing which improvement strategy to choose is that the feasibility of implementation is also considered. As was mentioned in Chapter 1, improving an enzyme by protein engineering can have the highest return in terms of reaction performance (even in excess of 100-fold), however this comes at the greatest cost in terms of both time and research investment. Instead, if there is only need to improve the enzyme moderately, and the option exists, then improvement through reaction engineering may be more pragmatic for the whole process development chain. Regardless, having a specific target for improvement (e.g. k_{cat} , K_M , substrate mass transfer, inhibition) is invaluable at the earliest possible stage so that a more efficient process development and faster implementation can be realised. Shorter development times are essential if biocatalysis is to better secure its position as a viable technology in, most importantly, the pharmaceutical industry due to short patent lifetimes (Truppo 2017). If the improvement strategy appears to be unfeasible, then the targets for reaction (exact synthesis step) can be revised with the help of retrosynthesis and the early-stage development cycle restarted (Turner and O'Reilly 2013; de Souza et al. 2017).

Following this protocol for conceptualising a biocatalytic reaction should instil confidence in eventual process implementation. Outside of this development cycle, Tufvesson et al. (2013) detailed systematic procedures for further improvements towards the fermentation step, biocatalyst, process and downstream. Here, there is more focus on physicochemical data and process models, which would otherwise be too detailed for the early-stage process development methodology presented here. Eventually, scale-up of the designed process should feature an adequately performing biocatalyst that is able to meet economic requirements.

7.2. Influence of scale when evaluating heterogeneous reactions

A factor to consider when evaluating biocatalytic reactions is that reaction performance at smallscale often may not reflect that of the large-scale under the same conditions. This is important because early-stage conceptualisation and reaction characterisations will always begin at the smallest allowable scale to allow minimal use of materials.

Often a major reason for such deviance across scales is due to substrate mass transfer. Enzymes as catalysts require at least some water to be present in their environment in order to be active (Adlercreutz 2008). Frequently organic substrates and products of synthetic interest are poorly soluble in an aqueous reaction medium (Meyer et al. 2013), which instigates mass transfer limitations – as is prevalent throughout this thesis. This limitation extends to gas-liquid systems, such as important enzyme-catalysed oxidation reactions (Hollmann et al. 2011) due to the similarly poor water-solubility of oxygen. On the other hand, conventional catalysis is most often performed in homogeneous gas-phase reactors or organic solvents. Under these circumstances reaction scale-up is more dependent on adequate heat transfer and reactor design, which is less relevant for biocatalysis performed under mild conditions. Interestingly, the mechanism for scale-dependence of gas-liquid mass transfer and more conventional liquid-liquid mass transfer differs (Table 7.1).

	Gas-Liquid Mass Transfer		Liquid-Liquid Mass Transfer	
	Dispersion (i.e. bubble size)	Hold-up [*]	Dispersion (i.e. droplet size / coalescence) [†]	Hold-up
Small-scale Large-scale	$\stackrel{\wedge}{\downarrow}$	$\begin{array}{c} \downarrow \downarrow \\ \uparrow \uparrow \end{array}$	$\uparrow \\ \downarrow$	N/A N/A

Table 7.1. Effect of scale on gas-liquid and liquid-liquid mass transfer. Arrows reflect improvement and hindrance for mass transfer.

Mixing is a function of power input, which can vary significantly with scale (Noorman 2016). Put simply, it is easier to thoroughly mix a smaller volume to achieve good dispersion (high power input per unit volume) than it is to mix a large volume. As dispersion increases so does the specific interfacial area of the second phase component (i.e. smaller bubbles or droplets), which leads to improved mass transfer. However when considering gas-liquid mass transfer, an even bigger influence is the increased gas-hold up at larger scales, allowing an increase in overall volumetric mass transfer coefficients ($k_L a$) (Stocks 2013). Liquid-liquid systems are not affected by hold-up because the phase component remains in the reaction volume unlike gases, which pass upwards through a reactor. Furthermore, liquid-liquid systems have a unique ability to be emulsified by adding surfactants which results in a homogeneous medium that is not dependent on mixing. Thus this makes liquid-liquid systems largely scale-independent. While solid-liquid systems are not the focus in this analysis, similar parallels can be drawn between these systems. However, they are less dependent on mixing to ensure a high interfacial area and are rather influenced more by particle size.

Another reason why some process metrics measured at small-scale may not reflect those of larger scales is because scale-up can afford better control over the reaction environment. This raises the question: what can we learn at different scales about prospective process performance

^{*} The extent of effect of hold-up on mass transfer is more or less pronounced for plug-flow or well-mixed regimes, respectively (Noorman 2016).

[†] Stable emulsions create a homogeneous medium that will be independent of mixing.

when a given reaction is limited by mass transfer? Table 7.2 summarises different types of apparatus at different scales, and the process metrics that can be reliably ascertained. It is advantageous to be able to quantify realistic process metrics in as small a scale as possible to allow fast and efficient process scale-up using less, often expensive, material.

Volume (Scale)	Example Apparatus	Types of Experiments	Types of Analytical Measurements	Process Metric
< 10 mL	Vials; Eppendorf tubes; Shake flasks; Microtitre plates	End-point analyses	GC/HPLC; Spectrophotometric	Reaction conversion; Reaction yield; Selectivity
10 mL – 1 L	Round bottom flasks (or similar); Small-scale stirred tank reactors	Time-course progressions [‡] ; End-point analyses	GC/HPLC; Online analytical methods (Chapter 5)	Reaction conversion; Reaction yield; Selectivity; Product concentration; Biocatalyst yield
>1L	Stirred tank reactors	Actual process performance	GC/HPLC; Online analytical methods (Chapter 5)	Reaction conversion; Reaction yield; Selectivity; Product concentration; Biocatalyst yield; Productivity; Specific productivity

Table 7.2. Experimental systems for the process evaluation of a biocatalytic reaction that is mass transfer limited (scale-dependent). GC – gas chromatography; HPLC – high-performance liquid chromatography.

At the smallest scales (e.g. Eppendorf tubes, vials, microtitre plates) the simplest and most common type of assessment is an end-point measurement made after a standardised reaction time. These experiments are an efficient means to gauge if an enzyme has activity towards a certain substrate as well as what conversion, yield and selectivity can be achieved after a certain length of time. However, it is difficult to decouple specific mechanisms for process performance because they are all aggregated into a single data point. Instead, time-course reaction progressions performed in a slightly larger scale with adequate control over the reaction environment (e.g. pH, temperature) ensures this more detailed knowledge of process performance. In addition, more convenient online methods of analysing reactions can be implemented at these larger-scales (Chapter 5). It is important to consider the scale at which these experiments are performed because some reactions that are limited by mass transfer (e.g.

[‡] Time-course reaction progressions can reveal kinetic effects (e.g. stability, inhibition, extent of mass transfer limitation) and can give indication to the space-time yield (Chapter 2).

gas-liquid systems) may not proceed at representative rates in small-scale apparatus. The most important metric to evaluate under these circumstances is the biocatalyst yield and maximum product concentration output. These indicate if the catalyst is sufficiently efficient and if enough product can be generated in a reasonable amount of time. In addition, if enzyme stability or substrate/product inhibition caused by high concentration occurs, this may also be identified using such time-course progressions before large-scale trials are conducted (Chapter 2). Timecourse progressions will also give an indication of the expected productivity of a reaction, and already at this stage, reactions that proceed too slowly may be earmarked for improvement. Naturally, only metrics assessed at large-scale can be considered true process metrics, however understanding how to gauge a reflection of the metrics at smaller scales is invaluable to the effectiveness of process development.

7.3. Non-aqueous biocatalysis

An alternative solution to the challenges posed in this thesis as well as the influence of scale during reaction assessments is to abandon water as a solvent altogether. Biocatalysis carried out in non-aqueous media has been long established, although is still limited in applications when compared to aqueous biocatalysis (Pollard and Kosjek 2008). Strictly speaking the term "nonaqueous" is not entirely accurate because enzymes must have water present on a molecular level in order to be active (Zaks and Klibanov 1988). Another more accurate name for this medium would be "monophasic organic solvents", which can be either water-immiscible (nearly anhydrous) or water-miscible where the organic solvent forms the predominant component fraction of the medium (Dordick 1989; Carrea and Riva 2008). The exact amount of water necessary to ensure activity differs from enzyme to enzyme. On one end of the spectrum, chymotrypsin retained full activity in an n-octane medium with only 50 molecules of water per enzyme molecule (Zaks and Klibanov 1986). This is indeed a miniscule amount of water when considering the size of most enzymes is about 50 000 Da. By comparison other enzymes such as horseradish peroxidase and polyphenol oxidase required 1.8×10^7 (0.25% (v/v)) (Zaks and Klibanov 1988) and 3.5×10^7 (0.5% (v/v)) (Kazandjian and Klibanov 1985) molecules of water per enzyme molecule in chloroform and toluene, respectively. Enzymes such as proteases, lipases, esterases, dehydrogenases and some cytochrome P450s are especially suited to low water conditions because they are naturally found in hydrophobic environments near or bound to cell membranes (Dordick 1989).

The most practical way of determining the water concentration in a medium will have either a molar, mass or volumetric basis. Enzyme activity in low water environments is however only a function of water actually bound to the enzyme molecule itself, and not how much water is in the medium as a whole (Zaks and Klibanov 1988). Instead, thermodynamic water activity or relative humidity (Halling 1990) has been accepted to be the best measure when studying the kinetics of enzymes in low water environments (Bell et al. 1995). A water activity of 1 reflects a completely aqueous medium and 0 reflects a dry organic medium. Water activity at equilibrium is the same in all phases even if the volumetric concentration of water differs. Therefore, it is most conveniently measured in the gas phase of a reactor by use of a sensor. This system can also be used for drying or wetting control of the medium (Won and Lee 2001; Petersson et al. 2007).

The key advantage of using non-aqueous solvent systems is the vastly increased solubility of nonpolar, hydrophobic substrates, such as those encountered in this thesis. Although there are also many other advantages of such systems, for example: thermodynamic equilibria can be shifted, side-reactions involving water are avoided, subsequent product recovery is made easier especially with the use of low boiling point solvents, biocatalyst recovery is also made easier by simple filtration since enzymes are often insoluble in organic media, enzyme thermostability is usually increased in low water environments, microbial contamination of the medium cannot occur, and lastly biocatalysis may be made more compatible with more traditional chemical processes through the use of similar organic media (Halling 1987; Dordick 1989; Klibanov 2001).

Despite these advantages a major disadvantage to using enzymes in low water environments is a tendency to reduced activity. Substrate solvation in more compatible organic environments reduces the binding availability of substrate with an enzyme, thereby effectively increasing the apparent K_M , even by two orders of magnitude (van Erp et al. 1991). It should be noted that lipases are exceptional cases where instead increased water activity can have an inhibiting effect on reaction kinetics (increasing the apparent K_M) because of their action at phase interfaces (Bovara et al. 1993; Valivety et al. 1993). The quantitative effect of solvation on kinetic parameters can be made through considering the thermodynamic properties of different solvents (Halling 1994).

The trade-off in process metrics under low-water conditions between increased solubility and reduced activity (through increased apparent K_M) is shown in Table 7.3 for a medium-value class product. Here it can be seen that the influence of decreased reaction rate due to solvation was not as severe because the required substrate concentration turnover is far in excess. For this case 11% more biocatalyst would need to be dosed in order to achieve the same activity as for a water-mediated reaction. Instead, there was a prominent 4-fold increase in overall space-time yield. This was due to the smaller reaction volume required because of the increased solubility of substrates in monophasic organic solvents. The influence of solvation on process performance is less and more pronounced for low- and high-value processes due to different substrate loading requirements. For these cases, approximately 5% and 60% more biocatalyst would need to be supplied to achieve the same 4-fold increase in space-time yield for the production of low- and high-value products, respectively. It is important to note that similar gains could be seen for oxidation processes due to the increased solubility of oxygen in organic media (Sato et al. 2014; Ramesh et al. 2016). This effect would be similar to the concept of using "oxygen vectors" to increase the rate of oxygen supply (and therefore reaction rate for oxygen-limited reactions) (Quijano et al. 2010). Enzymes are often vastly more thermostable in low-water conditions (Rupley and Careri 1991; Toscano et al. 1994), one exceptional case of α -chymotrypsin even maintaining full activity when exposed to 126 °C for 4 h (Mozhaev et al. 1991). This affords the possibility to further increase reaction rates by increasing temperature without destabilising otherwise more temperature sensitive enzymes, however only if the thermostability of reactants and products allows.

Table 7.3. Process metrics as a function of water activity for the conversion of poorly water-soluble substrates into medium-value products ($20 \in kg^{-1}$). Apparent K_M was assumed to increase by a factor of 100-fold in low-water conditions due to solvation (van Erp et al. 1991). The corresponding overall decrease in reaction rate under these conditions is countered through an elevated biocatalyst yield requirement to reach a standardised process rate constant of 3.33×10^{-3} min⁻¹. Reaction volume decreases with the use of monophasic organic solvents because of the vastly increased solubility of substrate in the medium (approximate value taken from the solubility of 2-hydroxybiphenyl in various solvents (Tomlin 2004)). Substrate/product molecular weight assumed to be 100 g mol⁻¹.

Water activity [-]	1	< 0.1
Product concentration [g L ⁻¹]	50	50
Substrate solubility [g L ⁻¹]	< 1	400
Apparent K_M [mM]	0.1	10
k_{cat} [min ⁻¹]	3.33 × 10 ⁻³	3.00×10^{-3}
k_{cat}/K_{M} [min ⁻¹ M ⁻¹]	33	0.30
Required biocatalyst yield [g g _{biocat} -1]	189	210
Reaction volume [L]	1000	250
Space-time yield [g $L^{-1} h^{-1}$]	10	40

Enzyme activity in non-aqueous solvents depends largely on their formulation. Water allows enzyme structures to be pliable. Therefore in the absence of water, enzymes have a high conformational rigidity. Biocatalysts prepared by lyophilisation for use in anhydrous media therefore exhibit a 'memory' of their previous conformation in the preceding aqueous preparation (Klibanov 1995). This memory influences their activity based on pre-lyophilisation exposure to, for example, pH optima or ligand additives. The conformational state due to pH especially influences the enzyme activity in non-aqueous media because pH is not a characteristic

property of organic solvents. Non-conventional means to control pH through "organic phase buffers" which make use of zwitterions and their sodium salts do, however, exist (Blackwood et al. 1994; Harper et al. 2000).

In most cases lyophilised enzymes are insoluble in organic media.[§] Insoluble biocatalyst suspensions in reaction media are actually desirable for process handling because biocatalyst recovery (and potential reuse) can be facilitated through simple filtration. In some instances enzyme agglomeration and consequential activity loss can occur when lyophilised preparations are directly added to organic solvents, although this is only possible at elevated water concentrations (Yamane et al. 1989; Stevenson and Storer 1991; Kim and Lee 1996). Sometimes the solid enzyme particulates forming the suspensions can bring about substrate diffusional limitations due to the enzyme molecules clumping together. This will mask the true kinetics of an enzyme's performance, as was discussed through mass transfer limitations in Chapter 2. In such cases immobilisation could be considered for better dispersing the effective surface area for catalysis for use in non-aqueous media. One aspect of immobilisation which is improved in nonaqueous systems is that activity loss through leaching of an enzyme into the reaction medium is less common due to the insolubility of enzymes in organic solvents. Therefore, more simple methods of immobilisation such as deposition onto glass beads can be used (Kazandjian and Klibanov 1985; Kazandjian et al. 1986). This avoids intraparticle diffusion limitations and structural efficacy loss caused by covalent bonding. However, "overcrowding" caused by multiple enzyme layers bound to the support must be avoided through optimisation to avoid internal diffusion limitations (Dordick 1989). Properties of the immobilisation support itself can also be used to help an enzyme in a non-aqueous environment. More specifically, some supports can be used to provide the necessary hydration for enzymes to work most effectively (Reslow et al. 1988; Adlercreutz 1992). Nevertheless, immobilisation should still be avoided if at all possible because the disadvantages of immobilisation covered in Chapter 3 still hold in non-aqueous systems.

Enzymes can also be engineered for improved stability in polar non-aqueous solvents (Arnold 1990). Previous studies have, however, only involved protein engineering one such enzyme, subtilisin, through site directed mutagenesis. Instead, a better target might be to engineer higher activity or substrate affinity since a largely reduced speed of reaction is almost always seen in non-aqueous environments. This is due to a higher apparent K_M caused by substrate solvation, as already discussed. Considering the power of modern protein engineering techniques such as directed evolution, there exists a huge potential for enzyme improvements for

[§] However, there are few examples of homogeneous enzyme catalysis in organic solvents where the enzymes were modified by pairing an ion from a surfactant with the protein structure to become soluble in the medium (Mozhaev et al. 1991; Paradkar and Dordick 1994).

use in non-aqueous environments. Indeed, this capability could be key for realising the implementation of many new enzyme classes beyond hydrolases in anhydrous solvents.

In conclusion, there remains great potential in monophasic organic solvents as media for improving the performance and application of biocatalysts in synthetic chemistry (Klibanov 2001). However as is evident from the lack of new literature, there is a need to revisit this field with renewed focus and also incorporating modern protein engineering techniques. In particular, there is a great need for more kinetic studies in the presence and absence of water to better understand the mechanistic specifics of implementing biocatalysts in monophasic organic solvents (e.g. Marty et al. 1992; Chatterjee and Russell 1992; Chatterjee and Russell 1993) especially with different enzyme classes (other than hydrolases). Moreover there is also the need to study the effect of solvation on specificity (Wescott and Klibanov 1993; Parida and Dordick 1993) for other enzyme classes. Monophasic organic solvents as media might prevent the use of ISPR in cases where product inhibition occurs. However, it would be of interest to study the phenomenon of product inhibition in low-water conditions because the conformational rigidity of enzymes might play a central role towards the mechanism of inhibition. Furthermore, the need for high product concentrations might not be as crucial when using monophasic organic solvents as media because the evaporation of water, which is also the most energy intensive step in the downstream, will no longer be necessary. Such studies will help clarify the underlying causes of activity loss in anhydrous media and how to prevent it, as well as many other fundamental questions (Klibanov 1989). Klibanov (2001) has stated on the prospects of using enzymes in neat organic solvents: "In fact, there is no fundamental reason why enzymes could not be more active in such media than in water." If this were to be realised, all the difficulties of biocatalytic reactions featuring poorly water-soluble substrates could be solved. In essence this would open the prospect for enzymes to be even better suited to industrial (organic solvent based) conditions.

Chapter 8. Conclusions

The aim of this thesis was to address the challenges of handling poorly water-soluble substrates in biocatalytic reaction characterisations. Furthermore, generic guidelines for assessing these reactions during early-stage reaction scoping were proposed in order to support the overall aim. Based on the work and arguments presented in this thesis, the following specific conclusions regarding the biocatalysis of poorly water-soluble substrates can be drawn:

Biocatalysis is now an established branch of synthetic chemistry in its own right. Within this field poorly water-soluble substrates are prevalent, and these substrates pose a number of challenges through the introduction of heterogeneous reaction media. Such challenges include: quantifying and dealing with limitations caused by poor mass transfer of substrate into the aqueous reaction medium, the representative sampling of heterogeneous media, as well as the stability of enzymes in media requiring co-solvents for improving substrate solubility.

In Chapters 2, 4 and 6 it was shown that reaction trajectory analysis can be used in early stages of reaction scoping to determine if a particular reaction is limited by substrate mass transfer (caused by poor aqueous solubility), enzyme kinetics or enzyme stability. Knowing which of these factors is the limiting bottleneck can direct further research and development efforts.

It is important to classify a biocatalytic reaction being considered according to its target end-use market value. In Chapter 3 it was shown that the value of the resultant product dictates the necessary process performance metrics of the reaction in order to meet process margin constraints. The production of low- and medium-value products requires more demanding reaction performance than that of high-value products to meet economically viable process margins. These process metrics include: product concentration (into the downstream), conversion, selectivity, space-time yield, and importantly biocatalyst yield. The biocatalyst yield requirement is further a function of the final intended formulation and the cost associated with its production, where lyophilised cell-free extracts are 4-fold more expensive to produce than using wet-whole cells directly from fermentation.

It is important to understand the primary limitation of a given system so that its influence on process performance metrics can be considered. Mass transfer can mask true enzyme kinetics and biocatalyst yields may be misleading under these limited conditions.

Benchmarking with target process performance metrics can reveal the simplest improvement strategy for a given biocatalytic reaction to satisfy all metrics, which is a prerequisite for industrial implementation. For example, in Chapter 4 it was found that by dosing the reaction with less enzyme, the reaction would then reach all process metric targets (including biocatalyst yield) even though the space-time yield would be reduced. Therefore, further increasing the product concentration from 74 g L⁻¹ through process development (e.g. ISPR to overcome inhibition), nor protein engineering or expression efforts would be strictly necessary to meet reaction performance requirements.

Special analytical online methods, such as oxygen measurements in the gas-phase of a reactor and sacrificial sampling activity assays, which were demonstrated in Chapter 5, are invaluable to following complex heterogeneous biocatalytic reactions. These accurate analytical procedures give confidence to the measured trends especially when poorly water-soluble substrates are involved, further improving the outcome of assessments using reaction trajectory analysis (as covered in Chapter 2).

The use of water-miscible and -immiscible solvents for improving substrate solubility must be carefully considered and the enzyme's tolerance (stability) and activity in their presence must always be quantified. The use of co-solvents is essential to realise a fast enough reaction when dealing with poorly water-soluble substrates through increasing their mass transfer into an aqueous medium for biocatalysis. For example, the biocatalytic Baeyer-Villiger oxidation of macrocyclic ketones could only proceed in the presence of a co-solvent. Here, an eventual space-time yield of 1.6 g L⁻¹ h⁻¹ was achieved with the addition of 20% (v/v) methanol as a co-solvent.

It has been previously shown that oxygen transfer is dependent on scale. Therefore, oxygen supply at smaller scales influences the results of characterisation experiments in oxidation reactions. Furthermore, mixing has a similar yet less pronounced effect on liquid-liquid mass transfer through dispersion. It is therefore important that the scale of experiments is always considered when performing experiments featuring poorly water-soluble substrates.

The success of biocatalysis towards industrial implementation is inextricably linked to its ability to meet target process performance metrics, and is easier for high-value products. Therefore for successful industrial implementation, reaction targets (or synthetic routes) must be chosen wisely so that an enzyme candidate is able to meet the required process performance metrics.

Overall this thesis shows the successful development and application of generic guidelines for assessing biocatalytic reactions involving poorly water-soluble substrates during early-stage reaction design, and proposes a pragmatic methodology for characterising the challenges of handling such biocatalytic reactions.

Chapter 9. Future perspectives

Since poorly water-soluble substrates are so prevalent in chemical processing the unique aspects they impose on biocatalytic processes will remain in the future. By no means have all these aspects been addressed in this thesis, and scope remains to explore those aspects that have not been studied and considered, further. Future avenues for research relevant to the studies and findings in this thesis are considered here. The initial recommendations are more significant and pertain to more general cases of biocatalysis, whilst the final perspectives only involve the specific case studies that were covered in this thesis.

9.1. Standardised methodologies for biocatalytic process conceptualisation and assessment

A systematic research flow for developing an enzyme-catalysed reaction from scratch through to later stages of process development was proposed in Chapter 2 and Chapter 7. A key outcome was the ability to formulate directed improvement strategies and assess their feasibility of implementation. It would be highly useful to see this methodology applied to further cases of biocatalytic reaction design to further establish whether it does indeed streamline the research process. Furthermore, the simple and efficient methodology relying on reaction trajectory analysis presented in Chapter 2 could be applied as the standard for reaction scoping and to measure process performance metrics in a reliable way in appropriate reactions.

At the heart of this development protocol is the need for representative process metric targets. Whilst they are intended to be used as simplified 'rules of thumb', their exact values should still be refined to better reflect the demands of industry. A more detailed study into the costing of biocatalytic processes would likely improve the fidelity of space-time yield and product concentration targets for different value-class products. This should take into account the different types of processing encountered for products of different values, for example: low-value products are typically manufactured in large volumes in dedicated plants and high-value products are more likely to be produced in several batch campaigns during the course of a year in rented plant equipment. A sensitivity analysis on the various metrics would also be particularly useful to distinguish if some metrics are more influential towards process economics than others.

9.2. Online analytical methods

For further biocatalytic reactions, alternative online methods should also be explored owing to their usefulness and accuracy in reaction characterisations. These online methods also hold potential for process monitoring and control because of their scalability. Standardised online analytics should therefore be prioritised in future biocatalytic reaction development instead of offline manual sampling methods, especially when heterogeneous mixtures are involved. Notable measurements could make use of colorimetric assays, conductivity, pH control, CO_2 consumption/liberation, to name a few. Where appropriate, these methods could also be indirectly coupled to a reaction of choice by using an alternative medium element (e.g. additional enzyme, indicator or carbonate buffer that produces CO_2 whilst controlling the pH as was done in Chapter 4).

9.3. Alternative water-free media for biocatalysis

Non-aqueous media or monophasic organic solvents are promising alternatives for reactions featuring poorly water-soluble substrates. However, future research should have more focus into the kinetic mechanisms underlying the general trend of enzyme activity loss in low-water environments – especially with different enzyme classes other than hydrolases. Effective protein engineering techniques such as directed evolution could be better applied to non-aqueous biocatalysis, ideally improving the performance of enzymes under these conditions. There also remains a huge potential for better cost- and performance-effective methods of immobilising enzymes. Ultimately, only if significant progress is made on both of these aspects of immobilisation could a generic technique that works for all biocatalytic applications be realised. Biocatalysts and their development have made massive strides since the bulk of research into non-aqueous biocatalysis was carried out. Therefore with these updated more efficient enzymes and techniques, the problems facing this field outlined in this thesis may be overcome in the future. In this way, poorly water-soluble substrates may no longer pose challenging for biocatalysis.

9.4. Biocatalyst formulation

All the cases studied in this thesis show the importance of target protein expression in the cellfree extract. Improving expression will have a direct impact on the specific activity of a given biocatalyst formulation, and therefore biocatalyst yield. This is because a higher expression of target protein (without the formation of inclusion bodies) will result in more activity within the cell. Furthermore, it is generally simpler to improve expression through fermentation optimisation than it is to engineer a protein to have an improved activity. Therefore, achieving decent expression should receive more focus during enzyme development. The cost of lyophilising cell-free extracts should also be decreased through optimising the processes of freeze drying and cell lysis. By doing so, the biocatalyst yield requirement from lyophilised cell-free extracts might be relaxed.

9.5. Case studies specific to this thesis

The esterase-catalysed desymmetrisation reaction is ready for scale-up based on this assessment which indicates adequate performance and enzyme robustness. However, the biocatalyst yield for such a high-value product must first be improved by dosing with less enzyme (at the expense of space-time yield, as discussed in Chapter 4). Alternative (and more complicated) methods for improving biocatalyst yield include: *in situ* product removal to overcome product inhibition and allow higher substrate turnovers, as well as protein engineering or optimising the expression of ECS-PLE06 in the lyophilised preparation. There is also the potential to further study increasing the reaction temperature and using co-solvents to help reduce the mass transfer limitation threshold.

On the other hand, the BVMO oxidation reaction suffered poor process performance in a number of aspects. Oxygen limitation was the most predominant, although there exist many routes to address this and potentially meet productivity requirements for low- to medium-value products: scale-up, the use of pure oxygen or pressurised reactors should all be considered. Even though the BVMO was robust (especially in comparison to most observed in literature), there is still the potential to improve its activity, and therefore biocatalyst yield, through optimising CDMO expression in the cell-free extract, protein engineering or selecting an alternative, more active candidate (e.g. polycyclic ketone monooxygenase (Fürst et al. 2017)).

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